THE GENETICS OF GILLES DE LA TOURETTE SYNDROME:-

A LINKAGE STUDY.

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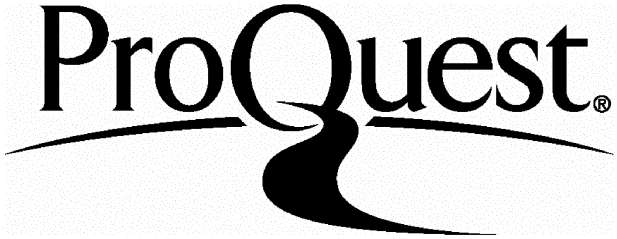

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ii ABSTRACT

Gilles de la Tourette Syndrome (GTS) is a neuropsychiatric disorder defined by the presence of multiple motor and one or more vocal tics and by the observation of a spectrum of symptoms which include involuntary thoughts, compulsive behaviours or movements including chronic motor tics (CMT). GTS and CMT are thought to share the same genetic aetiology when found within the same family. Segregation analyses of family data have shown an autosomal dominant mode of transmission with variable penetrance.

Since it is likely that single major loci contribute to the genetic susceptibility to GTS/CMT it was hypothesised that linkage analysis with genetic markers could localise a mutation to a specific chromosome. In order to test this hypothesis three approaches were adopted. The first approach was to try and detect non random segregation between marker alleles and disease locus using the lod method, in total over 70 polymorphic markers including blood groups, RFLPs, VNTRs, minisatellite and

microsatellite polymorphisms were genotyped. In the second approach it was hypothesised that a case of GTS in a patient carrying a chromosome 3 to 8 translocation was caused by the cytogenetic abnormality and that clues to the general aetiology of GTS/CMT might be derived from the specific chromosomal loci disrupted by the translocation. The third approach involved testing the role of known genes expressed in the CNS for their potential role in the aetiology of GTS. All three of these approaches demanded extensive linkage analysis and required the incorporation of variable disease models. Furthermore adjustment of penetrance parameters needed to be made in order to take into account the possible presence of non genetic cases or phenocopies and incomplete penetrance within the families studied. Two lod scores above 2.00 were found. One resulted from testing the hypothesis that random markers might show linkage to the disease and was found on chromosome one. The other positive lod score above 2.00 resulted from the hypothesis that the 3 to 8 translocation was involved in GTS and was found using markers localised to the region implicated on chromosome 3.

Linkage analysis was primarily carried out on a single large kindred, but where positive lod scores were found the sample was enlarged, and additional markers were used. When the hypothesis of chromosome 3 involvement was tested further in the enlarged sample a positive lod score of 2.98 was found on chromosome 3. A cell hybrid was produced from the GTS patient with the translocation in order to facilitate accurate mapping of linkage markers relative to the chromosome 3 and 8 breakpoints. It was found that the breakpoint on chromosome 3 was proximal to the marker loci used to obtain the positive lod score. Further work, including the use of chromosome-walking to obtain novel polymorphic microsatellite CA repeat sequences in the area, and repeated multipoint linkage analyses eventually excluded the whole region on chromosome 3 near the cytogenetic abnormality as well as the break-point on chromosome 8.

The third approach of investigating candidate genes focused on neuro-receptors and neurotransmitter enzymes. The dopamine D1, D2, D3, D4 and D5 receptors and the

dopamine pathway enzymes, dopamine-beta-hydroxylase and tyrosine hydroxylase, have all been hypothesised to be involved in susceptibility to GTS/CMT. All such candidate genes tested were excluded in two point and multipoint tests of linkage. In order to test the hypothesis that glutamate neurotransmitter receptor genes, such as the NMDAR1 receptor could be involved in the genetic aetiology of GTS further laboratory work needed to be carried out. A cDNA clone of GRIN1 (NMDA 1 subunit), a newly cloned neuroreceptor, was used to screen genomic libraries to find an associated RFLP or microsatellite marker for linkage analysis. A variable number tandem repeat (VNTR) was isolated and mapped by FISH and linkage analysis to chromosome 9q34.3.

The research failed to confirm linkage at the favoured loci on chromosomes 3 and 8 and did not support linkage at other loci. Work in other laboratories has also failed so far to implicate a major locus component in the genetic liability to Tourette Syndrome. The most likely explanation is that there is locus heterogeneity in GTS with several single major loci causing genetic susceptibility within any given family.

CHAPTER 1: General Introduction

Gilles de la Tourette Syndrome (GTS) is a neuropsychiatric condition characterised by multiple motor and one or more vocal tics. The syndrome was once thought to have a low prevalence in the general population and the literature up to the 1970's consisted mainly of case reports which concentrated on the bizarre physical and psychological manifestations. There was little speculation about the aetiology until the 1980's. Many approaches have been adopted to understand the aetiology of GTS and these have included initially psychoanalysis and subsequently neurological, biochemical, pharmacological and genetic investigations.

1.1 Gilles de la Tourette Syndrome (History)

The first description of a patient with GTS was by Itard in 1825. He described the Marquise de Dampierre, a French noble woman, who exhibited persistent body tics, barking sounds and uncontrollable utterances of obscenities. The disorder takes its name from Georges Gilles de la Tourette, who in 1885 published his classic report describing nine patients including the Marquise, who showed the triad of major symptoms and associated behaviours, these being multiple motor and vocal tics, a tendency to imitate (echophenomena) and coprolalia (inappropriate use of swearing). Gilles de la Tourette also noted that there was an association with obsessive compulsive behaviour and considered to have an hereditary component.

McHenry (1967) and Murray (1979) have argued convincingly that the prominent 18th century literary figure Dr Samuel Johnson was afflicted with GTS. Another early case is that of Prince de Conde as suggested by Stevens (1971), who was compelled to stuff his mouth with any nearby object, including a curtain to suppress an involuntary bark in the presence of Louis XIV. Shapiro et al (1978) traced the history of GTS from 1885 onwards and found mention of only about 50 cases. The literature now abounds with case reports and large cohorts (see Robertson 1989, 1994) as well as several large multiply affected families (Kurlan et al 1986; Robertson and Gourdie 1990; McMahon et al 1992).

1.2 Prevalence

At present there are many widely varying estimates for the population prevalence of GTS with the most widely accepted estimate being 0.5 per thousand (Bruun, 1984). However this rate may be an underestimate because tics, obsessions and compulsions in the general population are often not recognised. The disorder may have become more widely diagnosed as a result of increased knowledge of the disorder amongst clinicians. In 1973 there were only 174 patients identified in the U.S. and 53 in the U.K. (Abuzzahab & Anderson, 1973), however by 1984 that number had risen to about 110,000 in the U.S. (Bruun, 1984). There have been studies on the population prevalence of GTS by Lucas et al (1982), Burd et al (1986), Caine et al (1988) and Apter et al (1992). Kurlan et al (1988) suggest that all of these estimates may be too low because there are many cases of GTS which are very mild and are therefore not diagnosed. In addition many of the studies have been restricted to population studies of tic disorder and mounting evidence,

which is reviewed later, suggests that behavioural disorders such as Obsessive Compulsive Behaviours (OCB) are an integral part of GTS. There is also a debate as to whether or not GTS and Attention Deficit Hyperactivity Disorder (ADHD) are genetically related (Comings et al 1985; Pauls et al 1994; Eapen and Robertson 1992) and thus would occur in the relatives of GTS cases and in association with GTS.

1.3 The Phenomenology of GTS.

1.3.1 The syndrome of GTS

A valid and reliable method of diagnosis is an essential step in testing aetiological hypotheses for GTS and related disorders. The American Psychiatric Association (APA) has constructed operational criteria for diagnosing GTS/CMT. They include; the presence of multiple motor tics; the presence of 1 or more vocal tics; an age of onset before 21 years of age and duration of more than 1 year (DSM III, 1987). The World Health Organisation (1992) definition is the presence of multiple motor and one or more vocal tics.

Tics are brief involuntary movements (motor tics) or sounds (vocal/phonic tics) that occur out of a background of normal motor activity. There is a spectrum of tic disorders within GTS and these tics can be divided into simple and complex forms. Simple motor tics are abrupt, brief, isolated movements, such as a head twitch, shoulder shrug or facial grimace. Complex motor tics consist of more co-ordinated and complicated movements that often appear purposeful, such as touching, smelling and jumping. Repetitive sequences of

simple tics ~~can exist~~ which occupy a position between simple and complex motor tics have been documented (Singer and Walkup 1991).

Simple phonic tics include a variety of inarticulate noises and sounds, such as throat clearing, sniffing and grunting. Complex phonic tics consist of words, echolalia, palilalia (frequent repetition of a phrase) and coprolalia. Coprolalia is the most commonly known symptom of GTS, but it's presence is not required for the diagnosis of GTS. In fact coprolalia may be mild and transient and only occurs in a third of clinic populations, a minority of children and extended families (Robertson, 1989; 1994).

In some cases of GTS the tics may not be fast and abrupt, but consist of slower tonic movements that resemble dystonia (Fahn, 1982). The tic movements in GTS therefore, cover a wide range of involuntary behaviours, some of which appear quite unusual such as throwing objects and forced touching.

Another form of tic experienced by GTS patients are sensory tics, which are patterns of somatic sensations, such as feelings of pressure, tickle, warmth, cold or other abnormal sensations in skin, bones, muscles and joints (Shapiro et al 1988; Eapen et al 1994). Sensory tics are reported by about 40% of GTS patients. Kurlan et al (1989) report that the sensations are usually localised to specific areas and cause the patients to perform movements to attempt to relieve the uncomfortable sensations. The relief obtained is however only temporary and is therefore often repeated. These tics respond well to dopaminergic antagonists such as haloperidol.

Chronic motor tic disorder (CMT) is different from GTS in that motor or phonic tics are present, but not both. Reports from Golden (1978) and Pauls et al (1981) indicate that CMT and GTS are transmitted as hereditary traits in the same families and that CMT is a milder form of GTS. In family F24, described later in chapter 2, it can be seen that CMT in a parent can give rise to GTS in their off spring (F24/1 to F24/3) and vice versa (F24/42 to F24/44). The evidence is therefore against the hypothesis that GTS and CMT could be aetiologically heterogeneous disorders (Kurlan, 1988). Any such underlying heterogeneity could invalidate epidemiologic, genetic and therapeutic data.

1.3.2 Obsessive Compulsive Behaviour and GTS

Although motor tics are the most prominent clinical feature of GTS and are the basis for the diagnosis of the disorder, they may be accompanied by a range of related behavioural disorders. For example more than 50% of GTS patients suffer from OCB (Frankel et al, 1986; Pitman et al, 1987; Robertson et al 1988,1993; George et al 1992). Segregation analyses also point to OCB as being an alternative expression of GTS (Eapen et al 1993) because, under a dominant model its inclusion increases the penetrance levels for females (Pauls et al 1986) from 56% to 70%. In the large family (F24) studied in this thesis, OCB in a parent gave rise to either CMT or GTS in the off spring (F24/14 to F24/42 and F24/33).

Obsessive compulsive behaviours are characterised by the presence of recurrent, stereotyped behaviours and/or thoughts that intrude into consciousness or action and are typically experienced as senseless or alien. Common compulsive symptoms include

ordering and arranging habits, checking rituals, frequent counting, ritual decontaminations of objects or body parts and repeated handwashing. Common obsessive thoughts include fears or images of contamination with dirt or germs, of being responsible for the misfortunes of others, and fears of "loved ones" being harmed.

Some of the first comments on the association between GTS and obsessional behaviour were made by Gilles de La Tourette himself in 1889, subsequently numerous authors (Guinon (1886), Grasset (1890), Meige and Feindel (1907), Wilson (1927), Ascher (1948) and Bockner (1959)) noted the strong relationship. More recently there has been a growing literature on the relationship between OCB, obsessive compulsive disorder (OCD) and GTS. The following percentages of GTS patients were found to have OC symptoms, traits or illness: 11% (Kelman, 1965), 31% (Fernando, 1967), 32% (Comings & Comings, 1985), 33% (Abuzzahab & Anderson, 1973), 38% (Asam, 1982), and as high as 60% (Hagin et al, 1982), 66% (Montgomery et al, 1990), 68% (Nee et al, 1980), 71% (Morphew & Sim, 1969), 74% (Stefl, 1984) and 80% (Yaryura-Tobias et al, 1981). In controlled studies (Frankel et al, 1986; Green & Pitman, 1986; Van de Wettering et al, 1988; Comings & Comings, 1987; Robertson et al, 1993) GTS patients were found to have higher scores on OC inventories and to show more OCB than normal controls and depressed patients. The scores and behaviour ratings were as high as those of patients with OCD.

Robertson et al (1988) found that 37% of a group of 90 GTS patients reported OCB, and that coprolalia and echophenomena were significantly associated with OC phenomena. Others (Green & Pitman, 1986; Van de Wettering et al, 1988; Comings & Comings,

1987; Caine, 1985) have also found OC phenomena to be an integral part of GTS symptomatology. Cummings & Frankel (1985) comment on similarities between GTS and OCD, including age of onset, lifelong course, waxing and waning of symptoms, involuntary, intrusive, ego-alien behaviour and experiences, occurrence in the same families, and worsening with depression and anxiety.

One may also examine the association between OC symptoms and GTS from the perspective of OCD patients. Janet (1903) noted that in 15 of his 325 cases of OCD the illness began with tics. Several subsequent studies have reported tics in patients with OCD (Schilder, 1938; Grimshaw, 1964; Inouye, 1965). Rapoport (1988), in a review, notes that about 20% of OCD patients have tics.

Evidence for an overlap between GTS and OCB/OCD has been observed by Kurlan et al (1986), Pauls et al (1986a & 1986b), Comings & Comings (1987), and Robertson and Gourdie (1990) who all found that many relatives of GTS patients have OC thoughts and actions in the absence of tics or vocalisations. These authors suggest that GTS and OCD are genetically related.

1.3.3 Associated Behaviours and Psychopathology

1.3.3a Attention deficit hyperactivity disorder

There have been reports of many types of behaviour associated with GTS (see table 1.1). Attention deficit hyperactivity disorder (ADHD) is the most common of these and it has

been claimed that it occurs in 50-60% of children with GTS (Comings & Comings, 1987, 1988; Nee et al 1980; Pauls et al 1986). Moreover Comings and Comings (1984), argue that GTS and ADHD are aetiologically and genetically related because they co-segregate in some families. However other segregation analyses suggest that the two traits segregate independently because it was found that the rate of ADHD in relatives of probands with GTS and ADHD was eight times higher than for those with GTS alone (Pauls et al 1986; Pauls & Leckman 1986). It seems likely that GTS and ADHD are co-morbid conditions because of ascertainment bias and that the high incidence of ADHD in GTS patients is due to selective referral of subjects with both GTS and ADHD (Pauls et al 1986). The symptoms of ADHD usually precede the onset of phonic or motor tics by 2 to 3 years and may be more common in those individuals with more severe tics (Comings & Comings 1988).

1.3.3b Depression and anxiety

The psychopathology found in GTS includes depression (Robertson et al 1988, 1993) as well as anxiety (Coffey et al 1992; Robertson et al 1993) and many patients are subject to isolation and social under-achievement (Robertson 1989). The depression may be related to the duration of the disorder (Robertson et al 1988).

1.3.3c Self-injurious behaviour

Self-injurious behaviour (SIB) has been associated with GTS by several authors (Eisenberg et al, 1959; Eldridge et al, 1977; Lowe et al, 1982; Sandyk, 1986; Robertson

et al 1989). Robertson et al (1989) report on a sample of 90 GTS patients of whom 30 exhibited forms of SIB, of these two had cavum septum pellucidum possibly as a result of headbanging. They also discussed the case of a patient who died from a subdural haematoma as a result of headbanging and three patients who had permanent visual impairment from self inflicted eye injuries. The relationship between SIB and GTS is complex and not fully understood (Robertson 1992; Robertson and Yakeley 1993).

1.3.3d Sleep problems

Sleep disturbances seem to be common in GTS patients (Moldofsky et al, 1974; Nee et al 1980; Hagin et al, 1982; Stefl, 1984; Comings & Comings 1987; Caine et al, 1988; Robertson et al, 1988). The most commonly reported problems with sleep include insomnia, sleep-talking, nightmares, night terrors, sleepwalking, bruxism, enuresis, general restlessness, inability to take afternoon naps and difficulty with falling asleep. Polysomnographic studies have shown decreased REM sleep, but it is not clear if delta sleep is increased or decreased (Glaze et al 1983; Menddelson et al 1980). It has been suggested that the contributions of age, learning problems, use of medications, the presence of ADHD and the severity of tics have not been fully accounted for in the sleep disturbances found in GTS. For example the presence of ADHD may be a major determinant of sleep problems in GTS (Singer & Walkup 1991).

1.3.3e Aggression and GTS

Other associated behaviours include aggression (Modolfsky et al 1974; Corbett et al, 1969; Yaryura-Tobias et al, 1981; Stefl, 1984), inappropriate sexual behaviour (Moldofsky et al, 1974; Nee et al, 1980; Robertson et al, 1988) and the feeling of being "forced to touch" these are sometimes overlooked in psychiatric practice and a diagnosis of GTS is not made when it would be helpful to do so.

In some cases referral to a physician is due solely to the associated behaviour that a patient exhibits. It is generally accepted that many children that progress to GTS first express the illness as a series of behavioural disturbances, often labelled as ADHD. In 1987 Comings and Comings (1987a,~~1987b,1987c,1987d,1987e,1987f~~) published a series of papers on comparisons between 47 normal controls, 246 patients with GTS and 17 with ADHD using a self-reporting questionnaire. It was claimed that the findings suggested that ADHD, stuttering, dyslexia, conduct disorder, panic attacks, multiple phobias, OCB, depression, mania, and severe anxiety were 5 to 20 times more common in GTS patients than controls. Comings et al (1985) drew the conclusion that GTS was a very broadly based behavioural disorder and that the GTS gene can be expressed in a highly pleiotropic manner. Comings et al argued that GTS, and it's associated forms of expression, was one of the most common genetic disorders affecting humans. However this conclusion seems unwarranted in the light of potential ascertainment biases and an inadequately sized control group (Pauls et al 1994; Robertson 1989, 1994).

1.3.3f Transient Tic Disorder

Transient tic disorder (TTD), which has been defined in the DSM IIIR system as having a duration of less than a year (unlike GTS or CMT), is generally considered to be a mild variant of GTS (Kurlan et al, 1988). However Eapen and Robertson (1994) suggest that CMT and transient tic disorder (TTD) are not necessarily genetically related to GTS.

1.4 Pathophysiology

1.4.1 Neuroanatomic localisation

The exact neuroanatomic localisation of the CNS dysfunction in GTS is still speculative. The basal ganglia, frontal cortex and limbic system have been suggested as possible sites of pathology. Devinsky (1983) suggests that there is a possible midbrain involvement in the aetiology of GTS and that it is the periaqueductal gray and midbrain tegmentum which may be involved. This hypothesis was based on the fact that there is evidence of dopaminergic involvement in GTS and also on examination of the localisation of lesions found in encephalitis lethargica where GTS symptoms were present and on studies of the anatomy of vocalisation. Evidence for basal ganglia involvement arises from the role of the ganglia in other movement disorders such as Parkinson's disease and also as a result of the finding of minor structural alterations detected by computed tomography or magnetic resonance scanning in cases of GTS (Chase et al 1986; Harcherik et al 1985; Robertson et al 1990). One autopsy of a "classic" GTS patient showed arrested development of the striatum (Richardson 1982; Haber et al 1986). Lastly positron

emission tomography (PET) with labelled fluoro-deoxy-D-glucose shows that glucose utilisation in the basal ganglia is on average 16% above control levels, but there are also differences in the fronto-temporal areas (Chase et al 1984).

1.4.2 Biochemical abnormalities

1.4.2a Introduction

Previous studies of GTS have included investigations of neuroreceptor function, neurotransmitters and their metabolites as well as neurotransmitter related enzymes in an attempt to determine the pathophysiology of GTS. The neurotransmitter systems most often thought to be involved in GTS include those involving dopamine adrenaline, noradrenaline and serotonin.

Lately many genes for brain receptors have been cloned, some of these are strong candidate genes for GTS. Five dopamine receptors have been cloned by homology to each other or to other G-protein coupled receptors (Bunzow et al 1988; Sunahara et al 1990; Sokoloff et al 1990; Van Tol et al 1991; Sunahara et al 1991), some of these have produced negative results in linkage studies with GTS. In view of the finding of rare mutations, as in the amyloid protein for Alzheimer's disease, a thorough search in specific candidate genes for a role in GTS could include association as well as linkage studies using highly polymorphic microsatellites which give good power to detect allelic association. Furthermore a direct search for rare mutations in specific genes in GTS individuals could be justified. Other candidate genes include the glutamatergic and

tryptaminergic receptors and their associated metabolising enzymes have been cloned (Hollmann et al 1989; Werner et al 1991; Egebjerg et al 1991). More recently several NMDA receptor genes likely to be involved in memory have also been cloned in the rat and human (Moriyoshi et al 1991; Kumar et al 1991; Le Bourdelles et al, 1993; Karp et al, 1993; Brett et al, 1994). A recent report identifies a mutation in the alpha 1 subunit of the glycine receptor gene on chromosome 5 as the cause for the disease hyperekplexia, this is the first time a neuroreceptor has been found to be aetiologically responsible for a disease (Shiang et al 1993). Mutations in this gene are also good candidates for causing genetic susceptibility to GTS (Floeter & Hallett 1993).

Observations on the treatment of GTS patients have provided the most evidence in favour of a role for the dopamine system in the aetiology of GTS. The tryptaminergic pathways might also be implicated in GTS through the association between OCD and 5HT abnormalities. This is shown by the successful treatment of many OCD cases with potent serotonin uptake inhibitors assessed in double blind clinical trials (Cottraux et al 1990; Ananth et al 1979; Perse et al 1987; Goodman et al 1990). Possible abnormalities in serotonin metabolism in GTS have also been described by several groups (Cohen et al 1978; 1979; Butler et al 1979).

Neurotransmitter synthesising or degrading enzymes may also have a role in the susceptibility to GTS as well as the many different neurotransmitter transporter proteins. Despite considerable efforts in the past using non-genetic methods these enzymes and transporter proteins have yet to be implicated as useful vulnerability traits or markers in GTS.

Research reported in this thesis is intended to test the hypothesis that genes encoding proteins in the dopaminergic and serotonergic and other neuroreceptor pathways may contribute to the genetic aetiology of GTS.

1.4.2b Dopaminergic system

A possible link between the catecholamine system and GTS has been reported several times and has been reviewed by Caine (1985) and more recently by George et al (1992). Several studies have shown that dopamine antagonists and depletors modify symptoms in GTS patients (Sacks, 1982, Robertson and Eapen 1992, Jankovic et al 1984). However because such drugs act on many brain systems it is not clear from the work carried-out, whether the dopaminergic receptors themselves are aetiologically responsible for GTS or whether other neuroreceptor systems or metabolising enzymes in the catecholaminergic pathways are involved. Successful treatments used to ameliorate the symptoms of GTS employ predominantly D2 receptor antagonists such as haloperidol (Shapiro and Shapiro 1982), pimozide (Golden 1984) and sulpiride (Robertson et al 1990). However all these drugs have multiple actions on other brain transmission systems. There are now five recognised dopamine receptor genes which have been cloned and localised. It has been shown that commonly used dopamine antagonists have affinity for all five dopamine receptors (van Tol et al 1992), but that affinity for subtypes varies considerably. In addition it is known that dopamine antagonists have multiple actions on other brain transmission systems (Gilman et al 1992; Baldessarini 1980).

The effects of dopaminergic agonists, such as L-DOPA (Sacks, 1982), and CNS stimulants, such as methylphenidate (Golden, 1984; Robertson and Eapen, 1992), are deleterious and increase the number and severity of the symptoms shown by GTS patients, whereas the dopamine depletors, such as tetrabenazine (Jankovic et al 1984), do induce some improvement in the symptoms. These drugs provide evidence of the possible involvement of the enzymes controlling the synthesis and breakdown of dopamine. Dopamine beta hydroxylase (DBH) catalyses the conversion of dopamine to norepinephrine in the adrenal medulla (Kirshner, 1957). Tyrosine hydroxylase (TYH) is the first enzyme in the catecholamine biosynthetic pathway and is responsible for the conversion of L-tyrosine to 3,4-dihydrophenylalanine (DOPA) (Cooper, Bloom and Roth, 1991). Tyrosinase is involved in the conversion of tyrosine to DOPA to be used in the synthesis of melanin (Cooper, Bloom and Roth, 1991). These genes are, therefore, essential in the functioning of the dopaminergic pathways and a mutation in any one of them could have an impact on overall catecholamine metabolism. Another gene with a possible effect in GTS is the dopamine transporter protein (DAT) which is important in recycling used dopamine.

It is however not clear from the evidence whether the dopaminergic system itself is in some way aetiologically responsible for GTS, or whether the changes observed are the effect of another brain system dysfunction. Bornstein and Baker (1990) measured changes in several aminergic systems in a controlled study. Significant changes in beta-phenylethylamine (PEA), 5-hydroxytryptamine, p-tyramine and 3-methoxy-4-hydroxyphenylglycol were found in patients' urine. After control for the effects of drugs the most pronounced change was found in PEA.

PEA is produced by the decarboxylation of phenylalanine and has a mescaline-related psychotogenic action (Cooper, Bloom and Roth 1991). It is known that PEA has effects on brain dopamine, 5HT and norepinephrine (Jackson and Smythe, 1973; Sloviter et al., 1980). Abnormal levels of urinary PEA have also been reported in children with attention deficit hyperactivity disorder (Zametkin et al, 1984), which, as described earlier, is a comorbid disorder that is commonly diagnosed amongst patients affected by GTS (Robertson & Eapen 1992).

