MAPPING A BALANCED TRANSLOCATION t(1;11)(q42.2;q21) LINKED TO SCHIZOPHRENIA

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Declaration

I declare:

a) that this thesis was composed by myself and

b) that the work is my own, except where otherwise stated.

ABSTRACT

This thesis describes progress towards the mapping and cloning of the breakpoints of a balanced translocation t(1;11)(q42.2;q21) which is linked to schizophrenia and other major mental illness in an extensive Scottish pedigree. Schizophrenia is one of the principal forms of major mental illness; the lifetime risk for the disorder is approximately 1%. Very little is known about the aetiology of schizophrenia. The clustering of psychiatric illness within families and the results of twin and adoption studies indicate that major mental illness has a considerable genetic component. However the localisation, by linkage analysis, of genes that are responsible for psychiatric disorders is difficult. Mental illnesses exhibit genetic heterogeneity, phenocopies, assortative mating, incomplete penetrance, unclear mode of inheritance, uncertainties in diagnostic boundaries which all complicate linkage analysis. The process of gene localisation is greatly simplified by the presence of a chromosomal rearrangement. The balanced translocation which segregates with major mental illness in this family is therefore a very important, as well as a relatively rare, resource. In order to map the breakpoints of the translocation a series of somatic cell hybrids has been characterised by PCR and Southern analysis. This analysis has resulted in the determination of flanking markers of the breakpoints on both translocated chromosomes and in the production of a panel through which markers can be mapped, at high resolution, with respect to the chromosome 11 breakpoint region. To generate new markers for the region, microdissection and microcloning of the derived chromosome 1 was undertaken by others and I undertook to map the clones generated in this way. In total I have mapped 28 new microdissection markers, either to chromosome 1 or to a series of intervals on chromosome 11q. The markers which were found to map immediately distal to the chromosome 11 breakpoint were used to isolate YACs from that region. On the basis of co-hybridisation, fingerprint and pulsed field gel electrophoretic analysis, the YACs were assembled into a contig. Pulsed field gel electrophoretic analysis, of genomic DNA from an individual carrying the translocation, compared with DNA from a control individual, with a probe in the interval closest to the translocation breakpoint identified differences in three restriction fragments. This data was consistent with the translocation breakpoint being within approximately 550 kb of this marker.

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ABBREVIATIONS

A	adenine
ABS	absorbance
ACTN2	α-actinin2
ADROA2	adenosine receptor for adenine 2
ASP	affected sib pair analysis
BAC	bacterial artificial chromosome
bp	base pairs
BSA	bovine serum albumin
C	cytosine
Calc I	calcitonin 1
cDNA	complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humaine
CLG	fibroblast collagenase
CIP	calf intestinal phosphatase
cM	centimorgan
CMGT	chromosome mediated gene transfer
CT	computed tomography
°C	degrees centigrade
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dGTP	deoxyguanidine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytodine triphosphate
ddGTP	dideoxyguanidine triphosphate
ddTTP	dideoxythymidine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRD2	dopamine receptor D_2
DSM III R	Diagnostic and Statistical Manual of Mental Disorders III, revised
DDT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid disodium salt
EST	expressed sequence tag

FCS	foetal calf serum
Fes	feline sarcoma oncogene
FGF4	fibroblast growth factor 4
FISH	fluorescent in-situ hybridisation
G	guanine
GSTPI	glutathione S-transferase π
HBB	β-haemoglobin
HRAS1	Harvey rat sarcoma viral oncogene homolog 1
ICD	International Classification of Diseases 9
IPTG	Isopropyl β-D-Thiogalactopyranoside
kb	kilo bases
LMP	low melting point
LOD	logarithm of the odds
Mb	mega base
MD	microdissection clone
ME2	malic enzyme 2
Mod-2	malic enzyme, mitochondrial
MPA	minor physical abnormality
mRNA	messenger RNA
MRI	magnetic resonance imaging
NCAM	neural cell adhesion molecule
OMP	olfactory marker protein
PAH	phenylalanine hydroxylase
pBS	bluescribe
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEA	phenylethylamine
PFGE	pulsed field gel electrophoresis
PGA	pepsinogen A
PGBD	phorphobilinogen deaminase
PKU	phenylketonuria
PMSF	phenylmethylsulfonylflouride
pers. comm.	personal communication
RDC	Research Diagnostic Criteria
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROM1	rod outer membrane protein 1

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate
STMY1	stromelysin 1
STS	sequence tagged site
THY1	cell surface antigen thy1
T_m	melting temperature
TYR	tyrosinase
TRYLI	tyrosinase like
UV	ultraviolet
VNTR	variable number of tandem repeat sequences
XGAL	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YAC	yeast artificial chromosome

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CHAPTER 1

1. Introduction

1.1 Introduction

This thesis describes progress towards the mapping and cloning of the breakpoints of a balanced translocation t(1;11)(q42.2;q21) which appears to segregate with schizophrenia and other major mental illnesses in a Scottish pedigree.