1.4.2c Serotonergic system

Some clinical studies have emphasised the strong relationship between GTS and OCB. Current pharmacological evidence suggests that serotonin is the neurotransmitter most implicated in the pathogenesis of OCD (Rapoport, 1988) and at present the medications which have consistent anti-obsessional effects are the serotonin uptake blockers assessed by double blind clinical trials (Cottraux et al, 1990; Ananth et al, 1979; Perse et al, 1987; Goodman et al, 1990). A correlation has also been found between clinical response and a decrease in cerebrospinal fluid (CSF) levels of 5-HIAA, the primary metabolite of serotonin (Thoren et al, 1980). More compelling evidence for the role of serotonin in the pathophysiology of OCD comes from studies involving single-dose challenge studies of serotonin receptor agonists in untreated OCD patients (Zohar & Insel, 1987; Hollander et al, 1989; Charney et al, 1988). Abnormalities in serotonin metabolism in GTS have also been described in which CSF levels of 5-HIAA are decreased after Probenecid loading in patients with GTS (Cohen et al, 1978; Cohen et al, 1979; Butler et al, 1979). It has been

suggested that these changes in GTS patients are due to a mutation in the tryptophan oxygenase (TO) gene (Comings et al, 1991) because the TO gene is the rate-limiting enzyme in the oxidative degradation of tryptophan, thus controlling the amount of tryptophan available for conversion to serotonin. Neuroendocrine studies suggest that 5HT1, and particularly 5HT1a, receptor function may be disturbed in OCD (Montgomery et al, 1990). Hollander et al (1989) carried out double blind placebo controlled tests on patients and controls using m-chlorophenylpiperazine (mCPP) and fenfluramine. Fifty five percent of the patients with OCD experienced a transient exacerbation of symptoms with m-CPP but exhibited no change with fenfluramine. However the exact nature of the alterations in serotonergic function are uncertain. Due to the close relationship between GTS and OCB the serotonergic system may also be involved in the aetiology of GTS.

1.4.2d Glutamatergic system with special reference to NMDA

The glutamate receptors consist of two main classes, ionotropic and metabotropic. The metabotropic receptors are coupled to intracellular signal transduction through G-proteins (Schoepp et al 1990). The ionotropic receptors contain integral cation-specific ion channels and are divided into:- NMDA (N-methyl D-aspartate) receptors; AMPA (alpha-3-hydroxyl-5-methyl-4-isoxazoleproprionic acid) receptors, and kainate receptors (Monaghan et al 1889). The NMDA receptor is involved in memory and the mediation of excitotoxicity. There are 5 cloned subunits of the NMDA receptor; NMDAR1 (which has 7 isoforms A-G) and is designated GRIN1, and NMDAR2A, 2B, 2C, 2D. The functional receptor is a heteromer consisting of NMDAR1, which confers the NMDA properties of the receptor combined with the NMDAR2 subunits.

Moriyoshi et al (1991) demonstrated that NMDAR1 is the key subunit of the receptor as it exhibits the pharmacological and electrophysiological properties of the NMDA receptor. In the rat there are seven isoforms of NMDAR1 (A-G) formed by alternative splicing. The other constitutive subunits NMDAR2 (A-D) do not exhibit the intrinsic NMDA receptor properties, but have the ability to potentiate NMDAR1 activity (Kutsuwada et al 1992). It is thought that in vivo the different spatial and temporal expression of these subunit genes results in the generation of NR1 and NR2 heteromeric receptor subtypes (Kutsuwada et al 1992).

The NMDA receptor mediates many of the excitatory functions of glutamate, including long-term potentiation and long-term depression, these long-term changes in neuronal responsiveness could explain certain aspects of learning and memory (Collingridge et al 1990, Monaghan et al 1989). Additionally, in pathological situations, the excessive activation of excitatory synapses has been hypothesised to cause neuronal death in neurodegenerative disorders due to excitotoxicity (Choi, 1991). The excitotoxic effect is mediated by abnormal increases in intra-neuronal calcium ions. Although there is no direct evidence of glutamate receptor involvement in GTS in order to study this class of receptors from a genetic point of view in human disorders including GTS it would be helpful to identify polymorphic genetic markers within or near the NMDAR1 gene. Thus work in this thesis is reported which has enabled a very recently cloned human NMDAR1 gene to be investigated genetically in GTS.

1.4.2e Endogenous opioids

Several of the opioid peptides, such as dynorphin and metenkephalin, are localised within the basal ganglia. They are known to interact with dopamine neurons and have effects on movement (Nieuwenhuys 1985; Walker et al 1982; Li et al 1986; Broderick 1987). Opiate antagonists have been used in the treatment of GTS patients with mixed results, some investigators found a dramatic improvement in symptoms (Sandyk 1985; Gilman & Sandyk 1986), whereas others found only rare responders (Erenberg & Lederman 1989). In addition decreased dynorphin levels have been reported in the globus pallidus (Haber & Walter 1993).

1.4.2f Gamma amino-butyric acid (GABA)

The GABAergic neurones are inhibitory in function and present in both cortical and basal ganglia regions. They have their inhibitory effects enhanced by benzodiazepines which have some efficacy in tic suppression (Gonce & Barbeau 1977). However most pharmacological markers of GABA activity are normal in GTS (Singer et al 1990; Van Woert et al 1982).

1.4.2g Second messengers

The second messenger proteins and other molecules have also come under scrutiny in the search for the aetiological root of GTS. It has been shown that cyclic AMP is reduced in the cerebral cortex of adults with GTS (Singer et al 1990). Singer & Walkup (1991) have

suggested that there are many second messenger systems which might be involved in GTS. It is known that some of the neurotransmitter systems mentioned above interact with cyclic AMP and others activate or inhibit adenylate cyclase activity. Mutations affecting cyclic AMP or adenylate cyclase production could therefore both affect a wide variety of brain systems and also be responsible for the clinical variation that is to be found in GTS. Singer et al (1995) examined the levels of c AMP and phosphoinositide (PI) in post mortem GTS brains and in serum of GTS patients and concluded that there were no differences in the levels of these second messengers in GTS.

1.5 Chromosomal abnormalities

Cytogenetic abnormalities, identified in very rare cases of people suffering from specific genetic disorders sometimes give a good indication as to the chromosomal localisation of the genes generally involved in such a disease. A few cytogenetic abnormalities have been reported in rare cases of people suffering from GTS. Merskey (1974) reported a patient who was an XYY male. Singh et al (1982) reported on a XXX female with 9p mosaicism. Taylor (1990) reported on a patient with a 9p deletion. Comings et al (1987) reported 6 relatives suffering from GTS who carried a balanced translocation 46t(7;18)(q22;q22.1). Donnai (1987) reported an isolated case of GTS in a patient with a deletion on the long arm of chromosome 18 at 18q22.2. Kerbeshian et al (1984) described two GTS patients with fragile X syndrome. Down's syndrome has been reported in association with GTS in six different cases (Sacks 1982; Barabas et al 1986; Karlinsky et al 1986; Collacott and Ismail 1988).

Robertson & Trimble (1993) conducted a cytogenetic survey on 68 consecutive patients who satisfied DSM-III criteria for GTS and also reviewed the literature on chromosomal abnormalities in GTS. They found only three patients exhibiting abnormalities; a 47:XYY, a 46:XX 1qh+ with an extra heterochromatin band and a case of 46:XX, inv(9)(p11;q13). The last two were considered to be familial polymorphisms, which occur quite often in the general population (Hook & Porter 1977) and are probably not involved in the susceptibility to GTS. The Kleinfelter's anomaly (XYY) was also considered to be a chance finding and spuriously associated with the GTS phenotype. This is further supported by the observation that GTS demonstrates father to son transmission and can only rarely be sex linked in aetiology.

The cytogenetic evidence, to date therefore, provides no consistent evidence to localise a GTS mutant gene. The specific chromosomes implicated by the presence of abnormalities are two cases of XYY, chromosome 9 in three cases, chromosome 21 in six cases and chromosomes 7 & 18 with six cases (found in one family) and one isolated case of a chromosome 18 deletion. It can be argued that evidence for a chromosomal pathology causing GTS would be one of two scenarios. One would be a patient with the classic symptoms of GTS without a family history of GTS and without a family history of chromosomal abnormalities, thus implying that the GTS was caused by a de novo mutation involving the abnormal chromosome. A second favourable scenario would be the discovery of a family where a chromosomal abnormality consistently co-segregated with GTS. However none of the published cytogenetic abnormalities previously documented fitted these criteria.

GTS has been observed to cosegregate with Charcot Marie Tooth disease, in two families, (Pauls D., personal communication) and therefore raises the possibility of a common genetic lesion causing both disorders or that they are colocalised in a small deleted chromosomal region. Brett et al (1989, 1990, 1991) examined in detail the association of a reciprocal translocation, between chromosomes 3p and 8q, found in a case of GTS as described in this thesis.

Recently unstable DNA sequences comprised of a trinucleotide repeats within genes have been shown to expand in successive generations (Harley et al 1992) providing a molecular basis for differences in severity and age of onset among successive generations. This phenomenon known as "anticipation" has been found in myotonic dystrophy and fragile X syndrome (Yu et al 1991). Such unstable DNA sequences could be used to explain the complex familial segregation patterns for GTS and might be detectable in GTS families with the Repeat Expansion Detection (RED) method which uses the ligase chain reaction (Schalling et al, 1993).

1.6 The Genetics of GTS and CMT

1.6.1 Introduction

When Gilles de la Tourette (1885) wrote the original paper describing nine patients, he also mentioned that two patients had a family history of GTS. He suggested that the disease may be hereditary in nature. Many studies have been carried out on GTS families to determine whether or not there is a genetic factor involved in GTS. One family study

by Wilson et al (1978) examined familial recurrence to test the genetic hypothesis but found no significant excess of familial recurrence compared to controls. Only recently have more modern and sophisticated methods of analysis been used to investigate the genetic predisposition to GTS, these involve twin and family studies and complex segregation analyses on large family pedigrees.

1.6.2 Family Studies

Genetic research into GTS started in the late sixties and positive family histories were found in about 10% of GTS cases (Kelman 1965; Fernando 1967; Shapiro et al 1972). However these studies were not systematic and relied on current clinical samples. Friel (1973) reported on the first fully diagnosed multiply affected pedigree which two sisters affected with GTS had six children four of whom had multiple motor tics and one who had vocal tics. Frost et al (1976) performed the first extended family pedigree study on the family of a GTS patient. He interviewed fourteen of 17 maternal relatives, none were diagnosed as having GTS but 6 had tics, one paternal cousin was also found to have had a temporary tic syndrome for two years.

The first systematic family study reported was by Nee et al in 1980 and was performed to ascertain the mode of inheritance by means of segregation analyses and to collect material for a DNA linkage study. Direct interviews of all family members proved to be a more sensitive approach to assessing the relative risk of GTS (Pauls et al 1984). Since then there have been several systematic segregation analyses performed on GTS patients and families (Comings et al 1984; Devor 1984; Price et al 1984; Pauls & Leckmann

1986; Curtis et al 1992; Eapen et al 1993).

1.6.3 Twin Studies

The twin method has been used to assess the role of genetic factors in a trait by the comparison of concordance rates for the trait in monozygotic (MZ) twins and in same sex dizygotic (DZ) twins. MZ twins have identical genomes whereas in DZ twins they share on average 50% of their genes, as do normal siblings. A major assumption in twin studies is that MZ and same sex DZ twins share environmental factors to about the same extent, and that any differences observed between the two groups is due to their genetic make-up combined with intra familial or common environmental difference.

In 1980 Shapiro and Shapiro published their study of GTS in MZ and DZ twins stating that 7 out of 9 identical twins were concordant for GTS and that 3 out of 4 fraternal twins were discordant for GTS. Selection biases severely limit any conclusions from this study since the twins were not systematically ascertained and should not be quoted as offering evidence in favour of genetic effects, but genetic factors are compatible with the Shapiro study. Other research has shown evidence of variable phenotypic expression (pleiotropy) for any GTS susceptibility gene alleles. Harriman (1976) reported an identical twin who had GTS and found that the co-twin had a facial tic. This supports the argument that CMT are a variant expression of the GTS susceptibility alleles.

Jenkins et al (1983) and Waserman et al (1983) also report concordant monozygotic twins with GTS but once again such anecdotal evidence is little proof for or against

genetic aetiology. Price et al (1985) who used a questionnaire based approach, reported a study on an unbiased systematically ascertained sample of 43 pairs of same sex twins in which pair wise concordance rates for GTS were 53% amongst MZ twin pairs and 8% amongst DZ twin pairs. If the diagnostic criteria are broadened to include any tics in co-twins the pair wise concordance rates increase to 77% and 23% respectively for MZ and DZ pairs. Price used biometrical genetic methods to compute a "heritability" for GTS/CMT. The H'c statistic used as described by Kendler (1983) is expressed as.

$$H'c=1-Cdz/Cmz.$$

Cdz and Cmz are the probandwise concordance rates for the disorder in dizygotic (Cdz) and monozygotic (Cmz) twins. The higher the value of H'c the greater the aetiological value of genetic factors. This method makes the assumption that both same sex DZ and MZ twins share the relevant environmental factors to the same extent. The H'c for the twin data in GTS (Price et al 1985) varies from 0.70 for GTS to 0.85 for GTS and CMT. This increase could be due to a greater number of phenocopies rate for GTS when tics are included in the diagnosis, as suggested by Pauls (1992). The H'c values suggests that genetic factors play a major aetiological role in GTS and are higher than those for hypertension (0.58. Harvald et al 1965) and coronary heart disease (0.49. Berg 1981).

1.6.4 Adoption studies

The genetic component of a disease can also be detected in adoption studies where the expression of genetic factors can be observed in different rearing environments with more

certainty than in twins. One adoption study of GTS was carried-out by Shapiro et al (1988). These workers compared the adoptive families of 22 GTS patients with the biological families of 641 GTS patients. In the adoptive families they found no history of tics, but in the 641 biological families positive histories were found amongst first, second and third degree relatives in 302 families. This is probably an underestimate as family history data may detect fewer cases than a direct interview (Pauls et al 1986), nevertheless the differences in family history obtained provides a strong argument for a genetic component in GTS especially when taken together with the twin data.

1.7 Genetic Linkage analysis of GTS

1.7.1 Introduction

Linkage analysis consists of identifying polymorphic genetic markers that are sufficiently close on a chromosome such that they are inherited together with the disease mutation from one generation to the next. In such cases the marker and disease are said to be linked. The distance between the disease gene and the marker locus can be calculated by observing the number of recombinations that occur (recombination is the rearrangement or crossing over of alleles following exchange of material between pairs of homologous chromosomes during meiosis). The closer the disease locus is to a marker, the less likely recombination is to occur. Recombination is measured by the recombination fraction theta, which varies from zero to 0.5 or 50% (0.0<theta<0.5). A value of 0.5 indicates random segregation of the disease and marker alleles, a value less than 0.5 indicates that linkage may be present. Linkage maps of genetic distance along a chromosome use the

centimorgan unit of measure (cM), based on the frequency of recombination. Approximately one cM is equal to 1% recombination and the predicted length of a sex averaged human map is 3300 cM (Morton et al 1982).

The method adopted in determining linkage is the maximum likelihood estimate of the lod score at a specific recombination. The lod score is based upon the relative probability (Pr) of having observed the marker distribution within a family compared to the probability of observing 50% recombination in the family.

$$Pr = \frac{P(\text{family, given theta}=0\text{-}0.5)}{P(\text{family, given theta}=0.5)}$$

For convenience Pr is expressed as its logarithm. The Log10 of the relative probability is called the lod score (Morton 1955). The maximum likelihood estimate of the lod may be obtained by summing the lods for all the families studied at various values of theta. The maximum lod score is the value taken as showing the best estimate for linkage and will be found at a specific recombination fraction. At any specific value of theta, a lod exceeding 3.0 is said to confirm linkage and a value less than -2.0 rejects linkage. A comprehensive account of linkage analysis has been given by Ott (1985). By considering the relative lengths of all 22 autosomes it has been calculated that the prior odds of linkage for any two genes (ie. that two genes are syntenic) is 1:17.5 (Renwick, 1971). As a consequence a lod of 3 represents odds in favour of linkage of approximately 20:1 and not 1000:1.

There are now a large number of DNA markers and the effect of using many markers has been investigated in relation to the statistical significance of the lod. Thompson (1984) demonstrated, for the use of multiple markers used in independent tests of segregation that the interpretation of lod scores for a particular disease locus, requires the same level of significance as a single test.

Multipoint mapping (Lathrop et al 1984; Ott 1985) is a method of using linked markers to resolve gene order and linkage with disease loci. However Morton (1988) has criticised its use in relation to the mapping of disease genes because of the effects that genotyping errors may have on the multipoint lod with a disease gene. Nevertheless such likelihood analyses are widely used in the search for disease mutations.

1.7.2 Allelic association and genetic disease

1.7.2a Random markers

The approach of using random markers scattered about the chromosomes to detect linkage is now a widely used strategy since the advent of the Genethon panel of polymorphic dinucleotide repeat microsatellite markers (Gyapay et al 1994). Such markers were adopted in the search for genes for insulin dependent diabetes mellitus (IDDM). Davies et al (1994) conducted a genome wide search for susceptibility genes to IDDM by using 290 microsatellite markers to obtain a 10-20cM exclusion map in their search. In random searches of the genome in GTS Heutink et al (1990) excluded chromosomes 7 and 18, and Pakstis et al (1991) combined the exclusion data from

several groups to exclude more than 50% of the genome under the assumption of homogeneity of linkage, that is that there is only one gene causing all cases of GTS and related disorders.

1.7.2b Candidate genes

Another approach to linkage analysis is that of examining candidate genes which have already been localised and for which a function is already ascribed. Candidate genes in the case of GTS are genes which control some aspect of brain function, either as receptors or transmitters or relevant structural proteins. If the gene has been cloned and an associated marker polymorphism has been found, then it can be used for linkage and association analysis. With a family a candidate gene can be easily ruled out if a recombination occurs between the disease and the candidate gene. There have been several papers excluding candidate genes from involvement in the aetiology of GTS. Devor et al (1990) and Gelernter et al (1990) both previously excluded a role for the D2 dopamine receptor in GTS. Comings et al (1993) reported increased heterozygosity for the D3 receptor gene, but Brett et al (1993) failed to replicate this finding. Gelernter et al (1993) excluded a role for the D1 dopamine receptor and in 1995(Gelernter et al 1995) they excluded a role for the serotonin receptor gene 5HT7. Brett et al (1995 a & 1995b to be discussed later in this thesis) carried-out an extensive investigation of candidate genes in GTS in work reported in this thesis. Gelernter et al (1994) studied the effect of the D2 receptor gene alleles ~~on the severity of GTS~~ and found that the D2 gene had no influence on the severity of GTS.

1.7.2c Favoured loci

These are loci implicated by cytogenetic abnormalities (deletions, trisomies, translocations etc) or by disease localisations that could share a common gene locus between the disease and GTS. Some cytogenetic abnormalities can lead to the rapid cloning of a disease gene and mutation. For example, deletions have contributed to the cloning of some genes for inheritable cancers such as for familial adenomatous polyposis coli (FAP) on chromosome 5. Chromosome 5 was implicated because somatic cell mutations on chromosome 5q were observed in tumour tissue. Two individuals narrowed the search for the gene to 100kb through deletion mapping, three candidate genes were examined for mutations in 61 unrelated patients. One of these was the gene for FAP and sporadic colon cancers (Groden et al 1991).

1.8 Mode of transmission

To test the hypothesis that a disease has a particular mode of transmission the investigator must first ascertain a family sample by following strict rules (Thompson et al 1983). Subsequently, using maximum likelihood methods, a genetic model is chosen that takes into account the proportion of affected siblings and offspring. In addition the population prevalence and the presence or absence of Mendelian constraints must be specified. The main problems of such studies arise through the different methods of ascertaining families and affected individuals, pooling data from different sources, incomplete ascertainment, inaccurate diagnosis and genetic heterogeneity.

The comparison of maximum likelihood estimates between specific genetic models can provide evidence in favour of one model over another and hence an underlying genetic aetiology can be inferred. This information would be invaluable in genetic linkage studies, because demonstration of a major gene effect would provide impetus for the linkage strategy and greater precision in the estimation of the genetic parameters for that analysis. Identification of the mode of inheritance along with purely molecular genetic insights could help to elucidate the evolutionary and genetic mechanisms which have maintained GTS in the population. Lastly, a precise knowledge of genetic transmission would be useful in genetic counselling.

Complex segregation analysis as described by Lalouel et al (1983) can take into account reduced penetrance, phenocopies and multifactorial-polygenic (MFP) background effects. Reduced penetrance is interpreted as the incomplete or absent manifestation of a disorder in individuals who have the disease genotype. For GTS evidence of this is demonstrated from family studies and the finding of discordance in MZ twins. Phenocopies are those individuals who manifest the disorder even though they do not carry the disease genotype, possibly due to environmental or other genetic effects. MFP is interpreted as the presence of more than one gene of additive effect contributing to an individual's liability for the disorder acting jointly with environmental effects.

Two further factors which are important for the correct estimation of parameters for the transmission models in GTS are the classification of who is affected and any possible underlying genetic heterogeneity. A too restrictive definition of GTS would result in a large proportion of false negative cases, whilst a too inclusive definition would generate

many false positive cases. As described above, studies of twins have indicated that DSM III criteria for GTS provide an empirically useful approach for genetic studies. However the genetic models available do not easily allow for heterogeneity of linkage. In this case statistical methods to detect the fact that single major gene effects in GTS at several loci can operate independently to produce GTS must be employed.

The values of the genetic parameters that maximize the likelihood are the maximum likelihood estimates of these parameters. The ratio of the maximum likelihood under the null hypothesis (the observed pattern occurring by chance) to the maximum value of the likelihood under a particular genetic model is termed the ratio criterion. Therefore the smaller the likelihood ratio the less likely the null hypothesis is true. The statistical analysis consists of rejecting the null hypothesis by the ratio being smaller than that of a certain test statistic (for example a Chi-square value). If the underlying assumptions are varied, analyses may favour a number of models, none of which can be conclusively proven. In practise, conclusive evidence for one model cannot easily be found by segregation analysis because the extent of underlying locus heterogeneity of gene effects is unknown.

Several segregation analyses have been carried out on independent family samples (Comings et al 1984; Devor 1984; Price et al 1984; Pauls and Leckmann 1986; Pauls et al 1990; Curtis et al 1992). These studies support the theory that GTS is caused by a single major autosomal dominant locus which has a dominant effect. Segregation analyses can also help to determine the relationship of variable disease expression on to the underlying genetic transmission. Segregation analysis suggests that CMT and GTS appear

to be expressions of the same underlying major gene abnormality. OCB has also been investigated by Eapen et al (1993) on families ascertained through 40 unselected consecutive patients with GTS and found evidence for autosomal dominant transmission, they found that OCB was likely to be an alternative phenotypic expression of the putative GTS gene.

1.9 The Human genetic map

A large number of human genetic diseases have been found to be associated with single major gene effects. There are now approximately 10,000 recognised genetic loci or disorders (McKusick 1995). Recent advances in the techniques of molecular genetics have revolutionized linkage studies in man. Prior to the development of these methods, human linkage studies were severely hampered by the paucity of informative markers in any given family.

The use of restriction endonucleases, and the ability to clone and sequence human DNA has uncovered a wealth of hitherto inaccessible genetic variation. This variation can be detected by restriction fragment length polymorphisms (RFLPs), representing a point mutation in the genomic sequence, variable number tandem repeats (VNTRs) whose length variation is dependent on the number of tandem repeats of a short sequence (Jefferys et al 1986; Nakamura et al 1987) and microsatellites which consist of variable numbers of di-, tri- or tetranucleotide repeats. Such polymorphic sequences have dramatically increased the number of potential informative markers in any given pedigree. These methods will eventually define the organisation and even the nucleotide sequence

of the entire human genome. At present, many genetic disorders have become susceptible to efficient diagnosis at the genetic level, either through characterisation of mutant genes identified and isolated through aberrant metabolic pathways (forward genetics) or through linkage with other mapped loci (reverse genetics or positional cloning).

Just over 10 years ago the goal was to construct a human genetic map using RFLP's (Botstein et al 1980). Intensified mapping activity over the last decade has generated linkage maps for many human chromosomes (Drayna et al 1985, Donis-Keller et al 1987, Dracopoli et al 1988, Lathrop et al 1988, Nakamura et al 1988 a,b,c, O'Connel et al 1988, Tanzi et al 1988 and Rouleau et al 1989). With the development of these polymorphic markers the rate of acquisition of new mapping data has increased dramatically. A recent compilation has identified approximately 1800 expressed sequences and more than 2500 RFLP and other markers (Human Gene Mapping 11, 1991). More recently Genethon have completed a linkage map of the human genome at the 2-5 cM level using 5,000 microsatellites (Gyapay 1994). Using RFLP's, many human genetic diseases have been localized by linkage analysis and even more with the advent of microsatellites. The first was Huntington's chorea (Gusella et al 1983), but despite largescale collaborative efforts (Pritchard et al 1991) it took ten years after linkage was reported before the sequence of the abnormal gene was known.

A detailed genetic linkage map of the human genome will define positions of all known genetic loci, and allow the identification of markers linked to human genetic diseases. These will provide a starting position for the isolation of the relevant disease loci themselves by a variety of rapidly developing and improving physical methods, including

genomic walking and jumping and physical mapping with pulse-field gel electrophoresis (Rommens et al 1989). However a linkage map will not provide all relevant information on intragenic and regulatory regions of the genome that may affect the development of human diseases, nor will it remove the difficulties of chromosome walking between linked markers. These problems can be over come in part by the physical characterisation of the human genome and the development of a fine structure physical map. The ultimate goal of the development of a detailed physical map of the human genome is to obtain the ultimate map possible - that is the complete sequence of the genome. For the human genome roughly $3X10^9$ bp of DNA will have to be sequenced and this would be aided by improvements in technology (Prober et al 1987). The recent development (Burke et al 1987) of yeast cloning vectors with higher cloning capacity than the previously used cosmid vectors and the use of pulse field gel electrophoresis has enabled a preliminary ordering of the whole genome cloned into YACs. Olson et al (1989) designed a scheme to augment the combination of YAC and cosmid clone alignments by determining "sequence-tagged sites" (STSs) in the termini of all large cloned human fragments. Eventually a complete STS map of the human genome would be available, providing a set of overlapping, cloned and ordered sequences spanning an entire chromosome or portion of a chromosome.

1.10 Theoretical considerations for linkage analysis of GTS

A workshop organised by the MacArthur foundation (Merikangas et al 1989) has published recommendations aimed at resolving difficulties applying linkage analyses to the psychiatric disorders. The problems are often subsumed under the term "non-Mendelian

inheritance", but there are a number of different concepts which can be considered separately with advantage. These consist of difficulty in defining caseness, incomplete penetrance, presence of phenocopies, and genetic heterogeneity. In addition the mode of transmission is unknown and so the quantitative values attaching to each of the above values is unknown as is the gene frequency of the susceptibility allele. When a disease is inherited in a straightforward Mendelian fashion, this implies that it is possible to reliably diagnose the disease and that everybody who inherits a copy or, in the case of a recessive gene, two copies of the abnormal gene will go on to develop the disease at some stage during their life span and that everyone else will not. Because under these conditions, the mode of inheritance is probably known, it is also possible to make reasonably accurate estimates of the frequency of the abnormal gene in the population based on the prevalence of the disease. However a Mendelian mode of inheritance is not essential for modern methods of linkage analysis, and in fact only represents a special case of a more general model as implemented in the computer program LINKAGE (Lathrop et al 1985).

The general genetic model for detecting linkage between a disease and genetic markers depends only on the concept of there being some major gene effect, which means that the presence or absence of one or two copies of a certain allele at the susceptibility locus has a substantial effect in terms of altering the individual's liability to develop the disease. The genetic mode of transmission is then characterised by the probability of affection, or penetrance, conditional on each of the three possible genotypes (pAA, pAa and paa) and by the gene frequency of the abnormal allele conferring increased susceptibility. A fully penetrant dominant gene for a disease with no phenocopies is characterised by setting pAA, pAa and paa equal to 0, 1 and 1 respectively, while for a recessive gene the values

would be 0, 0 and 1. Where a disease is only partially penetrant, the probability of an individual inheriting the abnormal alleles developing the illness will fall to less than 1 and if phenocopies exist, that is to say if some cases have a non-genetic aetiology, then the probability of developing the disease despite having a normal genotype will rise to above zero.

It should be pointed out that there may be a difference between criteria used to produce an optimal strategy for defining cases within a family and those criteria which should be used in order to decide which families to select for study in the first instance. This is because the probability of an individual who has a minor abnormality being a "genetic case" is higher if he is known to have a close relative with the full-blown disease. To maximise the power of linkage analysis one wishes first to select families in which the disease gene is probably segregating, and then to select as cases those individuals within the family who are most likely carriers of the gene. This means that one may need to use conservative criteria to select families and more liberal criteria to define cases within them (Gurling et al 1991). It is important to note the sensitivity of the lod to misclassification (Skolnick et al 1984). The sensitivity moreover is very dependent on the genetic parameters specified during the linkage analysis. For example, one individual misclassified into affected/unaffected will influence the lod under both high and low penetrances to different degrees.

An issue that cannot be completely distinguished from the definition of caseness is that of heterogeneity of linkage in which some cases of an illness may be due to the actions of a particular disease gene whilst other cases may be associated with abnormalities at different

genetic loci. Linkage analysis will generally fail if such non-allelic or locus heterogeneity of linkage goes unrecognised. It is possible to analyse the data under the assumption that in only a proportion of families is the disease due to an abnormality at a particular locus whilst other families may be unlinked (Ott, 1986). However, such elaborations do considerably reduce the power of linkage analysis. In general, the presence of heterogeneity increases the recombination value at which the lod scores peak, by an amount that increases with the amount of heterogeneity (Cavalli-Sfortza et al 1986). There is a corresponding increase in the number of families necessary to establish linkage (Gershon et al 1987). Highly polymorphic markers however will greatly reduce the number of families required to establish linkage (Nakamura et al 1987). It is also true that using two genetic markers at a known genetic distance will increase the power of detecting heterogeneity (Lander et al 1986; Martinez et al 1989). A situation that is very problematic for the linkage approach is when non-allelic heterogeneity occurs within a family, that is when the multiple cases seen within a family occur as the result of actions of two different genes at separate loci. This could be the case with GTS, CMT and OCB being caused by genes at different loci. However, all the evidence outlined earlier points to the disorders all having the same genetic aetiology when found within the same family or sibship.

The application of linkage analysis to "non-Mendelian" illnesses may be seen formally as performing the analysis with an increased number of degrees of freedom. The lod score method depends on comparing the likelihoods of obtaining the observed distribution of cases and genetic markers under the conditions of linkage and non-linkage. The lod score is the log to the base 10 of the ratio of these likelihoods. With classical linkage analysis there is only one degree of freedom which is the marker-disease gene recombination

fraction or, equivalently, the map position of the disease gene. For a non-Mendelian disease with unknown mode of transmission each genotype-specific penetrance represents an added degree of freedom, as does the gene frequency, disease definition and, if heterogeneity is suspected, the proportion of families studied in which the disease gene is segregating. This would seem to add 6 degrees of freedom to the model and accordingly to achieve equivalent statistical significance to a lod score of 3 with the classical method one would have to obtain around double the lod score and use a threshold of around 6 (Kelsoe et al 1989). In fact this is rather a conservative approach, because varying the penetrances and phenotype definitions does not usually make much difference to the lod score.

The accepted practice of presenting the lod score under several representative conditions is perfectly valid. It is however essential to report that a range of models has been studied and to give some idea of the degree to which the lod score varies between the plausible models. The reader can then form an idea of whether or not the lod score is critically dependent on unjustified assumptions in the model - frequently it seems that this is not the case and that a positive lod score is quite robust to such parameters. An alternative or complementary approach to deal with the problem of unknown penetrance is to obtain a prior estimate of penetrance by a segregation analysis independent of any genetic marker data, for example using the computer programmes ILINK, from the LINKAGE package, or POINTER (Lalouel et al 1981). If this value is then held fixed throughout subsequent linkage analysis then penetrance no longer contributes to the degrees of freedom of the model. However there are problems with this approach, since families selected for linkage analysis may not be suitable for segregation analysis, and frequently it is desirable to vary

the penetrance and find a maximum likelihood estimate that does take account of linkage data.

Simulation studies have shown (Cox et al 1988) that linkage is likely to be detected even in the absence of knowledge of the mode of transmission, if a range of models are examined. An important effect of erroneous modelling is that it decreases the maximum lod (or at least the expected one), that can be obtained and furthermore the wrong genetic model for the disease may incorrectly reject linkage for a given recombination fraction. In no instances does erroneous specification of parameters artificially increase evidence for linkage (Clerget-Darpoux 1991). The greatest effect when the genetic model is badly specified is in estimating the recombination fraction which is highly sensitive to the mis-specification of the genetic parameters at the disease locus (Clerget-Darpoux 1991). The robustness of lod score estimates can be improved, however by using the information from several genetic markers simultaneously (multipoint linkage analysis) assuming all genotypes are error free.

A second method of linkage which does not require any assumptions about the mode of inheritance is the sib pair method (Green and Woodrow 1977; Weeks and Lange 1988). This method examines the distribution in members of a sibship of the alleles at the marker locus and disease phenotype. However the power of this method is a magnitude less than that of the lod score pedigree method. Furthermore, its power is closely related to the ability to determine whether the alleles of a sib pair are identical by descent, that is they represent the same parental chromosomal locus. This can be achieved only by studying highly polymorphic markers or including the parents. Linkage using this approach can be

detected in the presence of heterogeneity (Goldin and Gershon 1988), however, the power of the sib pair method for the detection and estimation of the extent of heterogeneity is much lower than the lod method.

CHAPTER 2: MATERIALS AND METHODS

2.1 Clinical Materials and Methods

2.1.1 Ascertainment procedure of pedigree F24.

The index case, who has GTS, presented at the Neuropsychiatric Clinic at the National Hospital for Nervous Diseases together with his mother who told the clinic doctor that she knew of a few other members of the family with similar symptoms. These were interviewed, and it became clear that the family would be of interest for the study of the genetics of the illness and in particular for linkage analysis. At this point the decision was made to interview and take blood samples from as many as possible of the members of the pedigree. The pedigree was thus originally selected for study on the basis that it contained multiple cases, and as such could not be considered ideal for segregation analysis. Nevertheless, subsequently, subjects were included regardless of their affection status, and there did seem to be a uniformly high density of cases throughout the pedigree rather than only in those branches which had led to its selection.

2.1.2 Clinical assesment of pedigree F24.

As many as possible of the pedigree members were interviewed personally. Those individuals who were not available for interview but about whom information could be gained from relatives were assigned "best guess" diagnoses. The pedigree contained 122

individuals of whom 85 were interviewed. A further 28 were assigned diagnoses on the basis of information from relatives.

The individuals in pedigree F24 (figure 2.1) were evaluated over a two year period. Subjects were interviewed to assess "caseness" using a semi-structured interview (Robertson and Gourdie, 1989). Patients were assigned to diagnostic categories as follows:

1. Definite GTS; satisfying DSM III (American Psychiatric Association, 1980) criteria on history and examination.

2. Probable GTS; symptoms observed but no history obtained, or history obtained but no symptoms observed.

3. Definite CMT; DSM III diagnosis on both history and examination.

4. Probable CMT; as for probable GTS but without vocalizations.

5. Obsessive compulsive behaviour; obtained on history and in the case of children, corroborated by parents.

The pedigree with diagnostic information is shown in table 2.1 and figure 2.1.

Table 2.1: Associated behaviours and features of 50 cases compared to 29 non-cases in F24.

Associated behaviour/ feature	N°. of "cases"	N°. of "non-cases"	Significance between"cases" and "noncases"
Sleeping disturbance	32	11	N/S
"Evening-up"	16	0	P=0.0001
Forced to touch	11	0	P=0.0035
Echolalia	10	0	P=0.0055
Aggressive behaviour	10	1	N/S
Arithmomania	8	0	P=0.019
Echopraxia	8	0	P=0.0174
Self-injurious behaviour	6	0	N/S
History of stuttering	4	1	N/S
Mental coprolalia	3	0	N/S
Coprolalia	1	0	N/S
Copropraxia	1	0	N/S
Palipraxia	1	0	N/S
Palilalia	0	0	N/S

P=Fisher's exact probability test. (After Robertson and Gourdie 1990)

Figure 2.1: Pedigree diagram of F24 showing the diagnostic data used for the segregation analysis and also subsequent linkage analyses.

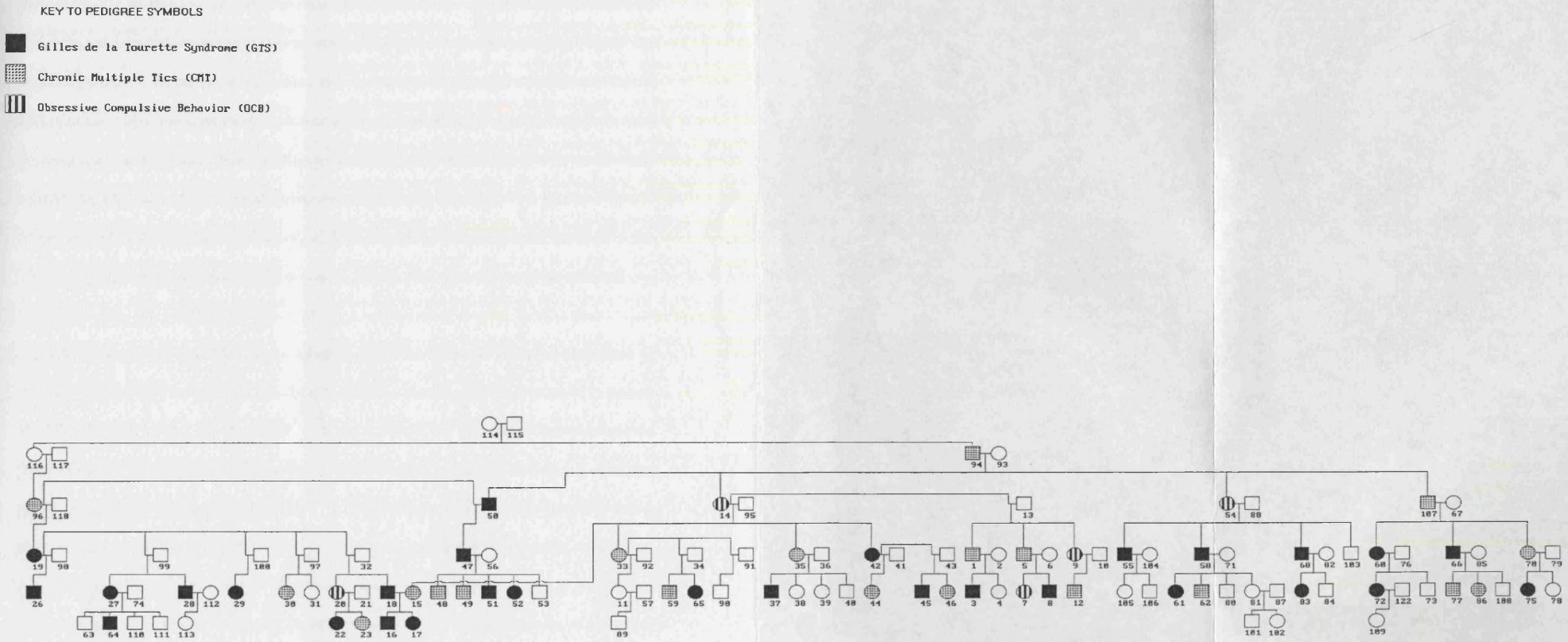


Figure 2.1: Pedigree diagram of F24 showing the diagnostic data used for the segregation analysis and also subsequent linkage analyses.

2.2 Methods used in segregation analysis.

In accordance with the procedure described by Morton et al (1983) the pedigree was divided up into nuclear families prior to analysis. Each nuclear family which did not have the original proband as a member was assigned a "pointer", this being defined as the affected person which had led to that family being included in the analysis. Because of the failure to use a systematic method for extending the pedigree the concept of a pointer was notional only. The proband was taken to be the pointer for the family of the most closely related affected case and then affected members of the latter's family were used as pointers to other families and so on until all cases had been included. Families were included in the analysis if they had an affected child or parent, but not if no members were affected.

Once the pedigree was divided into nuclear families the data was analysed according to the mixed model of Morton and MacLean (1974) as modified in the computer program POINTER (Lalouel and Morton 1981, Morton et al 1983). The mixed model proposes that affection results from the additive contribution of three factors: a random environmental component, multifactorial transmission (not necessarily genetic) and a single major locus effect. Each factor may or may not contribute to the liability to develop a particular disease. Multifactorial transmission may be due to a large number of independent factors, which may be polygenic or cultural or both, and results in a general tendency for the child to resemble the parent. Single major locus transmission occurs as a result of the inheritance of alleles at one particular locus. It is assumed that there are two alleles, a normal allele and a disease allele, and that the three possible genotypes at this locus (AA, Aa or aa) lead to significantly different liabilities to develop the disease.

Single major locus transmission thus depends on the discrete phenomenon of whether or not a particular allele is inherited from the parent, and as such it behaves somewhat differently from multifactorial transmission. For example there is a tendency for single locus effects to be "better preserved" than multifactorial effects as an extended pedigree is traversed - if a rare disease is due to multifactorial transmission then the risk to the relatives of secondary cases (those ascertained through a proband) is less than the risk to relatives of the proband, but if the disease is due to a single susceptibility allele then the risk to the relatives of all cases is the same.

POINTER allows constraints to be applied to the mixed model so that the likelihoods of different modes of transmission can be compared. If this is done with extended pedigrees (such as the one reported here) it is possible to test whether there is evidence for familial transmission, whether there is a evidence for a multifactorial component, and whether there is evidence for a single major locus component.

The pedigree was inspected and affection rates and sex distribution of the different syndromes were studied. The computer program POINTER was used to investigate different models of aetiology. Each analysis was performed taking either GTS or probable GTS as indicating affection, then was repeated taking GTS, probable GTS, CMT and probable CMT as indicating affection. The analyses were repeated using a wide range of different population prevalences and of sex ratios (male prevalence for GTS ranging from 10% to 0.05% and male:female ratio ranging from 1:1 to 5:1). On each occasion the prevalence for children of age seven or under was taken to be half the corresponding adult prevalence. To compare competing pairs of hypotheses iteration was performed for each

hypothesis and the log of the ratio of the maximum likelihood estimates of the two hypotheses was obtained. This was taken to approximate to a chi-squared statistic with the number of degrees of freedom equal to the number of constraints removed between one hypothesis and the next. The following pairs of hypotheses were compared:

1) No parent-child transmission against multifactorial transmission.

2) Multifactorial transmission against the mixed model (including a major locus effect and multifactorial factors).

3) Single gene (major locus) transmission against the mixed model.

4) Single gene recessive transmission against single gene dominant transmission.

The following pairs of hypotheses were compared using realistic values for population prevalence and sex ratio, and using the maximum likelihood estimates of other parameters derived from the earlier analyses:

1) Different gene frequencies producing either GTS alone on the one hand, or GTS and CMT on the other, against equal gene frequencies (i.e. possibly the same gene producing both illnesses).

2) Mendelian transmission probabilities against non-Mendelian probabilities.

The results of the former of these two analyses were also used to derive maximum likelihood estimates of gene penetrance. The second of the above analyses was performed as a test of goodness of fit.

2.3 Identification and investigation of a GTS patient with a balanced chromosome 3 to 8 translocation t(3:8)(p21.3 q14.3)

A GTS patient (BA) was identified in a clinic and because of the presence of physical dysmorphisms a cytogenetic abnormality was suspected. Following chromosome analysis the patient was found to be carrying a balanced translocation 46 XY, t(3:8) (p21.3 q24.1). Following this discovery a linkage study was performed as described in this thesis covering the areas on chromosomes three and eight implicated in the genetic aetiology of GTS in this isolated patient. There was little contact between the proband and his family when he was first identified, and it was not possible to investigate the relatives of the proband at this stage. However it did become apparent four years later that there were other cases of GTS in the family.

In order to study the regions involved in the translocation, 19 linkage markers on chromosome 3 and 19 linkage markers on chromosome 8 were used to test the hypothesis that there would be linkage at the loci implicated by the cytogenetic abnormality. Two point lod scores were calculated for each ~~probe~~ Marker. Multipoint lod scores, resulting from the recombinational data from several linked markers and the disease locus, were calculated in order to exclude large regions around the break-point.

After the initial finding (Brett et al 1990) suggesting possible linkage on chromosome 3 using three markers (THRB, E41 & RAF 1) the chromosomal region surrounding these loci was examined by using many more closely linked markers. Eleven markers were eventually used in constructing a series of FASTMAP lod scores. Chromosome walking was used to provide a more informative microsatellite marker at the THRB locus in the region of the original positive finding (Brett et al 1991).

Subsequently a blood sample was obtained from the patient carrying the translocation and a cell hybrid was produced which contained the abnormal chromosome 8 with the translocated section of chromosome 3p. The cell hybrids have been used to map the break-points relative to the probes used and it was possible to determine that the break point on chromosome 3 lies between the markers D3S1100 and D3S1312 and that the breakpoint on chromosome 8 the was between the markers D8S556 and D8S284.

2.4 Laboratory Methods

2.4.1 Sources of Chemicals

Ampicillin: Beecham Research Laboratories (Brentford, UK)

Calf intestinal phosphatase: Boehringer Corporation Ltd

Chemicals: Sigma Chemicals Ltd

Cetyl trimethyl ammonium bromide (CTAB): Sigma Chemicals Ltd

Ultra pure dATP, dCTP, dGTP, dTTP: Pharmacia Chemicals Ltd

DNA polymerase Klenow fragment: Boehringer Corporation Ltd

Fuji-RX film: Genetic Research Instruments

Hybond-N: Amersham International Plc

Kanamycin: Sigma Chemicals Ltd

SeaKem GTG agarose: Flowgen Instruments Ltd

Ultra pure LMP agarose: Gibco BRL

pUC18/19 DNA: Boehringer Corporation Ltd

mp18/19 M13 RF DNA: Boehringer Corporation Ltd

Restriction enzymes: Boehringer Corporation Ltd

T4 DNA ligase: Boehringer Corporation Ltd

ATP (special grade): Boehringer Corporation Ltd

T4 DNA polynucleotide kinase: Boehringer Corporation Ltd

Ampli-Taq (Taq pol): Cetus Perkin Elmer

Sequenase kit: United States Biochemicals Corporation

Femto-mol sequencing kit: Promega Corporation

Oligonucleotides synthesis: Oswel DNA services & HGMP resource centre (MRC)

Random hexanucleotides (pD6): Pharmacia Chemicals Ltd

Ribonuclease A: Sigma Chemicals Ltd

Sequagel kit: Flowgen Instruments Ltd

Agar: Difco laboratories

Yeast extract: Difco laboratories

Tryptone: Difco laboratories

Poly d(C-A).d(G-T): Pharmacia Chemicals Ltd

Poly d(C-T).d(G-A): Pharmacia Chemicals Ltd

DNA thermal cycler: Cetus Perkin Elmer

[alpha ^{32}P] dCTP 3000 ci/mMol: Amersham International Plc

[gama ^{32}P] ATP 3000 ci/mMol: Amersham International Plc

[gama ^{35}S] dATP NE0034S: Dupont NEN

2.4.2 Sources of probes

The human DNA minisatellite fingerprint clones were received from Professor A Jeffreys of Liecester University. The oligonucleotides used for priming the PCR of the microsatellites were produced by the MRC Human Genome Resource centre or Oswel DNA services.

2.4.3 Buffers and solutions

Ampicillin: prepared as a 100 mg/ml stock solution in water and stored at -20 °C. Used at a working concentration of 45ug/ml.

Blood lysis solution: 10 x, 100mM NaCl, 100mM EDTA pH approx 7.

Denaturization buffer: 1.5M NaCl, 0.5M NaOH

100x Denharts: 2% (w/v) bovine serum albumin, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) Ficoll 400 made up in water.

DNA loading buffer: 10 x, 0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll (type 400) in water.

Chloroform: chloroform and isoamyl alcohol mixed at a ratio of 24:1.

Ethidium bromide: solution of 10mg/ml in water. Stored 4°C.

IPTG: 100mM solution of isopropyl-B-D-thiogalactopyranoside (23.8 mg/ml of water). Store -20 °C.

Ligation buffer:10 x, 660mM Tris.Cl (pH7.6), 50mM $MgCl_2$, 50mM DTT, 6mM ATP. Store -20 °C.

Lysozyme buffer: 50mM glucose, 10mM EDTA, 25mM Tris.Cl (pH8.0). Lysozyme added to 4mg/ml.

Neutralization buffer: 1M Tris.Cl (pH7.6), 1.5M NaCl.

Proteinase-K buffer: 100mM NaCl, 10mM Tris.Cl (pH8.5), 25mM EDTA.

Phage lysis buffer: 2.5% SDS, 0.5M Tris.Cl (pH9.0), 0.25 M EDTA.

Phenol: dissolved at 65°C, saturated with water and then equilibrated with TE pH7.6.

Spermidine: 100mM solution in water. Store -20 °C.

SM buffer: 100mM NaCl, 10mM $MgSO_4$, 50mM Tris.Cl (pH7.5), 0.01% gelatin.

SSC: 20 x, 3M NaCl, 0.3M tri-sodium citrate.

T4 polynucleotide kinase buffer: 800mM Tris.Cl (pH7.6), 120mM $MgCl_2$, 60mM dithiothreitol (DTT), made up in water and stored at -20 °C.

TAE: 40mM Tris.Cl, 1mM EDTA in water, pH adjusted to 8.0 with glacial acetic acid.

Taq polymerase buffer: 10 x, 100mM Tris.Cl (pH8.3), 500mM KCl, 20mM $MgCl_2$, 0.1% gelatin. $MgCl_2$ varied in concentration from 1.0mM-2.5mM for different amplifications. Stored -20 °C

TBE: 90mM Tris, 90mM Boric acid, 1.25mM EDTA, pH8.3.

TE: 10mM Tris.Cl, 1mM EDTA in water, pH 7.5-9.0 as stated.

X-Gal: 2% solution made up by dissolving 20mg of 5-Bromo-4-chloro-3-indoly-B-D-galactoside in 1ml of dimethyl formamide. Store -20 °C.

2.4.4 Media

Glucose/minimal medium plates: 1.5% (w/v) minimal agar, 1 x M9 salts, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 1mM thiamine HCl, 0.2% glucose.

Luria Bertani medium (LB): 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 1% (w/v) sodium chloride made up in water and adjusted to pH7.5.

L-agar: 1.5% (w/v) Difco technical agar in LB.

H-agar: 1.2% (w/v) Difco technical agar in 1% bacto tryptone, 0.8% sodium chloride.

H-top agar: 0.5% agarose in 1% (w/v) bacto tryptone, 0.8% sodium chloride.

10 x M9 salts: per litre, 60g Na_2HPO_4, 30g KH_2PO_4, 10g NH_4Cl, 5g NaCl, stored at 4°C.

2 x TY: 1.6% (w/v) bacto tryptone, 1% (w/v) bacto yeast extract, 0.5% (w/v) sodium chloride made up in water.

All media were sterilized by autoclaving at 120°C for 20 minutes.