Schizophrenia is one of the most common major mental illnesses, affecting approximately 1% of the world's population at some point in their lives (Gottesman and Shields, 1982). It is a disabling disorder which begins in adolescence or young adulthood. The symptoms of schizophrenia have been divided into those which characterise the acute phase of the disorder (such as thought disorders, delusions, hallucinations, bizarre behaviour and catatonia) which are known as the positive symptoms. The negative symptoms, which tend to occur before and after the acute phase, include lack of motivation, social withdrawal, emotional apathy and attentional impairment (Michels and Marzuk, 1993).

Schizophrenia was defined as a distinct condition in the late 19th Century by Kraepelin (1919). Kraepelin divided severe mental illness into manic depressive insanity and dementia praecox. Dementia praecox was renamed "the schizophrenias" approximately 20 years later by Bleuler (1950) who argued that symptoms such as delusions and hallucinations were secondary to primary symptoms of thought association problems, ambivalence, affective abnormalities and autistic thinking (Gallant, 1990).

Currently there are a number of different diagnostic criteria for mental illnesses. Three major ones are the Diagnostic and Statistical Manual of Mental Disorders III, revised (DSM-III-R) of the American Psychiatric Association, the International Classification of Diseases 9 (ICD 9) and the Research Diagnostic Criteria (RDC). The diagnostic criteria for schizophrenia differ between these systems and a recent survey on the use of these by French and British psychiatrists found that different diagnostic criteria were used to diagnose schizophrenia in the two countries (van Os et al., 1993). Thus there is no consensus as to the precise definition of

schizophrenia and there is no biological marker for external validation. Consequently it is possible, if sufficient criteria are used, to produce a narrow definition of schizophrenia, which will allow reliable diagnosis. However, this will result in a high false negative rate.

The symptoms of schizophrenia are highly variable between individuals (Gottesman and Shields, 1982). It seems likely therefore that the term schizophrenia covers a group of disorders. Also, family (Baron et al., 1985; Kendler et al., 1985) and adoption (Kety et al., 1971) studies have shown that schizophrenia spectrum disorders (conditions which involve considerable overlap of symptoms with schizophrenia) such as schizotypal personality disorders, occur at high frequency in the relatives of schizophrenics, suggesting a genetic relationship between these disorders and schizophrenia. There is also doubt about the delineation drawn between schizophrenia and the affective disorders. The term affective disorders covers a group of illnesses which are characterised by disturbance in mood. The main forms of major affective disorder are unipolar major depression and bipolar disorder. In bipolar disorder (also known as manic-depressive illness) affected individuals suffer episodes of mania, as well as periods of depression. The controversy over whether schizophrenia and the affective disorders are biologically distinct is due to the existence of schizoaffective disorder, an illness which has features of both schizophrenia and affective disorder, and to the reports of monozygotic twins and triplets where one co-twin has been diagnosed as schizophrenic and the other as suffering from an affective disorder (McGuffin et al., 1982; Dalby et al., 1986; Farmer et al., 1987 and Lohr and Bracha 1992)

1.2 Epidemiological Aspects of Schizophrenia

1.2.1 Incidence

Schizophrenia is a very common disorder; the lifetime risk is approximately 1% (Gottesman and Shields, 1982). The incidence in males is slightly higher than in females (Gottesman and Shields, 1982). Unlike many diseases the prevalence of schizophrenia appears to be fairly similar world wide. There is evidence for areas of slightly higher prevalence, for example

in Northern Sweden, Western Ireland and Finland, but it is very difficult to assess these data with accuracy as different diagnostic criteria have been used in the different studies (Wyatt et al., 1988).

The incidence of schizophrenia is high considering that the fertility of schizophrenics is reduced (estimated to be 70% of that of socio-economically comparable unaffected individuals) (Wyatt et al., 1988). The reasons for the higher than expected incidence of schizophrenia are unknown. Hypotheses include a reproductive advantage to those who carry the gene (in the case of a recessive mutation) or to those who have the genetic predisposition but do not develop schizophrenia (in the case of a dominant mutation). There is some evidence for increased fertility in relatives of schizophrenics (Erlenmeyer-Kimling and Paradowski, 1966; Carter and Watts, 1971).