2.4.5 Southern Blotting and Hybridization analysis of Genomic DNA

a) Isolation of genomic DNA from peripheral lymphocytes

The peripheral lymphocytes from 10mls of blood were lysed in 40mls of 1 x blood lysis solution. The nuclei were pelleted by centrifugation at 2000g for 10 minutes, resuspended in 40mls of 1 x blood lysis solution and pelleted. The cleaned nuclei were resuspended in 10mls of proteinase-K buffer and the nuclear membranes lysed with 1ml of 10% SDS (sodium dodecyl sulphate). Protein digestion was carried out with proteinase-K at 55°C for 16-20hrs. The DNA was extracted with an equal volume of phenol and chloroform, and precipitated with one tenth the volume of 3M sodium acetate and 2.5 x the volume of absolute ethanol. The DNA was resuspended in 0.5mls of TE and dissolved at 4°C. The DNA concentration was measured by its absorbance at 260nm (OD of 1 equals 50ug/ml),and the samples equilibriated to 5ug/20ul.

b) Digestion of genomic DNA with restriction enzymes

5ug of genomic DNA was digested with 20 units of a restriction enzyme, at the temperature recommended by the manufacturer with the addition of 1mM spermidine and relevant buffer. The DNA was digested to completion overnight.

c) Southern blotting of genomic DNA and hybridization

Southern blotting (Southern 1975) was carried out using a modification of the protocol described by Amersham International plc for use with Hybond-N filter membranes. Digested DNA was separated by electrophoresis through 0.6-1% TAE agarose gels at 50V until the appropriate alleles had resolved (usually 18-48hrs). The gel was stained with ethidium bromide, photographed on a UV transilluminator and denatured with denaturization buffer for 1hr. Transfer to Hybond-N membrane was carried out overnight in 20 x SSC after which the DNA was covalently linked to the membrane by UV irradiation for 2 minutes. Membranes were prehybridized at 65°C in 20mls of 0.9M NaCl, 1% SDS and 50ug/ml autoclaved denatured salmon testes DNA for a minimum of four hours. Hybridization was carried out overnight at 65°C in 20mls of fresh solution including 10% (w/v) dextran sulphate as well as 100ng denatured oligolabelled probe at 2 x 10^6 cpm/ml. The membranes were then washed to a stringency of 1 to 0.1 x SSC, 0.1% SDS at 65°C. The filters were exposed against autoradiographic film at -70°C using intensifying screens. In order to reprobe the filters were treated as recommended by the manufacturer.

2.4.6 DNA Fingerprints using the minisatellite Lambda 33.6 probe

a) Probe preparation

The M13 probe DNA was prepared as for single stranded sequencing. The probes were labelled according to Hu and Messing (1982), 100ng of M13 single strand DNA was

hybridized with 2ng of M13 hybridization primer (Amersham International plc) at 65°C for 30 minutes in 10mM dithiothreitol,10mM Tris.HCl pH7.9, 60mM NaCl, and 6.6mM $MgCl_2$. The primer hybridizes after the polylinker. This was then cooled to RT before dATP, dGTP, and dTTP were added to a final concentration of 50uM each along with 3ul of [alpha32P] dCTP (3000Ci/mM). This was incubated in the buffer above with 1U of DNA polymerase Klenow fragment for 90 minutes at 15°C. The reaction being inhibited by the addition EDTA pH8.3 to 25mM, and the probe stored on ice until used.

b) Hybridizations

Genomic DNA was digested with HinfI overnight to completion. 5ug was size separated by electrophoresis in 1x TBE 24cm agarose gels run at 1 volt/cm with several changes of the buffer. The gels were run until the 4kb marker was within 2cm of the end of the gel. The gels were denatured for 1hr then neutralized for 1hr before the DNA was Southern transferred in 20 x SSC overnight onto Hybond-N. The DNA was UV fixed for 2 minutes. Hybridizations were carried out according to Jeffreys et al (1985). The Hybond-N filters were prehybridized at 65°C overnight in 1 x SSC, 5 x Denharts, 0.5% SDS and 1mg denatured herring sperm DNA (Sigma Chemical company). Hybridizations were in the same conditions but with 5% PEG(6000) overnight. Post hybridization washes were to a stringency of 1 x SSC, and autoradiography overnight at -70°C. Paternity of the families was checked by eye.

2.4.7 Plasmid and Cosmid Vector Preparations

solution 1: 50mM glucose, 25mM Tris-Cl and 10mM EDTA.

solution 2: 0.2M NaOH, 1% SDS.

solution 3: 147.2g Potassium acetate, 57.5 mls Glacial acetic acid. Resulting solution 3M Potassium ions and 5M acetate ions.

5ml overnight cultures prepared from single bacterial colonies were used to inoculate 500mls of sterile LB containing the appropriate antibiotic and grown overnight at 37°C with vigorous shaking. The bacteria were pelleted at 4200 rpm for 30 minutes at 4°C in a Beckman J6-B centrifuge and then resuspended in 50mls of solution 1. The cells were lysed by adding 100mls of solution 2 and the bacterial debri and chromosomal DNA was precipitated with the addition of 50mls of solution 3. After mixing, the precipitate was pelleted by centrifugation at 4200 rpm for 15 minutes at 4°C. The supernatant was filtered through nylon gauze and the plasmid DNA and bacterial RNA precipitated by adding 120mls of propan-2-ol. After centrifugation at 6000 rpm in a sorval GS3 rotor, the pellet was washed with 70% ethanol and resuspended in 5mls of TE pH8.0 before being transferred to a pre-weighed universal and made to 9g with TE pH8.0. To this was added 10g caesium chloride and 1ml of 5mg/ml ethidium bromide. The plasmid DNA was separated from the RNA according to density by centrifugation for 24-48hrs in a Beckman 70Ti rotor at 55000rpm at 20°C. The lower plasmid band was extracted from the gradient and made to 10mls with TE pH8.0. Plasmid DNA was precipitated with 20mls of absolute ethanol at room temperature and pelleted by centrifugation at 3000rpm for 20 minutes in a Beckman J6-B centrifuge. After washing with 70% ethanol and drying the DNA was

resuspended in 500ul TE and treated with 40U/ml RNase A for 15 minutes at 37°C. The protein was then extracted with phenol/chloroform and the cleaned purified plasmid DNA precipitated with ethanol.

2.4.8 Large Scale Phage DNA Preparation

Solutions:10% (w/v) maltose filter sterilized, 1M $MgSO_4$ (autoclaved),
3M Sodium Acetate pH5.2, PEG 6000 50% (w/v) mix overnight to dissolve fully.

The host bacteria (LE392) was grown overnight in 10mls of LB media with 10mM $MgSO_4$ and 0.2% maltose. The cells were pelleted by centrifugation and resuspended in 10mls of 10mM $MgSO_4$ and stored at 4°C for a maximum of three weeks. The optical density at 600nm was measured (OD 1=8 x 10^8 bacteria/ml). The titre of the phage lysate was also determined. In a total volume of 200ul containing 10mM $MgSO_4$ 10^{10} bacteria were incubated with 10^7 phage at 37°C for 20 minutes. After the absorption of the phage, the mixture was added to 500mls of LB, 10mM $MgSO_4$ and 0.2% maltose (in a smooth sided flask) and shaken vigorously overnight at 37°C. The flask was shaken for a further 30 minutes with 1ml of chloroform and the cell debris settled for 30 minutes. The lysate was removed leaving the chloroform behind and centrifuged at 8K and 4°C for 10 minutes (sorval GS3 rota). The cleared lysate was decanted into a clean flask and bacterial DNA and RNA were digested with 20ul of pancreatic DNase (20mg/ml) and 10ul pancreatic RNase (20mg/ml) by incubation at 37°C for 30 minutes. Sodium chloride was added to a final concentration of 1M dissolved and left on ice for 30 minutes after which 100mls of 50% PEG 6000 solution was added, mixed thoroughly and left on ice overnight to

precipitate the phage. The phage and other debris were then pelleted at 8K and 4°C for 10 minutes (sorval GS3 rota). The phage were then extracted with 3mls of SM buffer and spun at 8K for 2 minutes at RT (sorval HB4). The supernatant was collected and the remaining pellet was extracted once more with 1.5mls of SM buffer. To 0.5mls of the combined supernatants 5ul of 10% SDS and 10ul 0.25 M EDTA were added and the phage coats ruptured by incubation at 68°C for 15 minutes. This was extrated with an equal volume of phenol, then phenol chloroform and finally chloroform. The phage DNA was ethanol precipitated in the usual way.

2.4.9 Plasmid and Cosmid mini-preps with CTAB

The method described is a modification of Del Sal et al 1988. The bacterial cells from 1.5mls of an overnight culture were pelleted in an Eppendorf microfuge, and resuspended in 200ul of STET buffer (8% sucrose w/v, 0.1% TritonX-100 v/v, 50mM EDTA, 50mM Tris-HCl pH8.0). After the addition of lysozyme to break down the bacterial cell wall (4ul of 50mg/ml RT for five minutes) the samples were boiled for 45 seconds to precipitate proteins and cell debris. These were pelleted by centrifugation for 10 minutes and the supernatant removed to a fresh microfuge tube. The plasmid DNA was precipitated with 8ul CTAB (5% w/v) and centrifuged for 5 minutes at RT. The pellet was then resuspended in 300ul of 1.2M NaCl and the DNA reprecipitated with the addition of 750ul of absolute ethanol. The purified plasmid DNA was collected by centrifugation for 10 minutes, washed in 70% ethanol air dried and dissolved in 50ul of TE. For double stranded plasmid sequencing this DNA was further purified by several rounds of phenol/chloroform extractions.

2.4.10 Plasmid ligations

After restriction endonuclease digestion of vectors, an aliquot was run on a 0.8% TBE agarose gel to confirm that restriction of the DNA had gone to completion. The restriction endonuclease was inactivated at 65°C for 10 minutes. If the restricted ends were incompatible, the plasmid vector was precipitated with propanol and ammonium acetate (Maniatis et al 1982), otherwise the vector was treated with calf intestinal phosphatase (15-20U) for 60 minutes at 37°C then phenol and chloroform extracted and precipitated with absolute ethanol. Inserts were separated and purified in low melting point agarose in TAE, the correct band being visualised on a UV transilluminator and excised, for the NMDA receptor the fragment was treated with agarase to remove the agarose. 100ng of insert was ligated to 20ng of vector as a general rule in 1 x ligation buffer with 1U of T4 DNA ligase and the agarose diluted to 0.1%. Ligations were carried out overnight at RT.

2.4.11 Preparation of competent E.Coli cells

Bacteria were grown in 50 mls LB, 10mM $MgSO_4.7H2O$ and 0.2% glucose to mid logarithmic phase (OD 550nm of 0.4 for JM83 Rec-, and 0.3 for TG2). The cells were then left on ice for 10 minutes and then gently centrifuged at 1500g for 10 minutes at 4°C. The pelleted cells were resuspended in ice cold 0.5mls of the above solution, to this 2.5mls of 36% glycerin, 12% PEG (MW7500), 12 mM $MgSO_4.7H2O$ added to LB and sterilized by filtration, was added and mixed well without vortexing. The cells were aliquoted and stored at -80°C for up to 3 months.

2.4.12 Transformation of DNA into competent E.Coli

5ng of ligated vector was added to 100ul of competent cells and left on ice for at least 1hr. The bacteria were then heat shocked at 42°C for 3 minutes and left on ice. For plasmids an equal volume of 2 x LB was added and the cells incubated for 1hr to allow expression of the antibiotic resistance. The bacteria were briefly pelleted (15 seconds) and 1/10 and 1/100 dilutions were prepared in LB and spread onto an LB agar plate with the appropriate antibiotic and incubated overnight at 37°C. For PUC plasmid vectors X-Gal and IPTG were added to the agar to allow blue (non recombinants) white (recombinants) selection.

2.4.13 Random primer labelling of DNA probes

The method of Feinberg et al (1983) was used to label purified fragment DNA in low melting point agarose. 100ng was denatured by boiling for 7 minutes and quenched on ice before 10ul of labelling buffer, 30uCi of [alpha^{32}P] dCTP and 2 units of Klenow fragment were added to a final volume of 50ul. The reaction was incubated at 37°C for 1-2hrs. 50ul of TE buffer was added and the unincorporated radionucleotide removed by centrifugation (Beckman GRI) 2500rpm for 5minutes through a Sephadex G50 medium column. The incorporation was measured by Cherenkoff counting.

2.4.14 Competition of Human repeat containing probes

Probes that contain human repeat elements need competition with total human DNA before hybridization to genomic southerns. After the spun column probes were made up to a total volume 200ul with TE pH7.6 and to this 80ul of 1.2M sodium phosphate and 500ul of 2.5mg/ml sonicated human placental DNA were added. This mixture was boiled for 10 minutes and incubated for 5-8hrs at 65°C before being added directly to the hybridization solutions.

2.4.15 5' End labelling of Oligonucleotides

Oligonucleotides (25ng) were end labelled by T4 polynucleotide kinase in the presence of 120uCi of [gama^{32}P] ATP and 1 x kinase buffer in a total volume of 30ul. After 1hr at 37°C, the labelled probe was separated from the unincorporated isotope by Sephadex G50 (medium) spun column.

2.4.16 Hybridisation of Genomic Lambda Libraries

The library was titrated in a suitable host (LE392 for Clontech EMBL3 Sau3A1 partial HL1067j genomic library). 30,000 phage were plated out per 15cm petri dish and a total of 20 plates were prepared such that 4-5 genomes were screened (Clarke and Carbon 1976). The phage were made up to a total volume of 300ul with SM buffer and to this 300ul of $MgSO_4$ stock host cells were added and the mixture incubated at 37°C for 15-20 minutes for absorbtion to occur. This was mixed with 7mls of molten (45°C) top agarose

10mM $MgSO_4$ and poured directly onto LB agarose 15cm plate. The phage plaques were incubated overnight at 37°C (or until a near confluent phage plate had grown). Plaque lifts were taken directly onto Hybond-N and the plates stored at 4°C. Hybond-N nylon filters were laid directly onto the plates and left for 20-30 seconds and then carefully removed and placed onto filter paper soaked in denaturization solution for 5 minutes to denature the DNA. After neutralizing for a further five minutes the filters were washed gently in 2 x SSC and air dried. The DNA was fixed by baking at 80°C for 2hrs. Hybridizations were carried out in the same way as used for southern blots, with roughly seven filters per hybridization bag containing 100ng of labelled probe. After autoradiography (-70°C) usually overnight the positive clones were picked with the end of a sterile Pasteur pipette and stored in 500ul of SM buffer at 4°C. The filters were incubated at 75°C for 2hrs to remove the probe and were reused up to six times. Secondary screens were carried out by plating 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the SM stock using 200ul of plating cells per 9cm petri dish. Plaque lifts and hybridizations were as above. Further screens were carried out at 10^{-3}, 10^{-4} and 10^{-5} dilutions until a plate contained none but the purified clone. Plaques from these plates were stored in SM buffer at 4°C. Large scale phage DNA preparations of these clones were carried out.

2.4.17 Hybridisations of Cosmid Genomic Libraries

The cosmid glycerol stock was titrated. The volume equalling 30,000 colonies was added to 500ul of LB and spread onto a dried LB agarose 15cm plate containing the appropriate antibiotic (kanamycin for pcos2EMBL). The plates were incubated overnight at 37°C, and 15 plates were prepared representing approximately six genomes. Hybond-N filters were

placed onto the colonies and carefully lifted so as not to spread the bacterial colony. The plates were stored at 4°C. The filters were denatured for 5 minutes and then neutralized for a further 5 minutes before being gently washed in 2 x SSC. The filters were air dried and baked at 80°C for 2hrs. The filters were prehybridized at 65°C with 1mg of proteinase K overnight to remove the cellular debris (five filters per bag), rinsed and prehybridized again before hybridization as described above. Positive clones were picked with a sterile Pasteur pipette and stored in 500ul of LB at 4°C after a brief incubation at 37°C (5-10 minutes). Secondary screens were carried out on serial dilutions of the stock by plating 10^{-2} to 10^{-5} dilutions out on 9cm plates. Further screens were performed until a positive clone well isolated from other colonies could be picked. Mini and large scale preparations of cosmid DNA were carried out.

2.4.18 Shotgun cloning and sequencing of Microsatellites

a) Shotgun ligations

200ng of phage or cosmid DNA were restriction digested with Sau3A, AluI and HaeIII and combinations of these three. The digested clones were size separated in 1% agarose gels and southern blotted onto Hybond-N. The clones were screened for the presence of dinucleotide repeats using oligolabelled poly (dC-dT).(dG-dA) and poly (dC-dA).(dG-dT). Prehybridization of the filters was in 0.9M NaCl, 1% SDS and 40ug/ml tRNA at 65°C for at least 1hr. Hybridizations were carried out in the same buffer with the labelled probe overnight at 65°C. The filters were washed to a stringency of 1 x SSC at 65°C. Positive clones gave an autoradiographic signal after 1hr RT exposure to X-ray film. The

genomic clones were digested such that the microsatellite containing fragment was approximately 200-300bp. 100ng of this DNA was ligated to 100ng of prepared M13 mp18 vector DNA (prepared as plasmid vectors), BamHI cut mp18 for Sau3A ligations and HincII cut vector for AluI or HaeIII ligations. Ligations were carried out overnight at RT, in a total volume of 30ul.

b) Identification of microsatellite containing clones

The ligations were diluted to a volume of 100ul with TE pH7.6, and 10ul of this was added to 100ul of competent TG2 cells (grown originally on glucose minimal medium plates to express the F-pilus) and left on ice for a minimum of 1hr. After heat shocking for 3 minutes at 42°C, this was added to 100ul of logarithmic phase TG2 cells, 40ul X-gal, and 40ul IPTG mixed with 3mls of H-top agar poured onto warmed LB agar 9cm plates. The plates were incubated at 37°C overnight, clear plaques indicating recombinants (normally 100-200 recombinants/plate). Five plates for each genomic clone were routinely plated, and plaque lifts taken onto Hybond-N. The filters were denatured, neutralized and washed in 2 x SSC, air dried and finally UV fixed for two minutes. Recombinants containing microsatellites were identified by hybridization as described above. After 2hrs exposure to X-ray film positive M13 plaques could be identified. Alu elements with potential 3'poly deoxyadenylate tracts were identified by hybridizing Sau3A ligations with end labelled oligonucleotides representing the conserved regions of these elements. Hybridizations were carried out at 55°C in the same buffer as for dinucleotide repeats, with the filters washed to a stringency of 2 x SSC at RT. Positive clones were identified on X-ray film after overnight exposure.

c) Sequencing the microsatellites

A modification of the protocol described by Amersham in the booklet "M13 cloning and sequencing" was used to prepare single stranded DNA. An overnight culture of E.Coli TG2 was diluted 1:100 with 2 x TY medium and 1.5ml aliquots inoculated with a positive M13 plaque. After incubating with shaking for 6hrs at 37°C, the phage released into the medium was separated from the bacteria by centrifugation for 5 minutes in a microfuge. 1ml of the supernatant was removed and transferred to a new eppendorf tube. Phage particles were precipitated by addition of 200ul of ice cold 2.5M NaCl, 20% (w/v) polyethylene glycol 6000 (PEG) on ice for 15 minutes and pelleted by spinning in a microfuge for 5 minutes. All traces of PEG were removed from the phage pellet before resuspending in 100ul of TE pH7.6. The viral particles were lysed by extracting with phenol, then phenol/chloroform and finally chloroform, and the DNA template precipitated with ethanol overnight at -20°C before being finally washed, air dried and resuspended in 30ul of TE pH7.6. A check gel was run with about 1-2ul of phage DNA to ensure the presence of DNA for sequencing. DNA sequencing was performed by the chain termination method originally devised by Sanger et al (1977) and detailed by United States Biochemical Corporation for use with their Sequenase kit. Sequencing reactions were labelled with [^{35}S] dATP and run on 6% polyacrylamide gels in 1 x TBE for 2-18hrs. Gels were then fixed in 10% acetic acid and 10% methanol before drying at 80°C for 30 minutes and exposing to X-ray film at RT without intensifying screens. 3'Alu element ends were sequenced using the same protocol but replacing the primer with oligonucleotides representing the conserved regions of the elements.

2.4.19 Double stranded Plasmid sequencing

Plasmid DNA to be sequenced directly was prepared by the large scale preparation or more convenient CTAB mini preparation. 5ug of plasmid DNA was added to an equal volume of freshly prepared 0.4M NaOH and left at room temperature for 10 minutes. Denatured DNA was precipitated by addition of 0.1 volume 3M sodium acetate and 4 volumes ice-cold absolute ethanol and left at -70°C for 20 minutes. The DNA was recovered by centrifugation in a microfuge for 10 minutes, washed in 70% ethanol and air dried. The pellet was resuspended in 35ul of water and 7ul used in the sequencing reaction. An adaption of this process was used to sequence the NMDA clone using PCR cycling with the femto-mole sequencing kit from the Promega Corporation, and was performed on both the original lambda clone and also on the PvuII subclone PMB3.

2.4.20 PCR visualisation of microsatellites.

PCR was performed in 12.5 ul containing: 50 ng DNA, 12.5 pmoles of each primer, 1.0 mM $MgCl_2$, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 25 uM dATP and 200 uM of other dNTPs, 1 unit Taq polymerase (Perkin-Elmer/Cetus), 0.01% gelatin and 0.5 ul of ^{35}S-dATP at 500 Ci/mmol (Dupont). End labelled primers were also used for some microsatellite amplifications (100 pmoles of primer labelled in 10ul with 1ul gamma-^{32}P ATP at 3000Ci/mmol, 0.1 ul of this was used per reaction). Amplification was for 35 cycles with denaturation at 94°C, annealing at the appropriate temperature for the primers and 20 seconds extension at 72°C. The amplified product was separated by polyacrylamide gel electrophoresis using a urea denaturing gel. The gels were fixed in

10% acetic acid /10% methanol and vacuum dried on to 3mm paper. The gels were autoradiographed using Fuji X-ray film at -70 °C for 1 hour to 2 days depending on the primers used.

2.5 Method for making candidate or favoured loci informative for linkage analysis.

2.5.1 Genomic cloning and localisation of the NMDAR1 gene.

In order to follow the candidate gene approach in linkage analysis for GTS a polymorphic microsatellite marker for the NMDA (N-methyl-D-aspartate) receptor was sought. A cDNA clone of the NMDAR1 (isoform E)(Le Bourdelles et al 1993) gene was used to screen a genomic phage library and 3 different genomic cosmid libraries in order to find genomic clones in which a polymorphic marker for the locus could be found.

2.5.2 Increasing informativeness at the favoured locus on chromosome 3

Following the finding of a positive, but non-statistically significant, lod score of 2.9 on chromosome 3 (Brett et al 1990) more information from this locus was needed. Therefore the strategy of chromosome walking was used in order to find new polymorphisms in the region of interest. Chromosome walking allows the isolation of sequences whose functions are unknown but whose location is known. In this case the location for the chromosome walking was determined by the position of the positive lod score on chromosome 3. The aim of the walking was to increase the information available in this region for linkage calculations. Simple dinucleotide repeat polymorphisms consisting of CA_n or CT_n were sought. In addition a search for RFLPs was conducted by hybridising against a panel of genomic DNAs cut with 20 restriction enzymes. A major problem with using some RFLPs for genetic linkage is the limited amount of information obtained as a result of the limited heterozygosityof many polymorphic sites (Botstein et al 1980). The

easiest method of gaining more information in a single family is to increase the number of informative meioses for a specific locus and this usually entails finding a marker with a higher polymorphism information content (PIC).

A human genomic library in EMBL3 (Clontech) was screened with a human repeat free fragment probe of pBH302 at locus THRB (Gareau et al 1988). Chromosome walking identified a clone, lambda THRB-5. One Alu I fragment of lambda THRB-5, subcloned in M13, hybridised to poly(dC-dA). Sequencing of this subclone identified a $(GT)_n$ repeat which detects 5 alleles. The subclone was designated THRB-5GT. Flanking sequences were used to design PCR primers to amplify the repeat sequence.

To visualise the microsatellite PCR is performed in 12.5 ul containing: 50 ng DNA, 12.5 pmoles of each primer, 1.0 mM $MgCl_2$, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 25 uM dATP and 200 uM of other dNTPs, 1 unit Taq polymerase (Perkin-Elmer/Cetus), 0.01% gelatin. For this marker primer 1 was end labelled (100 pmoles of primer labelled in 10ul with 0.5ul of polynucleotide kinase and 1ul gamma-^{32}P ATP at 3000Ci/mmol, 0.1 ul of this was used per PCR reaction). Amplification is for 35 cycles with denaturation at 94°C, annealing at 57°C and extension at 72°C, all for 1 minute each.

2.6 Methods used for Linkage analysis

2.6.1 Phenotypes, Parameters and Calculations

For the purpose of the linkage analysis calculations, patients were assigned to the three following diagnostic categories as opposed to the five used for the segregation analysis described on page 47 chapter 1:

1. Definite or probable GTS; satisfying DSM III (American Psychiatric Association, 1980) criteria on history and/or examination.

2. Definite or probable CMT; DSM III diagnosis on history and/or examination.

3. Obsessive compulsive behaviours without GTS or CMT; obtained on history and, in the case of children, corroborated by parents.

Based on the results of a previous segregation analysis (Curtis et al 1992) autosomal transmission was assumed with a gene frequency of 0.0005, and heterozygote penetrances of 0.5 for GTS and 0.88 for GTS and CMT. To allow for occasional phenocopies, the normal homozygote penetrance was set to 0.001. The normal homozygote penetrances were later changed to 0.01 for GTS and 0.05 for GTS/CMT, making the calculations more conservative.

Table2.3: Showing the parameters used for lod score calculations.

	Homozygous Unaffected		Homozygous Affected
	faa	faA	fAA
GTS	0.01	0.50	0.50
GTS & CMT	0.05	0.88	0.88

Two diagnostic categories were used to indicate positive affection status, GTS only and GTS & CMT. No separate analysis was carried-out including the OCB cases as affected, because four out of five of the OCB cases were obligate carriers under the assumption of dominant transmission. Two-point and multipoint lod scores were calculated with the LINKAGE package of programs (Lathrop et al 1984) for the polymorphic markers. FASTMAP, a programme that combines lod scores on a fixed map taking into account the number of informative meioses for each marker (Curtis and Gurling 1993), was used to produce approximate multipoint maps for the exclusion of chromosomal regions examined. FASTMAP has the advantage over multipoint mapping in having much less requirement for computational time. However since FASTMAP is only an approximation to a multipoint, some three-point analyses were performed across regions where two flanking markers were used to obtain exclusions.

2.6.2 Lists of Polymorphic Markers used for Linkage Analysis of the Candidate Genes and Favoured Loci

Table 2.3:- Linkage markers and gene clones their location and allele frequencies used in the exclusion of candidate genes encoding serotonergic and catecholaminergic related proteins.

Probe	Locus	Chromosomal location	Alleles	Frequency
G-21	HTR1A	5cen-q11	A1	0.78
			A2	0.22
L599Ha	D5S76	5q11.2	A1	0.32
			A2	0.16
			A3	0.5
			A4	0.02
P599GT(n)	D5S76	5q11.2	A1	0.021
			A2	0.213
			A3	0.213
			A4	0.127
			A5	0.064
			A6	0.362
MNSs	MNS	4q31	M	0.53
			N	0.47
			S	0.31
			s	0.69
c-Ha-RAS1	HRAS	11p15.5		
THCA	TH	11p15.5		
pMS51	D11S97	11q13		
D2-5/D2-6	DRD2	11q22-q23	A1	0.15
			A2	0.47
			A3	0.22
			A4	0.16
DBH-CA	DBH	9q34.3		
HGR213-1	DRD1	5q34-35	A1	0.90
			A2	0.10
Mfd154CA	D5S211	5q33.3-qter		
D5CA	DRD5	4p16		
L214	DRD3	3q13.3	A1	0.72
			A2	0.28

The number of alleles was reduced to five in cases where markers had six or more alleles. A three-point analysis was performed between affection status HRAS and TH to test for linkage with the D4 locus (Gelernter et al 1992). A second three-point analysis was performed between affection status, DRD2 and D11S97 to exclude tyrosinase (Barton et al 1988) and a third three-point analysis was performed between affection status, DRD1 and D5S211 to test for linkage between the D1 gene (Wasmuth et al 1991) and GTS.

Table 2.2: The polymorphic linkage markers used to screen for linkage on chromosomes 3 and 8 with their locus symbols and allele frequencies used in linkage analysis.

Chromosome 3 linkage markers

Probe	Locus	Chromosomal location	Alleles	Frequency
DR82	D3S6	3pter-p21	A1	0.78
			A2	0.22
p627	RAF1	3p24	A1	0.74
			A2	0.26
L162-1	D3S18	3p21-24	A1	0.50
			A2	0.50
pBH302	THRB	3p21-24	A1	0.33
			A2	0.67
THRB-5GT	THRB	3p21-24	A1	0.07
			A2	0.08
			A3	0.33
			A4	0.42
			A5	0.10
pH3H2	D3F15S2E	3p21	A1	0.46
			A2	0.54
pH3E4	D3F15S2E	3p21	A1	0.50
			A2	0.50
pHF12-32	D3S2	3p21	A1	0.83
			A2	0.17
LIB12-37		3p21	VNTR	
R59A	D3S12	3p21-14	A1	0.33
			A2	0.33
			A3	0.33
R59B	D3S12	3p21-14	B1	0.50
			B2	0.50
E-41	D3S11	3p21-14	A1	0.08
			A2	0.69
			A3	0.23
	D3S1312	3p14.2-14.3	A1-A8	0.125
	D3S1284	3p13-p12	A1-A10	0.10
L892	D3S17	3p11	A1-A5	0.2

	D3S1281	3cen-q13	A1-A7	0.142
(phCP1)	CP	3q23-25	A1	0.39
			A2	0.61
DR-2	D3S5	3q21-qter	A1	0.31
			A2	0.69
Mfd17	D3S196	3q22-qter	A1	0.06
			A2	0.16
			A3	0.32
			A4	0.03
			A5	0.04
			A6	0.37
			A7	0.02

Chromosome 8 linkage markers

Probe	Locus	Chromosomal location	Alleles	Frequency
pHNFL	NEFL	8p21	A1	0.62
			A2	0.38
pSW508	D8S7	8p23	A1	0.84
			A2	0.16
pBS8.9	D8S11	8p23	A1	0.90
			A2	0.10
ptPA4352	PLAT	8p12-q11.2	A1	0.54
			A2	0.46
Puc9-2F	POLB	8pter-q22	A1	0.50
			A2	0.50
pCA15	CA3	8q13-22	A1	0.50
			A2	0.50
H25-3.8	CA2	8q22	A1	0.50
			A2	0.50
pHT 0.96	TG	8q24	A1	0.15
			A2	0.85
pCHT16	TG	8q24	A1	0.20
			A2	0.80
IRI-ULB	TG	8q24	A1	0.50
			A2	0.50
pMCT128.2	D8S39	8q	VNTR	
PyNM3	D8S38	8q	VNTR	

EMBL3.287	D8S8	8q	A1	0.41
			A2	0.53
			A3	0.03
			A4	0.03
Mfd31	Penk	8q23-24	A1	0.01
			A2	0.42
			A3	0.54
			A4	0.02
			A5	0.01
	D8S556	8q	A1-A8	0.125
	D8S284	8q	A1-A8	0.125
	D8S554	8q	A1-A9	0.111
	D8S272	8q	A1-A10	0.100
	D8S373	8q	A1-A6	0.166

For the lod score calculations markers with six or more alleles the number of alleles was reduced to five. The allele frequencies for many of the microsatellite markers and the markers compressed to five alleles were set to be equal, this has the disadvantage of inflating positive lod scores but will not affect the exclusions obtained.

CHAPTER 3: Segregation Analysis of Pedigree F24, Power Calculations and Investigation into a Translocation.

3.1.1 Results of Segregation anlysis

Tables 3.1 and 3.2 demonstrate that the male to female ratios for the prevalence of both GTS and CMT in our sample were approximately equal, with only a slight male excess. Goodness of fit tests showed that the true male to female ratiosfor the prevalence of GTS might be as high as 2:1, but are rejected at $p<0.001$ for a ratio of 3:1 or higher.

Table 3.1: Distribution by sex of Gilles de la Tourette syndrome in F24

	Male	Female	
GTS	16.0 15%	13.0 12%	29.0 27%
No GTS	44.0 41%	34.0 32%	78.0 73%
	60.0 56%	47.0 44%	107.0

Chi-squared = 0.030, 1 df, NS

Table 3.2:Distribution by sex of definite and probable chronic multiple tics in F24

	Male	Female	
GTS + CMT	26.0 24%	23.0 22%	49.0 46%
Neither	34.0 32%	24.0 22%	58.0 54%
	60.0 56%	47.0 44%	107

Chi-squared = 0.407, 1 df, NS

Table 3.3 shows that there was a slight, non-significant excess of women with

obsessive-compulsive symptoms. All 5 of the subjects who had a diagnosis of OCB or probable OCB without GTS or CMT were female. Since this is in line with previous work suggesting that OCB alone is more common in female than male relatives of patients with GTS, this finding has a one-tailed significance of 1/32, p=0.031.

Table 3.3: Distribution by sex of definite and probable OCBin F24

	Male	Female	
OCB	10 9%	13 12%	23 21%
No OCB	50 47%	34 32%	84 79%
	60 56%	47 44%	107

Chi-squared = 1.973, 1 df, NS

Inspection of the pedigree was suggestive of a dominant gene with partial penetrance, though in fact there are more affected individuals in the pedigree than would be expected under these conditions. Using POINTER for formal segregation analysis, the hypothesis that there was no transmission of a liability to affection from parent to child was rejected at $p<0.001$ for both GTS and CMT at all sex ratios and all population frequencies.

The hypothesis that there is no single major locus effect could be rejected at $p<0.01$ for all sex ratios if the true prevalence of GTS among males is 1% or less. This hypothesis could be rejected at $p<0.001$ if the true male prevalence of GTS is 0.5% or less, or if the male prevalence of CMT is 5% or less. In other words, there is good evidence in favour of the existence of single major locus transmission, and this evidence is not particularly

dependent on the population prevalence nor male:female ratio specified. When the model was unconstrained (i.e. the mixed model of transmission was specified) the maximum likelihood parameters obtained favoured a single locus mode of transmission without any additional multifactorial transmission. In addition for all male prevalence values for GTS of less than 5% a fully dominant mode of transmission was preferred, and when fully dominant transmission was compared with fully recessive the latter could be rejected at $p<0.001$.

With the exception of unrealistically high prevalences, the evidence for major locus dominant transmission was not contingent on the population prevalence or male:female ratio specified. However the same cannot be said about the estimates obtained for the frequency of the disease allele, which were highly dependent on these parameters. In fact once the disease prevalence is set to be 1% or less, the maximum likelihood estimate for the allele frequency is directly proportional to the male prevalence supplied. The value obtained for the constant linking the two is set out in Table 3.4 for the different male:female ratios and different disease models used.

Table 3.4: Value of constant, c, relating estimated gene frequency, q, to male population prevalence specified, Kp (for all male prevalences of 1% or less)

$q = c\,Kp$

m:f	GTS	GTS+CMT
1:1	0.85	0.58
2:1	0.63	0.39
5:1	0.52	0.14

The data was analysed in more detail using values thought to be close to the true values

for population prevalence: a prevalence among adult males and females of 0.0005 for GTS, and twice that for the combined syndromes of GTS or CMT. This yielded maximum likelihood estimates for the disease allele frequency of 0.0004 if GTS only was studied, or 0.0006 if GTS and CMT cases were included. Since logically if both diseases represent different expressions of the same disease allele then the allele frequencies must be equal in each case, the allele frequency was constrained to be 0.0005 and the analysis was repeated. The likelihoods of the models did not change appreciably, and so the data could be said to be consistent with the hypothesis that the different diseases represent variant expressions of a single dominant gene. The penetrance of this gene was estimated to be 0.50 if GTS is taken to indicate affection, or 0.88 if either GTS or CMT is indicative of affection. The estimates for the probability of affection in the absence of the disease allele (the normal homozygote penetrance) and for the probability of an affected individual not having the disease allele (the proportion of phenocopies) were both very low (less than 0.001). However these last two results do not refer to the population in general but only to individuals within the pedigree, and so little reliance should be placed upon them when compared to the results of other studies which have taken a wider sample of cases.

The constraints on transmission probabilities were removed to allow for non-Mendelian transmission. The models using the realistic values for prevalence as described above were tested under these conditions. In each case the unconstrained model was significantly more likely than the model constrained to Mendelian transmission, suggesting that the mixed model did not fit well with the data.

3.1.2 Power calculations estimate expected lod scores in F24.

Simulations were carried-out on the large pedigree F24 for a marker with four alleles of equal frequency tightly linked to a rare, highly penetrant dominant disease having the same pattern of affection as observed for the GTS/CMT model. The gene frequency was set at 0.0005 with penetrance values :-f0=0.001, f1=0.88, f2=0.88. In 100 simulations the lod scores obtained were in the range of 0.71 to 11.7 with a mean of 6.9, s.d. =2.23. Only one lod score was less than 2 and only 7 were less than 3. This implies that the large family F24 has the power of greater than 90% to produce a result of over 3.

3.1.3 Discussion of segregation analysis results

Previous workers have suggested that GTS is a genetically heterogeneous disease, with some cases being clearly familial and apparently having a significant single locus effect but with a substantial fraction of cases being sporadic and of unknown aetiology. Since GTS is rare it seems reasonable to regard the pedigree presented here as being representative of a single genetic subtype and therefore offering a particularly valuable opportunity to study a genetically homogeneous form of the disease.

There are strong theoretical objections to performing genetic studies and particularly segregation analysis on a pedigree which has been selected on the basis of containing multiple affected cases, because this introduces an obvious ascertainment bias. The likely effects of such a bias are impossible to quantify exactly because the pedigree was not sampled or extended in a systematic manner and so it is not possible to be completely

certain of the extent to which some of the results may be artefactual. However I believe that there are considerable benefits in being able to study the characteristics of a genetically "pure" form of the disease such as this pedigree seems to represent and that these benefits outweigh the disadvantages of having to use a method which is not strictly appropriate to the sample obtained. It is perhaps also important to realise that if there is genetic heterogeneity then the application of segregation analysis to a series of systematically ascertained probands would also be inappropriate. In such a case the parameters obtained would represent a compromise between subtypes rather than representing a true picture of the mode of transmission in any one family.

In the present case it is not thought feasible that the results obtained could be solely due to the methodological weaknesses outlined. The results of the analysis are in general very similar to those obtained by other workers who have analysed a systematic series of families (Pauls and Leckman, 1986) and one large pedigree (Pauls et al, 1990). The only slight anomaly in the data was that the excess of male cases of GTS which is commonly described was not detected. This may represent a genuine difference between the form of the disease occurring in this pedigree and other forms occurring in the population, or may be due to chance or some other cause. The subsequent specification of equal prevalences for both sexes meant that the penetrances derived for males and females were also equal. There was strong evidence for the action of a major autosomal dominant locus and this was not sensitive to the disease prevalence specified. It is not clear why this model was shown to fit poorly when the constraints on Mendelian transmission were removed, though this may possibly be due to the rather high frequency of cases commented on earlier. In general the results provide further support for the theory that

some cases of GTS are due to an autosomal dominant gene that can also be expressed as CMT, which seems therefore to be a milder form of the same illness.

Because there were only five cases of OCB occurring in the absence of GTS or CMT it was not possible to formally investigate a genetic relationship between the syndromes. Nevertheless the occurrence of five cases in a sample of this size represents a significant increase over the normal population prevalence. It is noteworthy that under the assumption of autosomal dominant transmission four of these five cases were "obligate carriers" for CMT and GTS - that is people who, while not having tics themselves, had affected parents and sibs and appeared to pass the syndrome on to their children. These observations might be taken to add weight to the suggestion that OCB alone can occur as a variant expression of the genetic abnormality which gives rise to GTS and CMT. There is also some evidence to support the previously mooted hypothesis (Pauls et al 1991) that such expression occurs more frequently in females than males, as all five such cases in our sample were female.

It is not possible to determine the extent to which the results from the study of one multiply-affected pedigree can be generalised to the population at large. Nevertheless there were no obvious phenomenological differences between these cases and those described in the literature and the genetic parameters derived from this pedigree conformed reassuringly closely to the results of similar segregation analyses which have employed different ascertainment procedures. It may therefore be the case that a substantial majority of GTS is due to the same genetic mechanism as that which is operating in this pedigree.

The indication that a single gene predisposition to GTS is operative in this large pedigree means that it is feasible to attempt to find genetic markers linked to GTS. The parameters that have been estimated in the segregation analysis can be employed in the models to be used for linkage analysis. The power calculations performed on F24 suggest that by using this large kindred a greater than 90% chance of finding linkage could be achieved. Therefore if markers linked to GTS can be found then ultimately this may lead to the cloning and sequencing of the responsible mutation.

3.2 Results of the Investigation of the GTS family with translocation.

A twenty year old male youth (BA) who was being treated for GTS and an associated behaviour disorder with haloperidol, was noted to have, in addition to head nodding and blinking, a small head, micrognathia, very fine hair on his head and no facial hair. Blood was therefore sent for chromosomal analysis because of the striking dysmorphic features. The patient was found to be carrying a balanced translocation 46 XY, t(3:8) (p21.3 q24.1) (figure 3.1)

When a detailed assessment and examination was conducted, it transpired that throughout his life and at interview, he had a wide repertoire of motor tics including a scalp movement, frowning, raising his eyebrows, blinking, eye rolling, nasal twitch, mouth opening, platysma tightening, hair out of the eyes flick, neck stretching, shoulder shrugging, looking at his watch as a habit, abdominal contractions, smelling things, spitting and adjusting his clothing. He also had several vocalisations including

sniffing, throat clearing, burping, clicking sounds, sucking noises, "raspberries" and various animal noises. He had possible coprolalia, but no copropraxia, positive palilalia and forced touching, and he satisfied (on history) diagnostic criteria as a child for ADHD. At the age of eighteen years GTS was diagnosed and he was treated with haloperidol.

On history, he was preoccupied with "sameness", brushed his teeth six times in a characteristic way and became obsessed with having baths. He was noted to be "ritualistic" over matters of cleanliness. He had periods of sexual disinhibition and used to touch his grandmother's breasts, expose himself and masturbate openly. He also indulged in self-injurious behaviour in that he hit himself regularly. The patient was born normally at term. He was noted to have been a healthy baby but had delayed milestones and his speech developed at the age of eighteen months. He had a disrupted childhood with many changes of home and carers. He lived with his maternal grandmother from birth until the age of four, with his parents from four to ten, was in care from ten to sixteen after which continued to live with his maternal grandmother. As a child he stammered, was enuretic and encopretic. During his childhood and teens he was noted to be badly behaved, disobedient and unusually aggressive, assaulting both family members and strangers. He also kicked doors and smashed ornaments in the home. At the age of five he was referred to a child guidance centre for assessment and was placed on amitryptiline "to shut him up". It appeared that he remained on this medication from the age of five to eighteen years. At the age of ten he was admitted to a child psychiatric unit for assessment and treatment. He was noted to have learning

difficulties and be educationally subnormal but never attended a special school. He was, however suspended from school because of his bad behaviour. He finally left school without any qualifications and attended a youth training scheme course. He attempted suicide twice, by overdosing and cutting his wrists and had been treated with various medications including amitryptiline, haloperidol, procyclidine and cyproterone.

On examination of his mental state he was neat and tidily dressed. He exhibited multiple vocal and motor tics. He played with his fingers in a manneristic fashion and avoided eye contact. His talk was monosyllabic and characterised by a stutter and explosive speech. He was euthymic, not suicidal nor psychotic, but he did have some pseudohallucinations. It was felt that he had poor insight.

Family history revealed that the patient's father, a manual worker, had been imprisoned many times for grievous bodily harm, theft and burglary. The patient's parents were divorced when the patient was 16 years old. It was not possible to interview his father. The mother, 38 years old, was depressed and repeatedly physically assaulted during her pregnancy with BA. The mother also took diazepam during the pregnancy and had postpartum psychosis at the time of BA's birth. The patient was an only child. Ten members of his maternal family were seen and examined and information was obtained on a further four. Caseness and pedigree are shown in figure 3.2.

The mother was a definite case of GTS and a blood sample was found to be positive for the 3:8 translocation. Further blood samples were taken from the grandfather,

uncle, aunt and cousins to see if the translocation co-segregated with the GTS in the family (see figure 3.2). The grandfather, aunt and uncle did not have the translocation but were diagnosed as having GTS. This suggests that the translocation was not the cause of the GTS seen in this family and was infact a de novo mutation in the gametogenesis which gave rise to the mother.

Figure 3.1: Diagrammatic representation of the translocation carried by the proband (BA) with GTS.

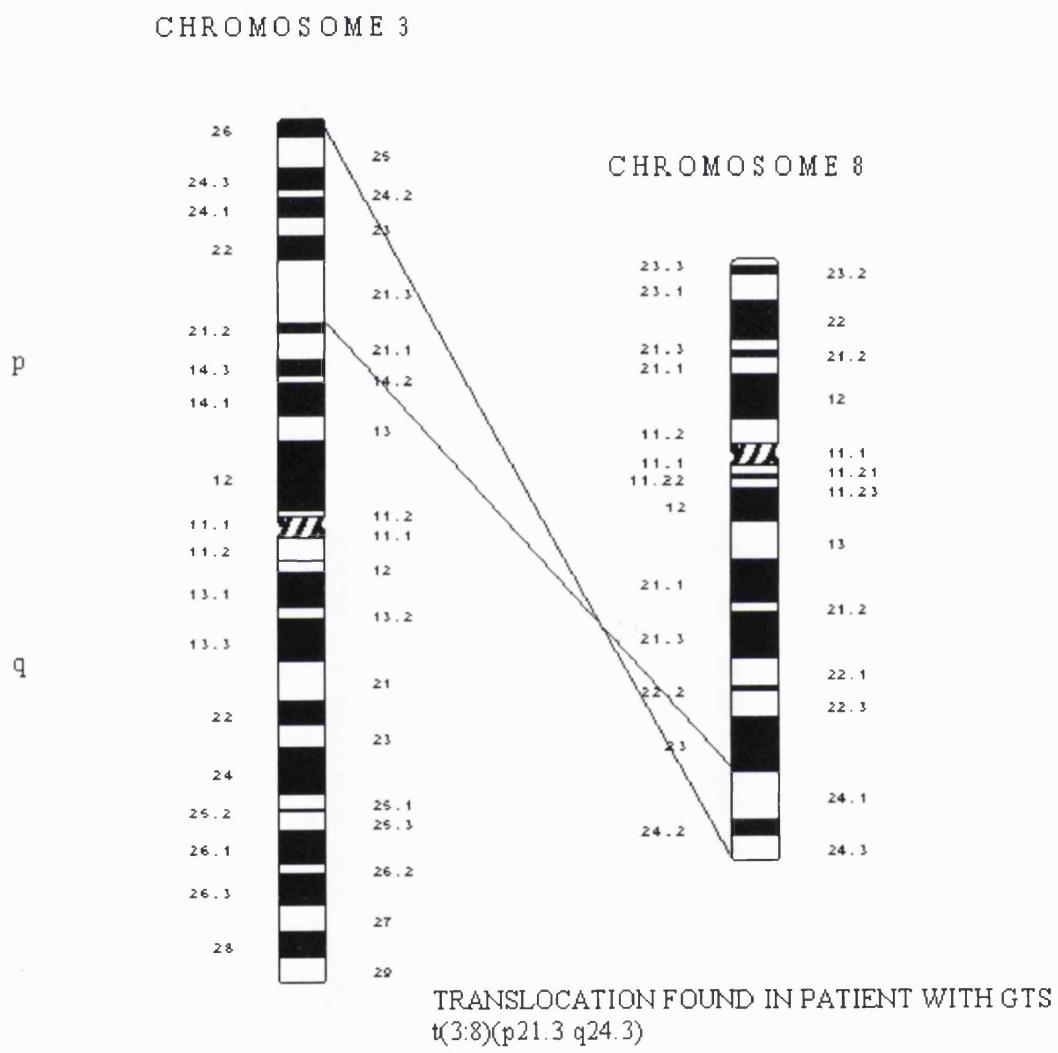
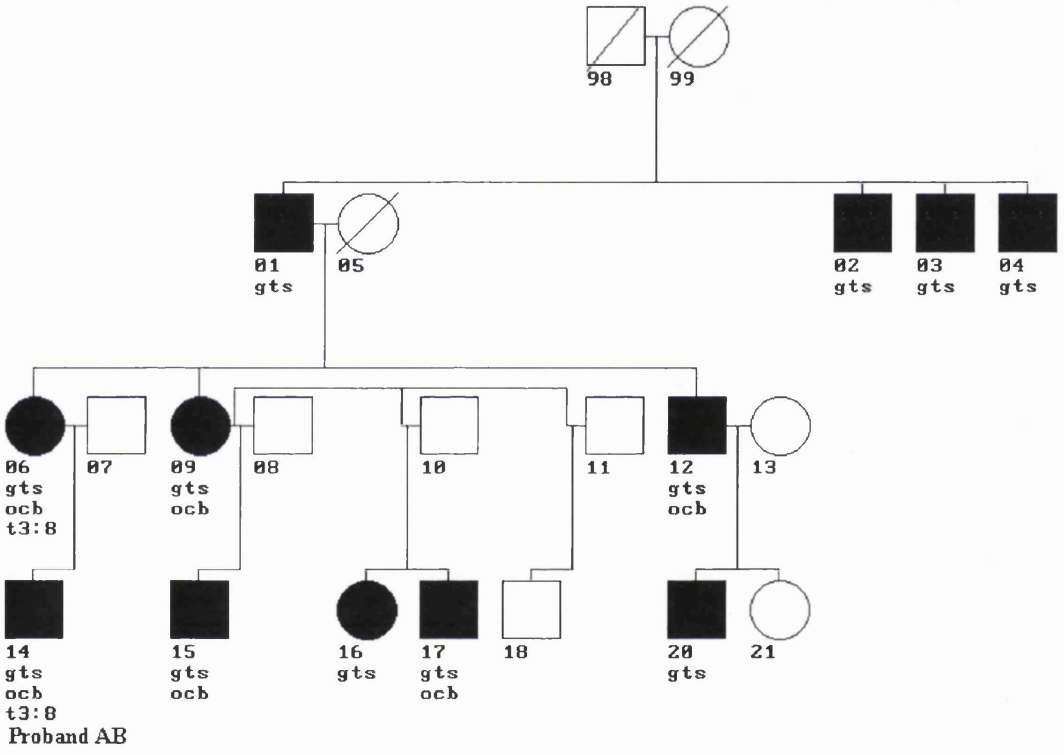


Figure 3.2: Pedigree diagram of F210 (AB's family) showing the diagnostic and cytogenetic data. Individuals 1, 6, 14, 9 & 12 were tested for the translocation as this was sufficient to detect co-segrgation with GTS in the family.



CHAPTER 4: Results of Chromosome Walking and Genomic Cloning

4.1 Isolation genomic clones for a neuroreceptor (NMDAR1)

A genomic phage clone was identified and sequenced and was found to contain some of the 3' coding regions of the NMDAR1 gene. This screening yielded only 1 positive clone from the phage library, supporting the observations of others about the inherent cloning difficulties of this gene (Henneberry, 1992). The clone (AD1) was used to localise the gene using fluorescent in situ hybridisation (FISH) to normal chromosomes and also to a lymphoblastoid hybrid cell line B00015 (described in Povey et al, 1992 and obtained from the European Cell Culture Repository, Wilts) which has the karyotype 46XXt(9;15)(q34.3;q24). FISH localised the gene to chromosome 9q34.3. The breakpoint on chromosome 9 has previously been shown to be distal to D9S14 and RXRA (Zhou et al 1992) and it has recently been determined that it is distal to D9S114 (Woodward 1993, personal communication). AD1 was screened for simple dinucleotide repeats using poly(dC.dA).(dG.dT) (Pharmacia) and for RFLPs using the radiolabelled AD1 on Southern blots restricted with 20 different enzymes.

Figure 7.1 shows that GRIN1 maps in the derivative chromosome 15, distal to the breakpoint and therefore lies distal to D9S114. A variably numbered tandem repeat (VNTR) was identified with over 12 alleles (figure 7.2) ranging in size from 2.1kb to 4.5kb, which were difficult to read due to the presence of non-specific repeat sequences elsewhere in the genome. The VNTR was not found in the cDNA clone demonstrating that the polymorphic sequence is located in an intron or at the 3' or 5' ends of the gene.