It may not be necessary to invoke a positive advantage for mutations which predispose to schizophrenia. It is possible that variable expression and lack of full manifestation of mutant alleles will reduce the ability of natural selection to remove these alleles from the population. Also the adult age of onset of schizophrenia implies that a proportion of individuals will have reproduced before the onset of schizophrenic symptoms. Finally, if there are a number of genes which when mutated have the potential to cause schizophrenia then, all other things being equal, the observed disease frequency will be higher than if only one gene were involved (Ridley and Baker, 1990).

1.2.2 Socio-economic Status

There is a higher than average incidence of schizophrenia amongst the lower socio-economic classes of large urban areas (Kohn, 1977). There are several possible explanations for this observation. These include "downward drift" of affected individuals; that greater stress is encountered in urban areas, which precipitates the disorder in susceptible individuals and that the crowding found in these areas would facilitate spread of any infectious causative agent (Wyatt et al., 1988)

1.2.3 Course and Outcome

There is considerable variation between individuals regarding the course and the outcome of the disorder (Michels and Marzuk, 1993). Some individuals (estimated at 14-30 % of patients) have a single episode followed by a full recovery. Most, however, have persistent or episodic psychopathologic symptoms and vocational and social difficulties. A proportion of these patients experience progressive deterioration. Females tend to have a better prognosis than males (Castle and Murray, 1991).

There are suggestions that the initial clinical symptoms of schizophrenia can be precipitated by stress (Wyatt et al., 1988). There is more substantial evidence that relapses are related to stress and that decreasing stress, particularly stress caused by the emotional atmosphere in the patient's home can decrease relapse frequency (Wyatt et al., 1988). Negative emotions such as criticism of the patient, over-involvement and hostility are measured in the form of an index of expressed emotion (EE). EE values are measured in close relatives of patients and it has been found that half of patients in high EE homes suffered a relapse over a nine month period compared with less than one quarter of patients in low EE homes. The highest levels of expressed emotion are generally found in cities in the West while rural communities in developing countries usually exhibit the lowest EE levels. Correspondingly, clinical and social outcome tends to be better in developing countries than developed ones (Leff et al., 1992).
The level of premorbid functioning (for example whether the person has a job) appears to be an important factor in predicting outcomes of schizophrenia (Eaton, 1991). Poor premorbid functioning tends to predict a poor outcome.

1.2.4 Season of Birth

Examination of the date of birth of schizophrenics indicates that schizophrenics are slightly more likely (7-15 %) to be born in late winter and spring. Most of this excess occurs within schizophrenics without a family history of psychiatric illness (Jones and Murray, 1991). The explanation for this well-established phenomenon is unknown. Hypotheses include a higher likelihood of *in utero* exposure to viral infections, which are known to vary

according to season and an increase in conceptions in late spring or early summer by parents predisposed to having schizophrenic offspring (Wyatt et al., 1988). A number of studies have been carried out to test whether prenatal exposure to influenza is likely to increase the risk of schizophrenia in adulthood (for example Mednick et al., 1988; Kendell et al., 1989; Bowler and Torrey, 1990 and O'Callaghan et al., 1991). These studies come to conflicting conclusions.

1.2.5 Age of Onset

The vast majority of schizophrenia cases exhibit adult onset; 90% of cases have an onset between 15 and 45 years of age (Roberts and Brunton, 1990). The peak age of onset differs between the sexes. In males the peak occurs between ages 18-25 whereas in females the peak is broader at 26-45 years (Stromgren, 1988).

1.2.6 Association of Other Psychiatric Illnesses with Schizophrenia

Varma et al. (1992) compared psychiatric morbidity in 1018 first degree relatives of schizophrenics to that found in the first degree relatives of a similar number of control patients. It was found that the relatives of schizophrenics were at a significantly increased risk. Psychiatric morbidity was observed in 34.8 % of first degree relatives of schizophrenic patients as compared to 9.2 % of first degree relatives of controls.

Kendler et al., (1985) studied psychiatric morbidity in 723 first degree relatives of schizophrenics as compared with that found in the relatives of controls. The following diagnoses were increased in the relatives of schizophrenics versus controls: chronic schizophrenia (3.7 % versus 0.2 %); schizoaffective disorder (1.4 % versus 0.1 %); paranoid disorder (0.9 % versus <0.1 %) and atypical psychosis (2.5 % versus 0.3 %). A similar study examined psychiatric morbidity in 376 first degree relatives of schizophrenics as compared with that found in the relatives of controls (Baron et al. 1985). The following diagnoses were increased in the relatives of schizophrenics versus controls: chronic schizophrenia (5.8 % versus 0.6 %); schizotypal personality disorder (definite) (14.6 % versus 2.1 %); schizotypal personality disorder (probable) (12.1 % versus 6.5 %) and paranoid personality disorder

(7.3 % versus 2.3 %). In a subsequent study, the relatives of schizophrenics that had diagnoses of major affective disorder were examined to see if this was associated with increased familial risk of schizophrenia and related disorders (Baron and Gruen, 1991). It was found that the relatives of schizophrenic patients with a family history of affective illness had a greater risk of schizophrenia and schizophrenia-related disorders when compared to the relatives of patients without such a family history.