A 2.3kb Pvu II fragment of AD1 was found to contain the VNTR sequence and was used to hybridise against the CEPH reference family Pvu II filters. Linkage analysis was performed, the polymorphism gave a maximum lod score of 20.09 at $theta_{m,f}$(0.00 and 0.03) with D9S7(table 7.1). It could not be precisely positioned on the CEPH chromosome 9 consortium map (Attwood et al 1994) at odds of 1000:1 but could be confidently placed distal to D9S67. The 2.3 kb PvuII fragment containing the VNTR was subcloned into the pCR-Script™ SK (+) phagemid (Stratagene) and called PMB3 in an attempt to remove the non-specific repeat sequences and make hybridisation more specific, however, this did not make the signals from Southern hybridisations easier to read. Some of the sequence was determined and analysed using the BLAST program (Altschul et al 1990). Sixty bases of the sequence obtained showed 100% homology to the 3' end of the published mRNA sequence for the human NMDAR1 subunit (Karp et al 1993), 94% with rat (Moriyoshi et al 1991) and 93% with mouse (Yamazaki et al 1992). The VNTR was hybridised to filters for F24, but due to the complexity of the allele pattern produced in such a large pedigree it was impossible to determine the genotypes.

Figure 4.1:- Hybridisation of the whole lambda genomic NMDA1R clone AD1 by FISH on a normal chromosome spread showing the presence of the cloned sequence on both copies of chromosome 9.

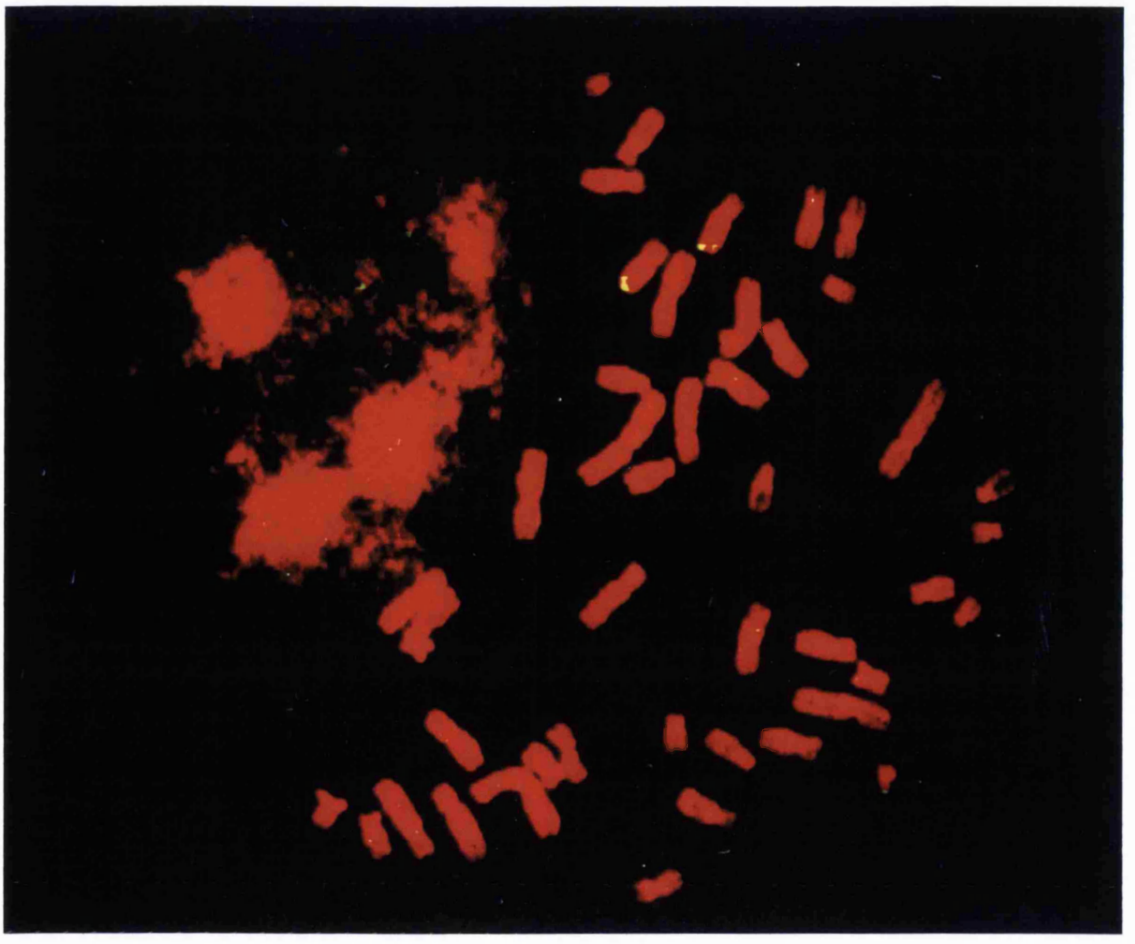


Figure 4.2:- Hybridisation of the whole lambda genomic NMDA1R clone AD1 by FISH on the lymphoblastoid cell line B0015 (karyotype 46XXt(9;15)(q34.3;q24)), showing the presence of the cloned sequence on the translocated chromosome 15 and normal chromosome 9.

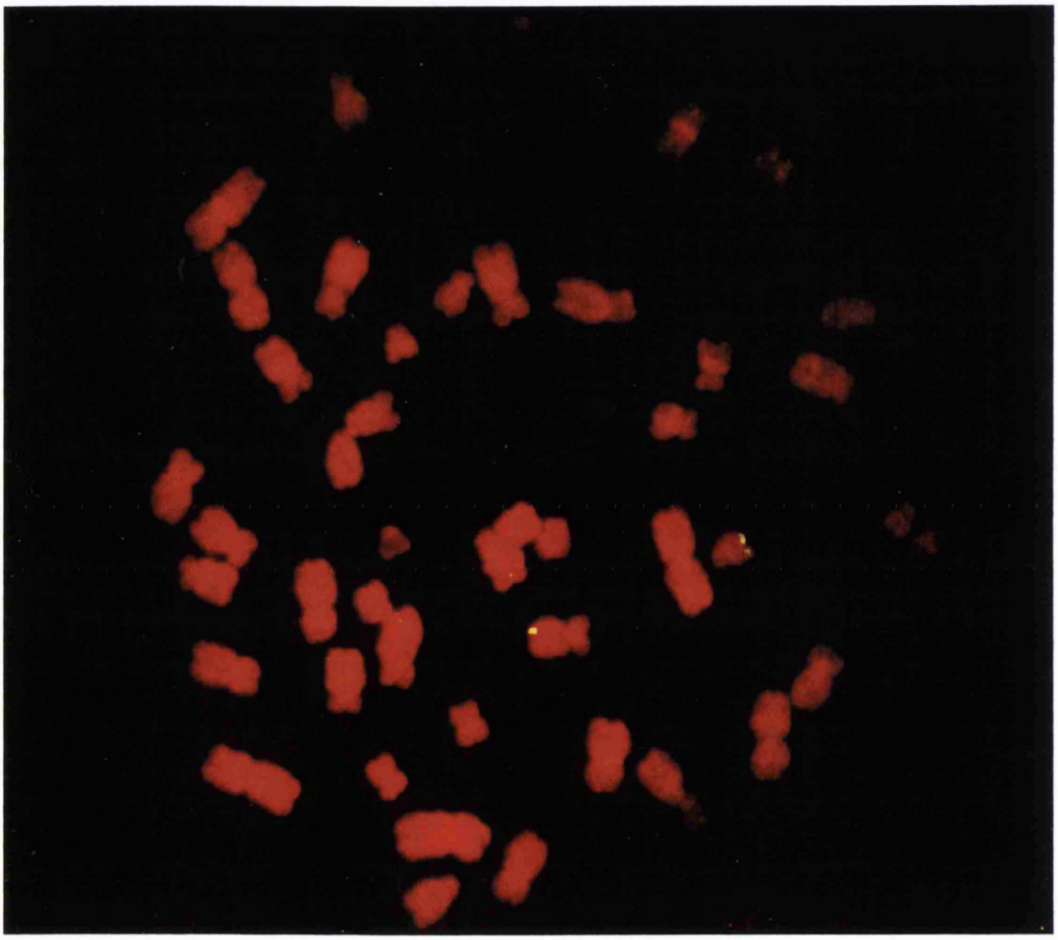


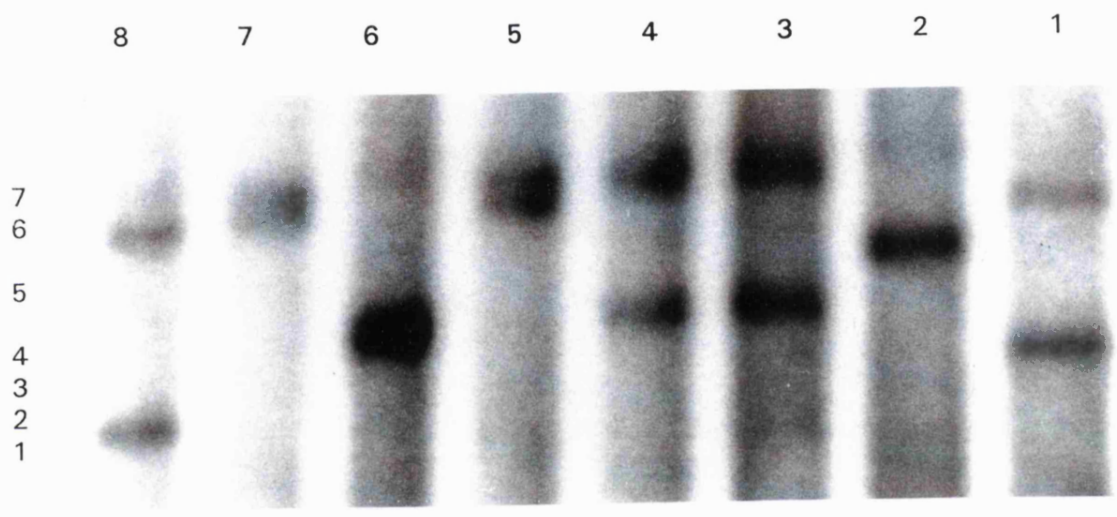
Table 4.1:- Two point lod score tables (female above and male below) between NMDAR1 and chromosome 9q markers. Markers shown on the same line do not recombine in the CEPH data and were treated as one during the analysis.

Theta	0.001	0.05	0.10	0.20	0.30	0.40
DBH/D9S66	-1.94	6.87	7.60	6.95	5.15	2.65
	-9.44	4.67	6.14	6.09	4.53	2.21
D9S14/D9S67	4.97	6.78	6.61	5.40	3.62	1.50
	5.72	8.21	7.82	6.27	4.20	1.80
D9S17	4.28	5.49	5.27	4.33	3.03	1.48
	1.24	2.73	2.75	2.29	1.54	0.66
D9S7	5.11	6.20	5.84	4.71	3.27	1.61
	13.83	12.78	11.65	9.19	6.42	3.29
D9S11	3.53	7.61	7.53	6.31	4.47	2.20
	11.02	10.30	9.44	7.46	5.14	2.52

Maximum likelihood estimates of recombination fraction (theta) at Z max for males and females.

	theta f	theta m	Zmax
DBH/D9S66	0.11	0.14	14.01
D9S14/D9S67	0.06	0.05	15.00
D9S17	0.04	0.07	8.28
D9S7	0.03	0.00	20.09
D9S11	0.07	0.00	18.69

Figure 4.3:- Southern hybridisation of the PMB3 subclone containing the VNTR sequence on a PvuII filter of a small nuclear family showing 7 alleles.



4.1.2 Discussion of cloning.

While this work was in progress, two other groups have reported assignment of GRIN1 to 9q34.3 (Collins et al 1993 and Karp et al 1993) using FISH, mentioning the possibility that GRIN1 might be a candidate locus either for Tuberous sclerosis (TSC1) or for Idiopathic Torsion Dystonia, both mapping to 9q34. However it was shown both by physical mapping and by genetic means, that GRIN1 lies distal to the distal flanking markers for these diseases (Kwiatkowski et al, 1993) and can therefore be excluded as a candidate gene. The distal position of GRIN1 makes it a valuable genetic marker for qter.

4.2.1 Chromosome Walking on Chromosome 3

The probes RAF1, THRB and D3S11 were used as starting points for the walking. No RFLPs were observed by screening with the clones obtained from the walking. Despite taking several steps by repeat genomic library screening in the walks for D3S11 and RAF1 no microsatellite sequences were discovered. One clone from the D3S11 screens did contain a polymorphic microsatellite sequence, which on investigation was found to be part of a double insert and the microsatellite was eventually found to come from chromosome 2 and not from sequences adjacent to D3S11 (Brett et al 1991; Brett 1992).

A clone was isolated from an EMBL3 (Clontech) human genomic library using a repeat free fragment of the probe pBH302 at the locus THRB (Gareau et al 1988) as the start of a chromosome walk. The clone lambda THRB-5 was found to contain a polymorphic dinucleotide designated THRB-5GT. The sequence flanking the repeat was ascertained

(Figure 4.2) and primers were designed for the PCR amplification of the repeat which showed 5 different alleles of varying frequencies (Table 4.3). This microsatellite for the THRB clone was used in the linkage analysis of chromosome 3. The dinucleotide repeat sequence was $(GT)_4$ (A) $(GT)_6$ $(G)_{13}$ $(GT)_3$ and is different from the sequence reported by Sakurai et al (1991).

Figure 4.4: DNA sequence surrounding the THRB microsatellite. The order of bases sequenced is G, A, T, C from left to right.

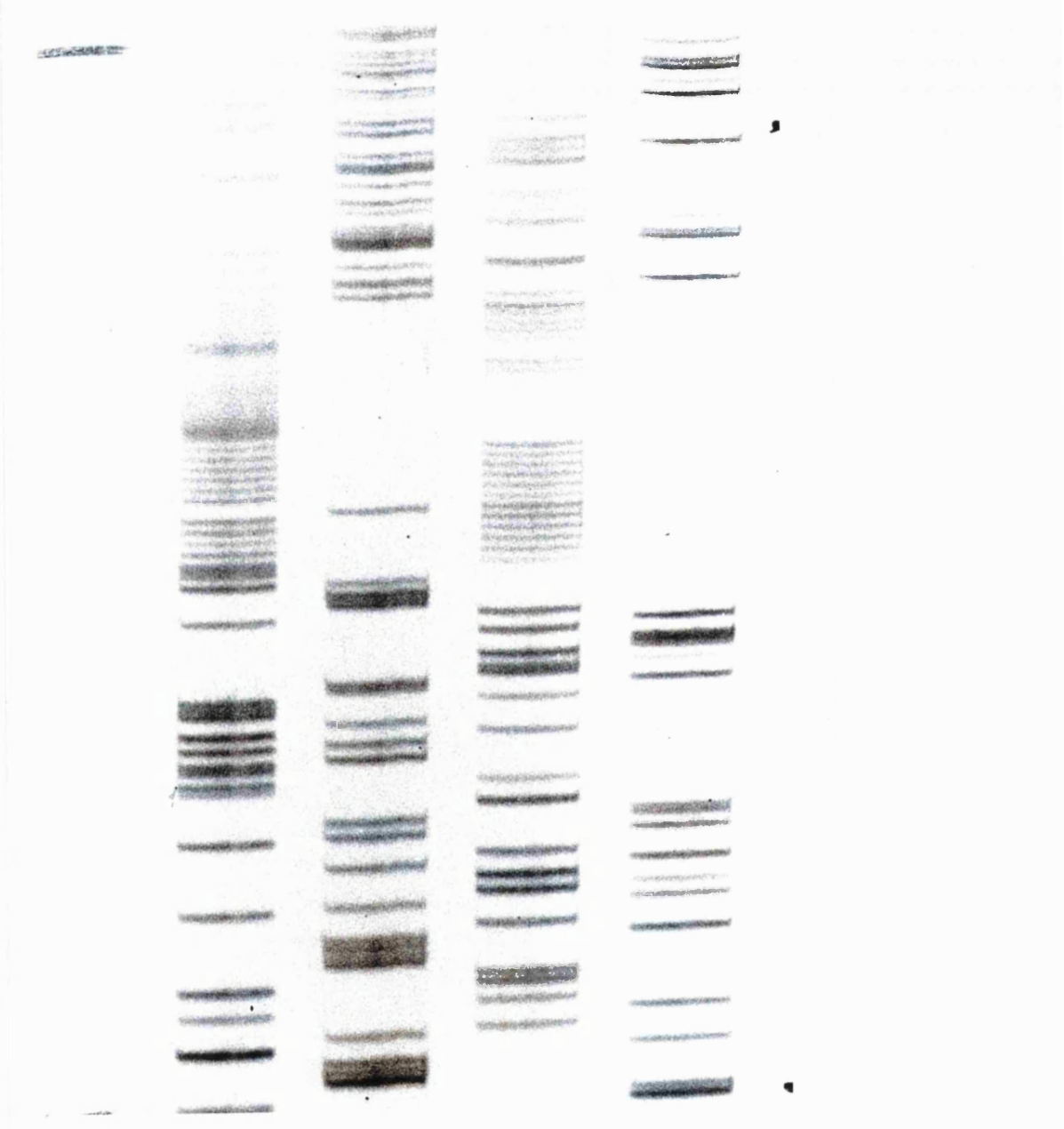


Table 4.2: Allele frequencies and sequence of the oligonucleotide primers for the THRB dinucleotide repeat, and the allele frequencies.

PCR primers:

THRB-5 1 5'-AACTGACTCTACTGACACCTG-3'

THRB-5 2 5'-ATGGTACCCTCATTCCTTAGG-3'

Allele Frequency of THRB-5GT polymorphism: Estimated from 42 chromosomes of unrelated European Caucasians.

THRB-5GT

Allele	Size (nt)	Frequency
1	189	0.10
2	191	0.42
3	193	0.33
4	195	0.08
5	201	0.07
Heterozygosity = 66%		PIC =0.64

4.2.2 Discussion

The microsatellite developed for the THRB locus was used for linkage analysis in F24 to provide exclusion data in the region of chromosome 3 implicated by the translocation in the patient with GTS. The data provided by the microsatellite was used in the FASTMAP calculations in Chapter 5. This demonstrates that the strategy of gene walking to find

more informative markers at a particular locus, as opposed to collecting more family material, is a feasible strategy.

CHAPTER 5 The Linkage analyses

5.1 Candidate Genes

5.1.1 Results of the linkage analyses in the catecholaminergic system

In order to test the hypothesis that genes encoding proteins in the catecholamine pathways may contribute to the genetic aetiology of GTS, polymorphic markers at or near the D1, D2, D3, D4, D5 neuroreceptor gene loci as well as at the genes encoding dopamine beta hydroxylase (DBH), tyrosinase (TY) and tyrosine hydroxylase (TYH) were studied in one large multiply affected pedigree. The results of this investigation, presented in this chapter exclude linkage of these candidate genes with GTS in the large pedigree F24.

The two-point lod scores for each marker used are shown in table 5.1. The genetic information from the probe for the D1 receptor gene was largely uninformative, but by using a tightly linked microsatellite at D5S211 it was possible to exclude the region around the DRD1 locus over a distance of 20 centimorgans with a two point analysis and over 40 centimorgans using a three-point lod analysis (fig.5.1). The gene for the enzyme tyrosinase is localised on chromosome 11q between the DRD2 and D11S97 loci and is excluded using a three-point analysis, (fig 5.2). A three-point analysis using markers at the HRAS and tyrosine hydroxylase loci was used to exclude the role of the D4 receptor in GTS (fig 5.3). All the other loci of interest, DRD2, DRD3, DRD5,

DBH and TH were excluded with two point analyses using their associated polymorphisms.

Comings et al (1993) reported an association between GTS and increased homozygosity of the MscI RFLP at the dopamine D3 receptor gene (DRD3), this reported association was investigated using classic linkage analysis in F24 and also by comparison of the allele and genotype frequencies in patients with GTS or CMT. Strongly negative lod scores were produced, which remained at less than -2 at all recombination fractions of 10% or less. These linkage results provide good evidence against cosegregation of the DRD3 locus with GTS and CMT in F24. In order to test the hypothesis that this locus might exert a modifying effect on the expression of another unknown locus the allele frequencies (table 5.1) of the individuals in the pedigree were compared to 4 control groups. No increase in homozygosity among the affected members of F24 was seen.

Table 5.1:- Distribution of DRD3 genotypes (alleles 1 & 2) in affected and unaffected individuals.

		Genotypes					
		number			*frequency*		
Controls	*N*	1,1	1,2	2,2	1,1	1,2	2,2
CEPH	91	40	46	5	44.0	50.5	5.5
UK	68	25	34	9	36.8	50.0	13.2
French	71	26	41	18	36.6	57.8	5.6
F24	28	8	15	5	28.6	53.6	17.9
Cases							
GTS	19	5	12	2	26.3	63.2	10.5
GTS/CMT	33	11	18	4	33.3	54.5	12.1

Table 5.2. Two point lod scores for linkage between affection status and nine markers for two diagnostic models.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for the RFLP marker c-Ha-RAS1 at the HRASlocus.								
GTS	-6.946	-6.201	-4.281	-2.541	-1.860	-1.117	-0.574	-0.201
GTS & CMT	-16.199	-14.280	-10.219	-5.835	-3.912	-1.993	-0.815	-0.194
Two-point lod scores for VNTR marker pMS51 at locus D11S97.								
GTS	-9.443	-8.260	-6.232	-4.142	-2.925	-1.523	-0.776	-0.394
GTS & CMT	-21.255	-19.568	-14.169	-7.693	-4.791	-1.984	-0.723	-0.235
Two-point lod scores for the microsatellite THCA at the tyrosine hydroxylase locus TH.								
GTS	-10.909	-10.006	-7.289	-4.243	-2.561	-0.967	-0.208	0.074
GTS & CMT	-23.638	-22.435	-16.944	-9.437	-6.036	-2.760	-1.078	-0.239
Two-point lod scores for the microsatellite D2-5/D2-6 at the dopamine 2 receptor locus DRD2.								
GTS	-12.201	-11.777	-9.280	-5.529	-3.595	-1.748	-0.889	-0.396
GTS & CMT	-13.600	-13.029	-10.175	-5.576	-3.185	-1.048	-0.255	-0.048
Two-point lod scores for the microsatellite DBH-CA at the dopamine beta hydroxylase locus DBH.								
GTS	-8.903	-7.726	-5.455	-3.145	-1.946	-0.692	-0.173	-0.013
GTS & CMT	-8.539	-6.614	-3.554	-1.104	-0.241	0.439	0.636	0.445
Two-point lod scores for the RFLP marker HGR213-1 at the dopamine D1 receptor locus DRD1.								
GTS	0.806	0.804	0.784	0.698	0.591	0.387	0.213	0.083
GTS & CMT	0.016	0.023	0.085	0.271	0.383	0.408	0.312	0.169
Two-point lod scores for the microsatellite Mfd154CA at locus D5S211.								
GTS	-10.655	-10.443	-8.914	-5.782	-3.951	-1.985	-0.954	-0.358
GTS & CMT	-14.928	-14.087	-11.419	-6.917	-4.246	-1.777	-0.697	-0.202
Two-point lod scores for the microsatellite D5CA at the dopamine D5 receptor locus DRD5.								
GTS	-12.858	-11.914	-9.427	-5.769	-3.862	-1.908	-0.784	-0.183
GTS & CMT	-22.939	-20.703	-16.289	-10.531	-7.102	-3.598	-1.610	-0.481
Two-point lod scores for the RFLP marker L214 at the dopamine D3 receptor locus DRD3.								
GTS	-3.204	-3.071	-2.516	-1.659	-1.126	-0.567	-0.264	-0.088
GTS & CMT	-6.263	-5.720	-4.195	-2.267	-1.345	-0.601	-0.300	-0.128

Figure 5.1:- Multipoint lod scores on chromosome 5 between GTS/CMT and D5S211 and DRD1 to exclude the dopamine D1 receptor gene. DRD1 is at 0cM and D5S211 is at 5cM.

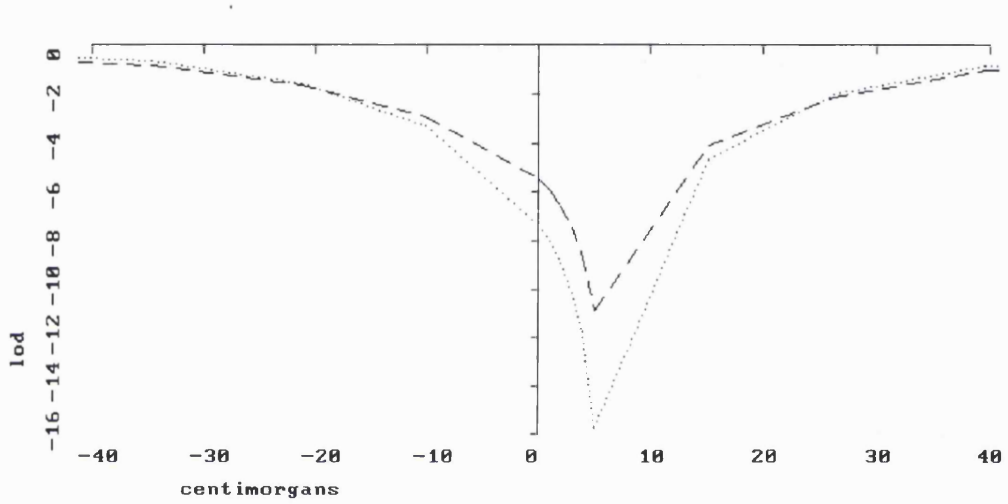


Figure 5.2:- Multipoint lod scores on chromosome 11 between GTS/CMT and D11S97 and DRD2 to exclude the gene for tyrosinase. D11S97 is at 0cM and DRD2 is at 40 cM, with the tyrosinase gene lying at 25 cM from D11S97 and 15cM from DRD2.

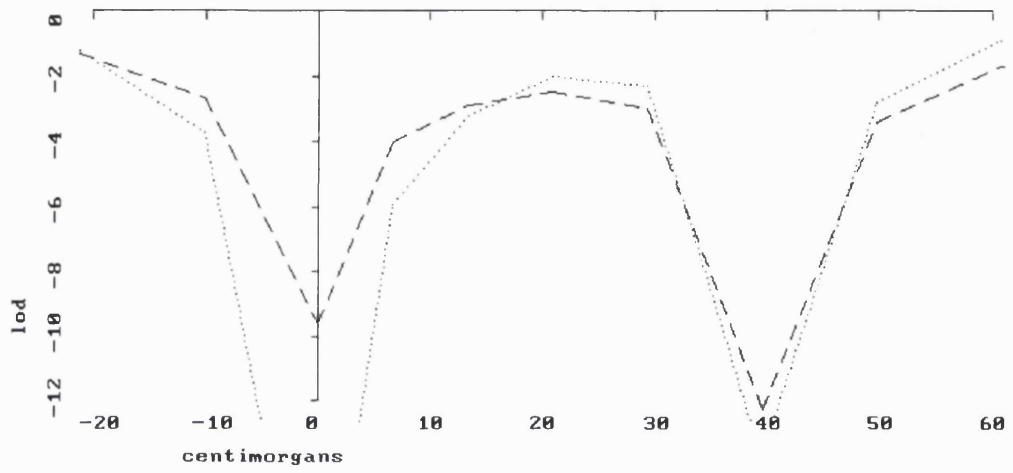
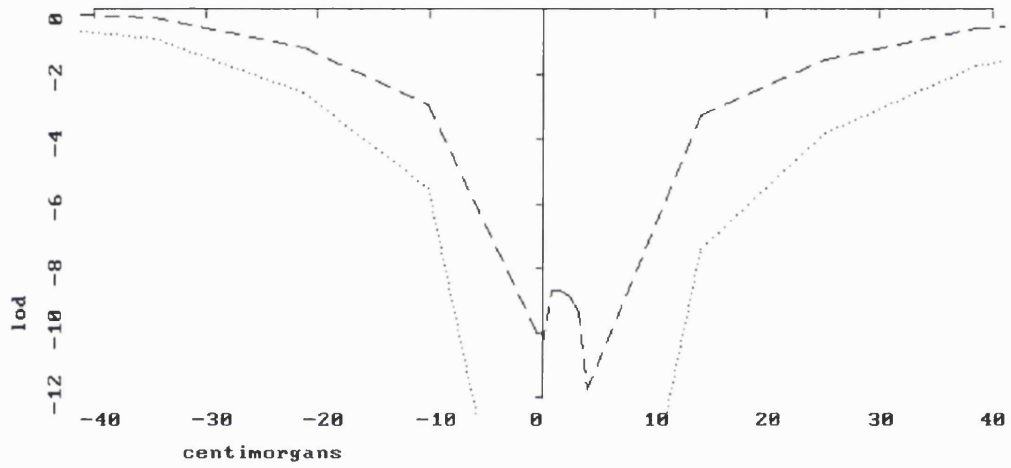


Figure 5.3:- Multipoint lod scores on chromosome 11 between GTS/CMT and TH and HRAS to exclude the gene for D4 dopamine receptor. TH is at 0cM and HRAS-1 is at 3.8cM, with the DRD4 gene lying 2cM, either proximal or distal, of HRAS-1.



5.1.2 Discussion of Catecholamine Linkage Analyses

Given the difficulty experienced so far in localising a susceptibility gene for GTS and related disorders, methods to overcome the possibility that there is heterogeneity of linkage in the genetic predisposition to GTS must be considered. We have chosen the approach used by Gelernter et al (1990, 1993), of primarily studying a single large kindred, in which it may be assumed a single locus is responsible. Weakly positive lod scores in the main kindred were followed up by studying the smaller families. The results suggest that genetic mutations in any of the cloned human dopamine receptor genes and the catecholamine metabolising enzyme genes studied are not involved in the aetiology of GTS in the family studied by us, however these genes could be responsible in other families affected by GTS.

In excluding these genes we have only investigated a small number of the potential candidate genes of neurological relevance that have recently been cloned. The involvement of serotonergic system genes has been proposed (Crosley, 1979), some of these genes have been examined in the next section. In addition there are many second messenger systems which might be involved in the aetiology of GTS (Singer & Walkup 1991).

5.1.3 Results of the linkage analyses in the serotonergic system

The Taq I RFLP for the 5HT1a receptor gene locus (Melmer et al, 1991) was used for the study and was combined with the RFLP and the microsatellite identified at the D5S76

locus (Sherrington et al, 1991; 1992). The blood groups MNS on chromosome 4 were used to provide linkage information for the tryptophan oxygenase (TO) locus.

From published linkage data (Melmer et al, 1991) the 5HT1A (HTR1A) locus maps to within 2cM of the locus D5S79 on chromosome 5. Therefore markers at this locus can be used to exclude 5HT1A if sufficient exclusion data is obtained from the linkage analysis. Two markers were used, an RFLP (L599Ha) and a microsatellite ($P599GT_{(n)}$) (Melmer et al, 1991; Sherrington et al, 1992). The TO gene is less than 1cM distance from the MNS blood group (Comings et al, 1991), therefore this locus was used as a marker for the TO gene itself. For the MNS blood group two allelic systems (MN and Ss) were defined with zero recombination between them.

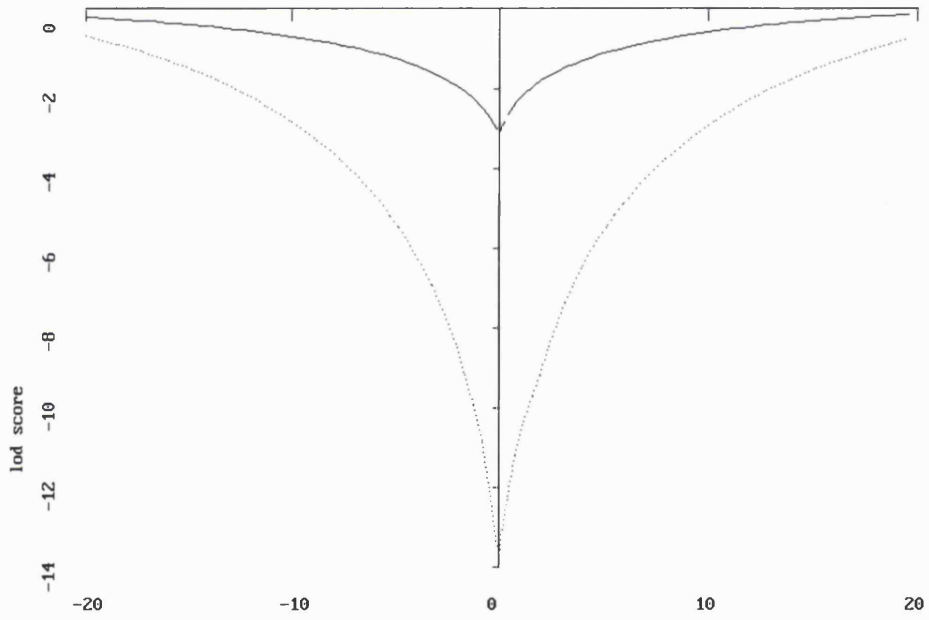
When linkage analysis was carried out between the RFLP and microsatellite polymorphism at D5S76 a maximum lod score of 8.66 at a recombination fraction of zero was found. The two point lod scores for D5S76 (RFLP & microsatellite) and G-21 were combined into an overall map (fig 5.4) using FASTMAP (Curtis and Gurling 1993). The results reported are for the marker order which gives the lod scores of lowest magnitude and so are conservative. The 5HT1a probe G-21 gave results which were only weakly informative and detected no recombinants with GTS. However, when CMT cases were also included as affected, a lod score of -2.0 at zero recombination was obtained (table 5.2). Counting only GTS cases as affected resulted in an exclusion (lod less than -2) up to a recombination fraction of 1% with D5S76, and when CMT cases were also included an exclusion of over 10% was achieved (table 5.2). This certainly excludes all of the region to which the 5HT1a gene has been mapped. With the MNS blood group an

exclusion of nearly 20% was obtained for GTS and of over 30% for CMT (table 5.2). No positive lod scores of any note were produced by any of the markers at any recombination fraction.

Table 5.3. 2-point and 3-point LOD scores between affection status and markers employing two diagnostic models.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores between affection status and the G-21 TaqI RFLP.								
GTS	0.908	0.905	0.884	0.793	0.685	0.492	0.320	0.159
GTS & CMT	-2.000	-1.751	-1.057	-0.398	-0.111	0.133	0.200	0.153
Three-point lod scores of between affection status and the L599Ha RFLP and P599GT(n) microsatellite at D5S76.								
GTS	-2.901	-2.732	-2.102	-1.366	-0.840	-0.103	0.213	0.193
GTS & CMT	-14.311	-13.345	-10.274	-5.852	-2.970	-0.498	0.298	0.344
Three-point lod scores between affection status and blood group markers MN & Ss on chromosome 4.								
GTS	-12.365	-11.975	-9.855	-6.152	-4.086	-1.958	-0.938	-0.385
GTS & CMT	-22.059	-19.349	-14.323	-9.208	-6.798	-3.986	-2.147	-0.864

Figure 5.4: Estimated multipoint lod scores produced by FASTMAP based on two-point lod scores at D5S76 and 5HTR1A. The solid line represents lods for the GTS diagnostic model and the dotted line for GTS and CMT. D5S76 has been placed at a map position of 0 centimorgans and 5HTR1A at 2 centimorgans.



5.1.4 Discussion of the linkage analysis of serotonergic related genes

There have been several previous linkage analyses of GTS using similar models of linkage to those used in this thesis (Brett et al, 1989; Brett et al, 1991; Brett et al, 1990; Gelernter et al, 1990; Pakstis et al, 1991; Heutink et al, 1990; Devor et al, 1990) reviewed in the introduction. This analysis eliminates two possible hypotheses, that the 5HT1a receptor and the tryptophan oxygenase genes are responsible for the genetic susceptibility to GTS in one very large multiplex kindred when an autosomal dominant mode of transmission is assumed. Other candidate genes involved in the serotonergic system such as the 5HT transporter and the 5HT1b, 5HT1c and 5HT2 genes have been cloned (Sparkes et al, 1991; Julius et al, 1988) and these should also be examined for a possible role in the susceptibility to GTS.

5.2 Favoured Loci for GTS

5.2.1 Summary of Investigation into a Translocation

A positive lod score of 2.9 was obtained on chromosome 3 with markers at the loci RAF1, THRB, and D3S11. Subsequently the genetic map of this region was improved and new polymorphic markers close to the original three markers were identified. In addition the family members of the translocation GTS proband were traced. Further linkage analysis did not support the presence of a major gene effect on the short arm of chromosome three and other members of the family with GTS were identified who did not share the translocation.

5.2.2 Results of the Linkage Analyses on Chromosomes 3 and 8

The markers used and their allele frequencies are listed in the methods chapter. Two-point lod scores were calculated with the LINKAGE package of programmes (Lathrop et al 1984) using the parameters set out in the methods chapter. Multiple two point lod scores were combined into an overall map using the FASTMAP programme.

Since the results for chromosome 3 were reported in correspondence to the Lancet (Brett et al 1990) many more probes linked in this region have been used and their lod scores calculated (table 5.3). In the map used for the FASTMAP analysis the position of one of the original three markers (D3S11) was omitted due to lack of certainty in the actual distances from flanking markers. With the new map order and new markers the region

from D3S17 to D3S196 was excluded (lod <-2.00) as shown in figure 5.7. The microsatellite D3S1100 which flanks the breakpoint was not included in the FASTMAP due to uncertainty of the position relative to the older RFLP markers.

Some of the markers used on chromosomes 8 produced several small positive lod scores (table 5.4), these were investigated further by performing a FASTMAP analysis across some of the p-arm and all of the q-arm of chromosome 8 with linked markers, this gave negative lods of less than -2.00 for the whole map (figure 5.8) and therefore the whole region between NEFL at 8p21 to D8S373 at the telomere of 8q was excluded.

Table 5.4: Two-point lod scores for linkage between affection status and markers on chromosome 3.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400

Two-point lod scores for the THRB microsatellite against the THRB RFLPs.

	2.558	2.554	2.509	2.297	2.003	1.366	0.753	0.285

Two-point lod scores for the RFLP marker pBH302 at the THRB locus.

GTS	-2.095	-1.784	-1.059	-0.498	-0.325	-0.218	-0.146	-0.065
GTS & CMT	-4.669	-3.761	-2.315	-1.063	-0.553	-0.136	0.018	0.052

Two-point lod scores for the microsatellite THRB-5GT at the THRB locus.

GTS	-6.856	-6.372	-5.395	-3.664	-2.411	-0.916	-0.179	0.075
GTS & CMT	-15.000	-13.956	-9.948	-5.329	-3.269	-1.161	-0.124	0.197

Two-point lod scores combining the data from the THRB microsatellite and RFLP.

GTS	-6.869	-6.384	-5.344	-3.438	-2.186	-0.754	-0.068	0.132
GTS & CMT	-15.220	-13.902	-9.586	-4.974	-2.969	-0.948	0.025	0.273

Two-point lod scores for the RFLP marker p627 at the RAF locus, "B" system of alleles.

GTS	0.505	0.516	0.648	1.062	1.134	0.822	0.400	0.110
GTS & CMT	-2.098	-1.840	-1.139	-0.136	0.465	0.673	0.449	0.187

Two-point lod scores combining the data from the two allele systems of p627 at the RAF locus.

GTS	1.768	1.776	1.907	2.338	2.365	1.865	1.131	0.444
GTS & CMT	-3.291	-2.766	-1.363	0.265	1.107	1.398	0.998	0.421

Two-point lod scores for VNTR marker R59 "A" system of alleles at D3S12.

GTS	-1.240	-1.231	-1.152	-0.841	-0.571	-0.264	-0.110	-0.032
GTS & CMT	-0.636	-0.631	-0.586	-0.452	-0.367	-0.292	-0.222	-0.115

Two-point lod scores for VNTR marker R59 "B" system of alleles.

GTS	-2.362	-2.355	-2.265	-1.700	-1.200	-0.647	-0.329	-0.123
GTS & CMT	-2.938	-2.933	-2.825	-1.991	-1.345	-0.701	-0.353	-0.125

Two-point lod scores combining the data from the R59 marker.

GTS	-2.673	-2.665	-2.560	-1.914	-1.338	-0.705	-0.354	-0.132
GTS & CMT	-5.269	-5.002	-4.187	-2.697	-1.788	-0.938	-0.495	-0.188

Two-point lod scores for the RFLP marker pH3H2 at locus D3F15S2E.

GTS	0.246	0.281	0.493	0.784	0.821	0.661	0.423	0.196
GTS & CMT	-3.831	-3.435	-2.164	-0.850	-0.306	0.072	0.139	0.095

Two-point lod scores for MFD17 microsatellite at locus D3S196.

GTS	-10.351	-9.899	-7.958	-5.013	-3.429	-1.759	-0.827	-0.275
GTS & CMT	-16.025	-14.888	-10.438	-5.995	-4.072	-2.203	-1.192	-0.472

Two-point lod scores for the VNTR marker L892 at locus D3S17.

GTS	-8.784	-7.895	-5.351	-2.702	-1.515	-0.372	0.154	0.263
GTS & CMT	-14.237	-12.135	-8.151	-4.337	-2.494	-0.688	0.013	0.179

Two-point lod scores for the RFLP marker E41 at locus D3S11.

GTS	0.399	0.437	0.667	1.012	1.100	0.997	0.733	0.376
GTS & CMT	0.591	0.856	1.534	2.020	2.065	1.785	1.298	0.676

Two-point lod scores for the RFLP marker DR-2 at locus D3S5.

GTS	1.344	1.342	1.319	1.212	1.069	0.766	0.461	0.190
GTS & CMT	-1.219	-0.854	-0.075	0.539	0.719	0.690	0.468	0.184

Two-point lod scores for the RFLP marker phCP1 at the caeruloplasmin locus CP.

GTS	-4.020	-3.854	-3.186	-2.241	-1.605	-0.755	-0.300	-0.086
GTS & CMT	-5.998	-5.464	-4.003	-1.967	-0.851	-0.056	0.078	0.013

Two-point lod scores for the VNTR marker L162-1 at locus D3S18.

GTS	-2.554	-2.378	-1.596	-0.648	-0.294	-0.080	-0.027	-0.007
GTS & CMT	-5.199	-4.904	-3.867	-2.172	-1.219	-0.387	-0.061	0.039

Two-point lod scores for the microsatellite at locus D3S1312.

GTS	-7.768	-7.425	-6.019	-3.743	-2.601	-1.351	-0.638	-0.233
GTS & CMT	-12.280	-10.989	-7.365	-3.779	-2.053	-0.486	-0.010	0.035

Two-point lod scores for the microsatellite at locus D3S1284.

GTS	-6.467	-5.934	-4.059	-1.773	-0.755	-0.066	0.033	-0.024
GTS & CMT	-11.685	-10.440	-7.339	-3.553	-1.543	0.031	0.422	0.289

Two-point lod scores for the microsatellite at locus D3S1281.

GTS	-6.421	-6.152	-4.894	-2.531	-1.206	-0.172	0.124	0.132
GTS & CMT	-16.675	-14.597	-10.200	-5.473	-3.062	-1.003	-0.216	0.029

Two-point lod scores for the VNTR marker LIB12-37.

GTS	-7.758	-7.504	-6.189	-3.726	-2.386	-1.122	-0.504	-0.156
GTS & CMT	-9.191	-8.730	-6.791	-4.100	-2.707	-1.216	-0.508	-0.143

Two-point lod scores for the microsatellite at locus D3S1100.

GTS	-9.217	-8.759	-6.734	-3.894	-2.557	-1.155	-0.435	-0.099
GTS & CMT	-17.725	-16.148	-10.912	-5.796	-3.679	-1.675	-0.627	-0.129

Table 5.5: Two point lod scores for linkage between affection status and the markers used on chromosome 8.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400

Two-point lod scores for the RFLP IRI-ULB at the TG locus.

GTS	-0.881	-0.881	-0.879	-0.833	-0.622	-0.171	0.008	0.020
GTS & CMT	-1.819	-1.666	-1.095	-0.451	-0.133	0.179	0.206	0.080

Two-point lod scores for the RFLP marker pHT-0.96 at the TG Locus.

GTS	0.205	0.204	0.199	0.177	0.151	0.105	0.065	0.030
GTS & CMT	0.204	0.203	0.198	0.175	0.149	0.103	0.064	0.030

Two-point lod scores for RFLP marker H25-3.8 at the CA2 locus.

GTS	-2.838	-2.714	-2.207	-1.570	-1.170	-0.616	-0.282	-0.088
GTS & CMT	-1.261	-1.093	-0.514	0.061	0.277	0.411	0.378	0.237

Two-point lod scores for the RFLP marker pCA15 at the CA3 locus.

GTS	-3.852	-3.734	-3.199	-2.144	-1.441	-0.734	-0.371	-0.149
GTS & CMT	-4.333	-4.320	-4.061	-2.880	-2.012	-1.082	-0.570	-0.237

Two-point lod scores for the RFLP marker pHNFL at the NEFL locus.

GTS	-2.070	-2.061	-1.872	-0.989	-0.453	0.011	0.153	0.128
GTS & CMT	-2.371	-2.229	-1.692	-1.053	-0.559	0.080	0.283	0.218

Two-point lod scores for the RFLP marker ptPA4352 at the PLAT locus.

GTS	0.333	0.332	0.324	0.285	0.233	0.129	0.048	0.006
GTS & CMT	1.375	1.371	1.339	1.197	1.020	0.689	0.408	0.185

Two-point lod scores for the VNTR marker pMCT128.2 at locus D8S39.

GTS	-10.130	-9.681	-7.724	-4.653	-2.935	-1.260	-0.484	-0.118
GTS & CMT	-9.577	-8.968	-6.776	-3.453	-1.644	-0.130	0.353	0.339

Two-point lod scores for the VNTR marker PYNM3 at locus D8S38.

GTS	-0.906	-0.733	0.027	0.875	1.183	1.253	0.962	0.479
GTS & CMT	-2.935	-2.783	-1.809	-0.244	0.380	0.748	0.654	0.366

Two-point lod scores for the RFLP marker pBS8.9 at locus D8S11.

GTS	-0.880	-0.783	-0.347	0.163	0.344	0.414	0.343	0.200
GTS & CMT	-1.119	-1.016	-0.562	-0.017	0.192	0.301	0.266	0.162

Two-point lod scores for the RFLP marker pSW508 at locus D8S7.

GTS	0.663	0.663	0.665	0.656	0.620	0.512	0.385	0.224
GTS & CMT	-0.383	-0.382	-0.371	-0.307	-0.166	0.087	0.156	0.105

Two-point lod scores for RFLP marker EMBL3.287 at locus D8S8.

GTS	-0.377	-0.208	0.351	0.809	0.886	0.765	0.541	0.282
GTS & CMT	-4.797	-4.339	-2.670	-0.932	-0.253	0.216	0.294	0.202

Two-point lod scores for the microsatellite marker Mfd31 at the PENK locus.

GTS	-1.877	-1.684	-0.862	0.075	0.384	0.474	0.360	0.188
GTS & CMT	-4.536	-4.174	-2.901	-1.069	-0.274	0.299	0.383	0.244

Two-point lod scores for the microsatellite marker at D8S556.

GTS	-5.411	-5.133	-4.079	-2.550	-1.480	-0.390	0.075	0.179
GTS & CMT	-16.521	-15.026	-10.862	-6.251	-3.854	-1.613	-0.494	0.000

Two-point lod scores for the microsatellite marker at D8S284.

GTS	-5.392	-5.081	-3.770	-1.700	-0.779	0.040	0.316	0.262
GTS & CMT	-13.358	-12.033	-8.015	-3.751	-2.027	-0.474	0.161	0.269

Two-point lod scores for the microsatellite marker at D8S554.

GTS	-8.539	-7.546	-5.649	-2.778	-1.112	0.144	0.405	0.261
GTS & CMT	-14.038	-12.347	-9.446	-5.697	-3.128	-0.662	0.217	0.320

Two-point lod scores for the microsatellite marker at D8S272.

GTS	-7.477	-7.071	-5.140	-2.111	-0.715	0.346	0.563	0.363
GTS & CMT	-10.264	-7.396	-3.617	-0.075	1.212	1.898	1.677	0.961

Two-point lod scores for the microsatellite marker at D8S373.

GTS	-10.097	-9.745	-7.998	-4.636	-2.834	-1.218	-0.510	-0.163
GTS & CMT	-15.047	-13.714	-9.430	-4.646	-2.632	-0.941	-0.222	0.049

Figure 5.5: FASTMAP lod scores for chromosome 3 from D3S17 to D3S196 with the breakpoint lying between D3S1100 and D3S1312 (Map order and distances from the Genome Database and Gyapay et al 1994). D3S17 is at 0cM; D3S18 at 3cM; RAF1 at 6cm; THRB (RFLP and microsatellite) at 26cM; D3S12 at 30cM; D3F15S2 at 42 cM; D3S1312 at 57cM; D3S1284 at 77cM; D3S1281 at 97cM and D3S196 at 137cM.

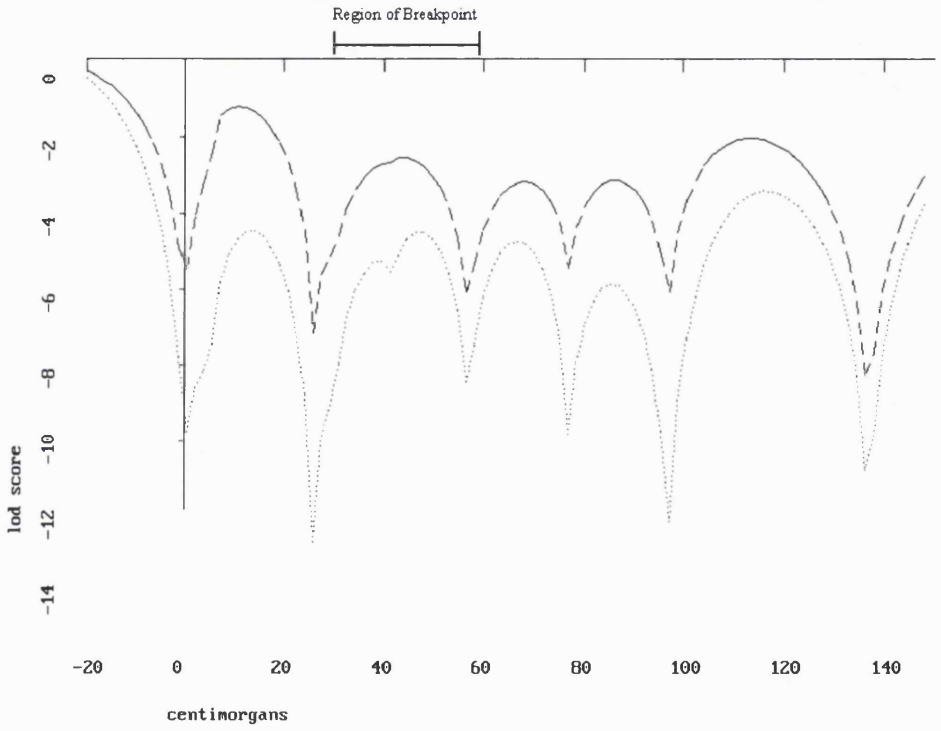
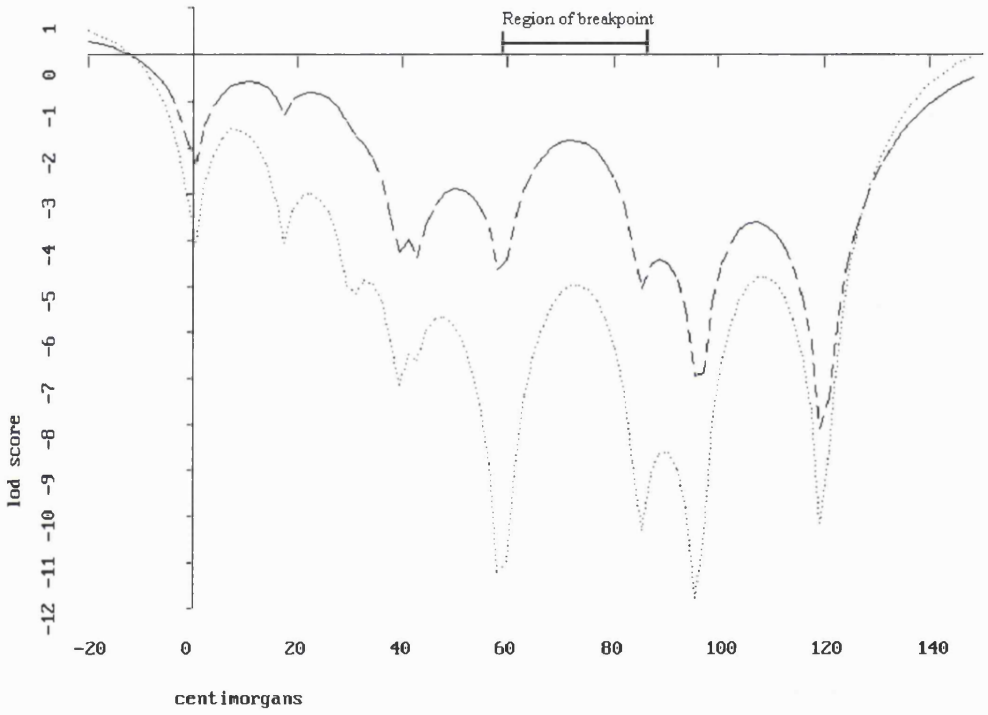


Figure 5.6: FASTMAP lod scores for chromosome 8 from NEFL to D8S373 with the breakpoint lying between D8S556 and D8S284 (Map order and distances from the Genome Database and Gyapay et al 1994). NEFL is at 0cM; PLAT at 8cM; PENK at 17cM; D8S8 at 30cM; CA3 at 39cM; CA2 at 43cM; D8S556 at 59cM; D8S284 at 85cM; D8S554 at 97cM; D8S272 at 98cM and D8S373 is at 121cM.



5.2.3 Discussion of Chromosome 3 and 8 results.

From the results obtained using improved map data and further markers in the region surrounding the original positive lod score it was concluded that the original lod score did not reflect any involvement of the relevant chromosomes 3 and 8 in the aetiology of GTS in the families studied in this thesis. The cytogenetic screens carried-out showed that the proband and his mother were the only carriers of the translocation. From this the conclusion drawn was that the translocation was less likely to be involved in the aetiology of the GTS and related disorders in this family. Furthermore, from work carried-out on the cell hybrids from the proband we found that the actual chromosome 3 break-point was localised proximal to the region producing the positive lod score.

The results must, however, be interpreted with the possibility of heterogeneity in mind. This means that these exclusions may not apply to all GTS families being studied by linkage methods. To date approximately 85% of the genome has been covered in the search for the predisposing gene in GTS (Pakstis et al 1991) assuming homogeneity. Considerably less has been excluded assuming heterogeneity.

The approach of examining candidate genes and regions implicated by cytogenetic abnormalities is a potentially fruitful one in the current situation where a confirmed linkage has yet to be found. There have been a few other instances of reported associations between GTS and cytogenetic abnormalities but these have not resulted in the localisation of the predisposing gene, for example on chromosome 9p (Taylor 1990; Singh 1982). The validity of this method has not been lessened by the present experience with the

chromosome 3 and 8 translocation. With much of the genome already screened further randomly chosen markers should soon localise a susceptibility gene for GTS but this is likely to be within single large kindreds.

5.3 Random Loci

5.3.1 Results of Linkage Analysis with Random Markers

The lod score results for many random markers (Blood groups, RFLPs, VNTRs and microsatellites) which were used in the project with no a priori reason follow in this section. This was part of a wider strategy of using random markers scattered around the genome in collaboration with other groups studying GTS (Pakstis et al 1991) in order to exclude the whole genome under the assumption of genetic homogeneity.

Many types of genetic marker were used in the random search, these include; blood group data, RFLPs, VNTRs and microsatellite markers. In total 75 markers have been used in this project on 7 different chromosomes. All the markers reported were used for two-point lod score calculations with only one positive lod score on chromosome 1 being produced . This region was excluded with a multipoint analysis for the region around the Duffy blood group using the VNTR markers MUC1 and SPTA which flank this region. The blood grouping was performed with the help of Mr Len Dobson of the Blood Transfusion Service at Hither Green Hospital. The genotyping and linkage calculations were performed using the methods described in the methods chapter 2.

Table 5.6: Two-point lod scores for linkage between affection status and 6 blood group markers.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for the AGO blood group.								
GTS	-4.508	-4.329	-3.479	-2.270	-1.627	-0.912	-0.476	-0.188
GTS & CMT	-4.150	-3.784	-2.570	-1.345	-0.838	-0.397	-0.175	-0.047
Two-point lod scores for the Rhesus-D blood group.								
GTS	-1.225	-1.222	-1.197	-1.069	-0.884	-0.521	-0.250	-0.080
GTS & CMT	-0.998	-0.995	-0.972	-0.860	-0.719	-0.469	-0.265	-0.109
Two-point lod scores for the Rhesus blood group.								
GTS	-0.520	-0.409	0.043	0.476	0.587	0.669	0.596	0.350
GTS & CMT	-2.062	-1.804	-0.787	0.310	0.698	0.884	0.757	0.426
Two-point lod scores for the Kind (JKA) blood group "A" only.								
GTS	-0.724	-0.713	-0.619	-0.329	-0.125	0.047	0.076	0.048
GTS & CMT	-0.386	-0.379	-0.322	-0.129	0.017	0.130	0.121	0.061
Two-point lod scores for the Kind (JKAB) blood group.								
GTS	0.613	0.609	0.576	0.436	0.283	0.066	-0.032	-0.040
GTS & CMT	1.068	1.062	1.013	0.797	0.544	0.132	-0.082	-0.094
Two-point lod scores for the Kell (KK) blood group.								
GTS	0.815	0.814	0.804	0.757	0.696	0.558	0.399	0.216
GTS & CMT	-2.549	-2.503	-2.162	-1.347	-0.845	-0.337	-0.096	0.004

Table 5.7: Two-point lod scores for linkage between affection status and two minisatellite VNTR markers from Cellmark Diagnostics.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two point lod scores for pMS43.								
GTS	-3.343	-3.111	-2.183	-1.066	-0.585	-0.215	-0.091	-0.036
GTS & CMT	-3.345	-3.004	-1.831	-0.648	-0.198	0.080	0.108	0.067
Two-point lod scores for pMS51.								
GTS	-9.443	-8.260	-6.232	-4.142	-2.925	-1.523	-0.776	-0.394
GTS & CMT	-21.255	-19.568	-14.169	-7.693	-4.791	-1.984	-0.723	-0.235

Table 5.8: Two-point lod scores for linkage between affection status and three markers on chromosome 1 including the Duffy blood group.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for the VNTR marker MUC 10.								
GTS	-7.236	-7.006	-5.713	-3.655	-2.475	-1.255	-0.544	-0.146
GTS & CMT	-9.745	-9.294	-7.626	-5.275	-3.572	-1.815	-0.935	-0.367
Two-point lod scores for the VNTR marker SPTA1 at the Alpha Spectrin locus.								
GTS	-1.677	-1.669	-1.539	-0.951	-0.621	-0.390	-0.267	-0.130
GTS & CMT	-5.606	-5.323	-4.102	-2.315	-1.317	-0.412	-0.051	0.063
Two-point lod scores for the Duffy blood group locus FYAB.								
GTS	0.491	0.489	0.470	0.387	0.297	0.223	0.199	0.108
GTS & CMT	2.264	2.260	2.223	2.045	1.803	1.282	0.747	0.278
Two-point lod scores between FYAB and MUC10 to show close proximity to each other.								
MUC & FYAB	5.407	5.407	5.405	5.273	4.923	3.858	2.522	1.103

Table 5.8: Two-point lod scores for linkage between affection status and 4 microsatellite markers on chromosome 4.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for the GABRA1 associated microsatellite.								
GTS	-3.581	-3.451	-2.385	-0.159	0.821	1.287	1.043	0.545
GTS & CMT	-7.316	-7.146	-5.714	-2.730	-1.153	0.132	0.431	0.304
Two-point lod scores for D4S174.								
GTS	-12.305	-10.119	-7.838	-4.891	-3.227	-1.513	-0.665	-0.218
GTS & CMT	-13.060	-11.703	-9.423	-6.232	-4.203	-1.866	-0.654	-0.096
Two-point lod scores for D4S1558.								
GTS	-2.716	-2.547	-1.716	-0.503	0.019	0.338	0.330	0.196
GTS & CMT	-0.750	-0.694	-0.327	0.370	0.668	0.733	0.552	0.295
Two-point lod scores for D4S398.								
GTS	-1.768	-1.753	-1.624	-0.941	-0.239	0.366	0.451	0.286
GTS & CMT	-3.999	-3.818	-3.072	-1.716	-0.743	0.187	0.439	0.329

Table 5.10: Two-point lod scores for linkage between affection status and several Genethon microsatellite markers on Chromosome 5.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for the microsatellite at D5S119.								
GTS	-2.055	-1.975	-1.454	-0.443	0.047	0.348	0.335	0.201
GTS & CMT	-3.921	-3.859	-3.385	-2.084	-1.190	-0.317	0.007	0.076
Two-point lod scores for the microsatellite at D5S207.								
GTS	-6.489	-6.414	-5.905	-4.456	-3.277	-1.702	-0.773	-0.259
GTS & CMT	-4.658	-4.623	-4.331	-3.294	-2.260	-0.872	-0.186	0.048
Two-point lod scores for the microsatellite at D5S211.								
GTS	-8.443	-8.339	-7.598	-5.583	-3.928	-2.014	-0.980	-0.370
GTS & CMT	-9.155	-8.867	-7.560	-4.988	-3.279	-1.475	-0.613	-0.194
Two-point lod scores for the microsatellite at D5S82.								
GTS	-1.971	-1.938	-1.692	-1.107	-0.705	-0.223	-0.035	0.007
GTS & CMT	-3.141	-3.103	-2.812	-1.930	-1.132	-0.214	0.095	0.116
Two-point lod scores for the microsatellite at CSF1 locus.								
GTS	-4.578	-4.340	-3.203	-1.344	-0.425	0.227	0.288	0.122
GTS & CMT	-5.093	-4.571	-3.091	-1.356	-0.506	0.215	0.375	0.208
Two-point lod scores for microsatellite at the GRA1 locus.								
GTS	-8.649	-7.849	-5.848	-3.184	-1.749	-0.450	0.013	0.087
GTS & CMT	-8.254	-7.279	-5.684	-3.402	-1.996	-0.521	0.036	0.106

Table 5.11: Two-point lod scores for linkage between affection status and two RFLP markers on chromosome 9.

Theta	0.000	0.001	0.010	0.050	0.100	0.200	0.300	0.400
Two-point lod scores for PA3.								
GTS	-0.363	-0.357	-0.309	-0.159	-0.053	0.033	0.043	0.026
GTS & CMT	-0.292	-0.288	-0.252	-0.140	-0.061	0.003	0.017	0.015
Two-point lod scores for ALDH1.								
GTS	-1.568	-1.524	-1.240	-0.695	-0.410	-0.141	-0.009	0.037
GTS & CMT	-3.483	-3.246	-2.359	-1.308	-0.864	-0.520	-0.344	-0.162

5.3.2 Discussion of random loci results.

The strategy of using random markers has become a realistic strategy with the advent of microsatellite markers, with their high heterozygosity and their accurate mapping to the genome by the Genethon collaboration (Gyapay et al 1994). It is possible to scan the whole genome using the minimum of markers at 10-20cM intervals along all the chromosomes using 290 microsatellite markers (Davies et al 1994). This approach will yield several chromosomal regions which will require further examination to either be excluded from having any involvement in the aetiology of a disease or will reveal the location of the gene responsible for disease susceptibility. The results must, however, be interpreted with the possibility of heterogeneity in mind. This means that these exclusions may not apply to all GTS families being studied by linkage methods and one can only be sure that the exclusion is valid for F24. These random markers can be used in conjunction with the results of the markers at candidate gene and favoured loci to test for linkage at regular intervals along all the chromosomes. Such a step is being planned for this project.

CHAPTER 6: GENERAL DISCUSSION

This study used three different methodological approaches to performing genetic linkage studies to test the hypotheses set out in chapter 5. The candidate gene approach was used to examine the possible involvement of relevant proteins in the dopaminergic and serotonergic neurotransmitter systems. The favoured locus approach was used to test the hypothesis that chromosomal regions implicated by cytogenetic abnormalities were involved. Lastly the random marker approach was used to systematically screen chromosomes. The candidate gene approach produced no positive lods. The latter two approaches produced one lod of above 2.00 and one lod above 1.00. In both cases however the positivity of the lod scores was due to a lack of informative meioses in F24 caused by low levels of heterozygosity in the markers used and the positive lods became significantly negative after further analyses using linked markers. All of the three approaches used in this thesis are equally valid in the search for genetic linkage and as shown by this study are not mutually exclusive as specific markers for candidate genes can be included as the first steps in a genome wide search. The speed of genotyping has however made it more practical to perform a complete genome search than previously. Davies et al (1994) completed a whole genome search by typing 291 microsatellite markers in approximately 400 individuals in less than a year utilising fluorescent labelling and automated sequencers to genotype the individuals. This technology is improving all the time, particularly with the use of tetra nucleotide markers which are more accurately genotyped and as a result genome searches will increase in speed.

Several research groups have been involved in a collaborative effort to localise the GTS susceptibility gene (Devor et al 1990; Heutink et al 1990; Gelernter et al 1990,1993 & 1994;

Pakstis et al 1991) and have so far excluded approximately 85% of the genome (Personnal communication TSA US). This failure to find linkage could result from the gene being localised in the remaining 15% of the genome, or more likely due to the nature of the exclusion map which was constructed under the hypothesis of homogeneity of linkage in the families studied. The exclusion map was constructed using linkage data from several cohorts of families and no one single large family was tested with every marker. The power provided by a cohort of families used in linkage studies to detect heterogeneity of linkage may be limited and positive lod scores could be missed if specific families are not large enough to yield significant lod scores on their own. Even though GTS is a complex disorder exhibiting pleiotropy, the evidence from segregation analyses suggest that the susceptibility gene is inherited in an autosomal dominant manner, which means that the maximum power for linkage will be derived for multigenerational families with unilineal transmission. It is for these reasons that the research in this thesis was targeted at just one large unilineal kindred.

The underlying mode of transmission of a genetic susceptibility to a disease has an important role in the design of experiments to detect linkage in any given family sample. Values for penetrance, disease gene frequency, allele frequencies of markers and disease classification are incorporated into the linkage calculations. Some of these parameters, such as penetrance and disease gene frequency, can be estimated from segregation analyses which also take into account the population prevalence of GTS and CMT. The values used in this thesis were derived from the segregation analysis described earlier and differ slightly from the values determined by other research groups. This difference was found in the frequency of GTS or CMT in males and females. Previous research showed a greater proponderance of males with GTS than females. In F24 no such difference existed. Therefore in contrast to our own work

other laboratories have used different penetrance values for males and females (see table 2.1). In addition only one diagnostic category (GTS & CMT) was used by the GTS collaborative research group in exclusion studies.

Table 6.1:- The penetrance values used by the collaborative groups for linkage analysis.

		faa	faA	fAA
GTS & CMT	male	0.0002-0.005	0.90	0.90
	female	0.000-0.0001	0.60	0.60

Gene frequency=0.003-0.005

These differences in penetrance and phenocopy rates should not make a significant difference in the lod scores obtained in this study or the collaborative groups exclusion studies. The differences would make the calculations in this thesis more conservative than those of the collaborative group and therefore less likely to produce a false negative lod scored due to the higher phenocopy rates and lower penetrances. Despite the failure to find linkage to GTS, once the methodological short falls of the collaborative group are ironed out, it seems likely that the GTS susceptibility loci will soon be found.

By paying attention to underlying heterogeneity of linkage and heterogeneity in the mode of transmission it should be possible to localise a GTS susceptibility allele by genetic linkage in affected families to a map resolution of one to five million base pairs or less. This can be achieved by the cloning of markers that "step over" recombinants by virtue of being genetically closer than previously used markers. This will need to be carried out with the use of samples biased heavily towards affected cases and not relying on unaffected possibly non

penetrant cases for linkage information. This approach will only provide robust estimates of the recombination fraction if non-allelic (locus) heterogeneity is not too great. The inclusion of unaffected cases would bring about uncertainty because apparent recombinants may in fact be unaffected carrier individuals. The diagnosis of the affected individuals is obviously of great importance as mis-diagnosis could lead to either a false positive or negative result. Because of uncertainty over the relationship between the aetiologies of GTS and CMT two models of affection (GTS and GTS + CMT) were used for the calculations in this thesis. The diagnostic criteria employed were those that have been found to be reliable and valid in diagnostic studies by other research groups. Yet some doubt must remain as to the use of diagnostic criteria for CMT in which it may be possible that some non genetic "phenocopies" are present in family linkage samples. Alternatively a dummy quantitative variable can be used to deal with multiple affection categories in genetic linkage analysis (Curtis and Gurling 1991).

The genes encoding susceptibility loci for a number of human diseases with some if not all of these complicating factors have been mapped using linkage analysis. A clinically defined entity among the progressive myoclonus epilepsies has been mapped by linkage to chromosome 21q22 (Lehesjoki et al 1991). Two loci have been identified for the autosomal dominant disorder Tuberous Sclerosis on chromosomes 9q34 (Fryer et al 1987) and 11q14-23 (Smith et al 1990). Linkage analysis in mice has identified two potential loci for non-obese diabetes (Todd et al 1991). A gene in the insulin-IGF2 region on chromosome 11p has been identified for HLA-DR4-dependent diabetes susceptibility (Julier et al 1991). Close linkage between the candidate gene glucokinase on chromosome 7 to early onset non-insulin dependent diabetes mellitus (Froguel et al 1992) has been found. Following studies in the NOD mouse Davies et al (1994) and Hashimoto et al (1994) performed genome wide searches

for susceptibility genes for type 1 diabetes (IDDM) using the affected sib-pair method and found 4 loci with good evidence for involvement in the disease aetiology and several more with slight evidence. Linkage has also been demonstrated between the common inherited disorder familial combined hyperlipidaemia (prevalence of 0.5%-2%) and the apolipoprotein gene cluster on chromosome 11q23-24 (Wojciechowski et al 1991). Although the genetics of hypertension generally seem to favour polygenic inheritance, a mutation consisting of a chimaeric 11 beta hydroxylase and aldosterone synthase gene causes a Mendelian subtype of the disorder (Lifton et al 1992).

At present there is a dichotomy of opinion as to the best approach to finding linkage to the genes causing complex disorders. One side argues in favour of a strategy of studying nuclear families with affected sib-pairs as Davies et al (1994) and Hashimoto et al (1994) have used in their search for IDDM genes, the other opinion favours the search for linkage in large multiplex kindreds. Each approach has its own advantages and disadvantages. Thomson (1994) has reviewed the use of the affected sib-pair method which looks for increased sharing of parental alleles over the expected 50% and suggests that this method will detect genes of only a moderate effect on disease pathogenesis which would be missed by the more stringent lod score method used for monogenic diseases. Suarez et al (1994) have shown, using computer simulation, that if a number of loci each with a moderately small effect on disease are implicated then linkage will be difficult to detect and duplicate, with both heterogeneity between data sets within a study and between studies expected. The other advantage of the affected sib-pair analysis is that no assumptions are made about the mode of transmission which, if misspecified can cause linkage to be missed. However Curtis et al (1995) argue that affected sib-pairs do not provide much power for the mapping of disease loci and that

methods exist which can detect linkage in extended pedigrees as well as sib-pairs without making assumptions concerning the mode of transmission (Weeks and Lange 1988; Curtis and Sham 1994). Fortunately for those that analyse exclusively with the lod score methodVieland et al (1992; 1993) found that, when a disease is controlled by two or more Mendelian loci, it can be modelled in a linkage analysis as a single locus mendelian disease with reduced penetrance in extended pedigrees without any loss of power or chance of a false negative result.

Field et al (1994) describe a modification to the lod score method where the mode of transmission is unknown on affected and discordant sib pairs from extended pedigrees. As a complex segregation analysis was performed on pedigree F24 and estimates of the parameters for the transmission model ascertained which agreed closely to those of other groups this suggests that linkage would be detected using the parameters described in table 2.1, even if two or more genes were acting epistatically to cause GTS. Simulations have also been carried-out on F24 to find the power of the pedigree in producing significant lod scores should linkage be present, the results of this (3.1.2) suggest that F24 provides sufficient power to generate a lod score over 3.

As described earlier a maximum lod score of 3 (an odds ratio of 1000:1) is required to assert linkage. This criterion is quite strict due to the low prior probability that the two traits are linked. It has been calculated that the prior probability that two randomly selected loci lie less than theta=0.3 apart is only 2% (Elston & Lange 1975). Therefore the posterior odds for a lod of 3 is the prior odds of linkage (0.02) multiplied by the odds provided by the data (1000:1) which gives a posterior probability of linkage of 95% and a posterior probability of

5% for a false positive.

Following the successes of positional cloning as a strategy to localise genes causing disorders with simple Mendelian patterns of inheritance geneticists are using the same approach for disorders with complex segregation patterns. Guidelines for the numbers of pedigrees required to detect linkage assuming heterogeneity have been simulated (Boehnke 1990). The problems of the extra degrees of freedom introduced by varying penetrance, affection status, and phenocopy rate (Clerget-Darpoux 1991) in the linkage analysis can be accounted for by increasing the level of significance. This may however increase the likelihood of type II statistical errors to an unacceptable level. A number of improvements have been proposed which should increase the power of linkage analysis. The methods of interval mapping and simultaneous searches can reduce the numbers of families needed to detect linkage and heterogeneity (Lander and Botstein 1986). The adverse affects of varying penetrance can be overcome by limiting the analysis to affected individuals only, however this will reduce the power of the analysis. To identify genes affecting the risk of developing GTS or genes say for a polygenic mode or oligogenic mode of inheritance the affected-sib-pair method of linkage analysis could be used (Weeks and Lange 1988; Amos et al 1990; Bishop and Williamson 1990). The disadvantage of this method is a loss of power compared to classical linkage analysis and the problem that heterogeneity of linkage between families cannot be demonstrated easily.

The autosomal dominant form of Retinitis Pigmentosa is both clinically and genetically heterogeneous. Subdivided into two groups based primarily on age of onset, there is considerable within-family variation in expression, variable penetrance and late age of onset

cases. Nevertheless in three separate studies each using single large multiplex families it was possible to detect three susceptibility loci have been identified. Chromosome 3 (Farrar et al 1990), chromosome 8 (Blanton et al 1991) and chromosome 6 (Farrar et al 1991) this demonstrates a clear advantage for using single large pedigrees.

Alzheimer's disease is also genetically heterogeneous (St George-Hyslop 1990) with some arbitrarily chosen early age of onset families (< 65) showing linkage to chromosome 21. The amyloid precursor protein (APP), a strong candidate gene for Alzheimer's disease, was mapped to the same region of chromosome 21. APP is the precursor of amyloid beta peptide the major component of amyloid plaques. This candidate was originally excluded by the demonstration of recombinants in a few families (Van Broeckhoven et al 1987) but subsequent families which were compatible for linkage with APP identified rare mutations causing the disease (Goate et al 1991; Chartier-Harlin et al 1991). Following on from the APP linkage, Alzheimers dementia has been found to be linked to mutations on three more chromosomes (van Broekhoven et al, 1992; St George Hyslop et al, 1992; Strittmatter et al, 1993).

A number of techniques have recently been improved with the use of PCR which allow the rapid identification of mutations in a candidate genes. Single strand conformation analysis (SSCP; Orita et al 1989; Poduslo et al 1991) detects changes in non-denaturing polyacrylamide gels because the electrophoretic mobility of single stranded DNA depends not only on its size but also on its sequence. Denaturing gradient gel electrophoresis has been improved by the addition of GC clamps which alters the melting properties of the fragment. This greatly increases the fraction of possible mutations that are detected (Abrams et al 1990; Traystman et al 1990). Heteroduplex DNA with mismatches denatures at different levels of

denaturant due to changes in the helix,and as a consequence are electrophoretically retarded. Another method uses RNase A to cleave at mutations between cRNA and genomic DNA (Kaufman et al 1990) alternatively chemical cleavage of mismatch heteroduplexes can also identify point mutations (Forrest et al 1991; Dianzani et al 1991). Identified mutations are then checked by direct genomic sequencing using PCR. Unfortunately, positive linkage results do not lead inevitably to the rapid identification of the disease gene. For the autosomal dominant disorder myotonic dystrophy (DM), linkage was first demonstrated to the Lewis blood group and secretor loci in 1954, but despite chromosome 19 being small and containing many polymorphic markers the disease gene has only recently been cloned and sequenced (Brook et al 1992).

GTS does present problems for molecular genetics research, but they do not really present "special" problems which are qualitatively different from those encountered in the genetic investigation of other diseases. Such research always demands a heavy investment of time and resources and it is reasonable to suppose that if adequate effort is applied then at least some of the genes involved will eventually be identified. It could be argued that the potential benefits that would accrue are relatively greater than for non-psychiatric illness, because so little is now known about the aetiology of GTS. Even a small increase in our understanding will represent a large step forward in neuroscience. The localisation, sequencing and cloning of a susceptibility locus with the subsequent identification of its product would mean that the whole disease process of this complex syndrome could be fully understood. The cloning of a gene or genes causing genetic susceptibility to GTS will provide unique insights into brain function and complex neuropsychological processes.

CHAPTER 7: REFERENCES

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