Many groups have investigated the genetic relatedness of schizophrenia to the affective disorders (reviewed in Taylor, 1992); it was found that schizophrenia and affective disorders do co-occur in a proportion of families with affected individuals.

The existence of schizoaffective disorder also indicates that the separation of schizophrenia and affective illness may not be valid. There is considerable variation between DSM-III-R, ICD -9, and RDC diagnostic criteria for schizoaffective disorder (reviewed by Lapensee, 1992). It appears that there are a number of different illnesses or subtypes encompassed by the term (Lapensee, 1992). The relatives of patients with schizoaffective disorder appear to be at a higher risk to both schizophrenia and affective disorder (Lapensee, 1992).

Examples of identical twins who are discordant for psychiatric illnesses also exist. McGuffin et al. (1982) reported identical triplets two of whom were schizophrenic while the third had bipolar illness. Dalby et al. (1986) reported a pair of monozygotic twins, one of whom was diagnosed as suffering from schizophrenia and the other from mania. A similar case was reported by Lohr and Bracha (1992). A larger twin study found that of 26 monozygotic twin pairs where at least one co-twin was schizophrenic in five cases the co-twin had been assigned to a DSM-III affective disorder category (Farmer et al., 1987).

These studies clearly indicate that there is a genetic relationship between schizophrenia and other psychiatric illness.

1.3 Pathology of Schizophrenia

Post-mortem studies of brain weights of schizophrenic patients have found that the disease is associated with a decrease in total brain weight and length (Roberts and Brunton, 1990). Reductions were also suggested in cerebral hemispheric and cortical volumes (Roberts and Brunton, 1990). More consistently, studies find increases in ventricular size and reductions in size of medial temporal lobe structures associated with schizophrenia (Waddington, 1993).

Magnetic resonance imaging (MRI) and computed tomography (CT) scanning are non-invasive *in vivo* methods which have been used in a number of studies (summarised in Jones and Murray, 1991) to compare brain structure in schizophrenics and matched controls. Many CT studies have found that a large proportion of schizophrenics have enlarged cerebral ventricles, although there is controversy over the proportion of cases and the extent of the enlargements. It is clear however that any ventricular enlargement is present at the onset of positive symptoms, is not related to illness length and shows no evidence of progression on follow-up (up to 8 years).

MRI studies have confirmed the increase in ventricular size in a proportion of schizophrenics. These studies also found that temporal lobe structures, in particular the hippocampus, were reduced in size. One study reported that the superior temporal gyrus was decreased in volume, particularly on the left side (Barta et al., 1990). The reduction in size of this region, which is an auditory association area, appeared to correlate with the severity of hallucinations.

Where sex differences have been reported, usually greater deviations from normal have been noted in males than in females (Jones and Murray, 1991). Monozygotic twins discordant for schizophrenia have been shown to have differences in ventricular volumes (Jones and Murray, 1991). Two studies have found that in the majority of cases the schizophrenic twin had larger cerebral ventricles. The second study also found that the volume of grey

matter in the left temporal lobe was smaller in the schizophrenic twin than the unaffected twin.

Schizophrenia-like symptoms occur at a higher than expected frequency in temporal lobe epilepsy (Jones and Murray, 1991). Such findings prompted studies of the limbic temporal lobe of schizophrenics. Decreases from expected sizes were found, particularly in the hippocampus.

Three studies have examined the frequency of minor physical abnormalities (MPAs) in schizophrenics versus controls (reviewed in Jones and Murray, 1991). MPAs are trivial abnormalities in ectodermal development, such as abnormalities in hand morphology. All three studies found an increased incidence of MPAs in schizophrenics relative to controls, the highest frequency being found in familial schizophrenia cases. MPAs are indicative of pathological processes in foetal life (Bracha et.al., 1991). The period of ectodermal development affected can be deduced from the nature of the ectodermal abnormalities. These studies suggest the influence of factors during the fourth and fifth months of gestation (the first two thirds of the second prenatal trimester) (Bracha et al., 1991). This is the period during which neural migration to the cortex occurs (Bracha et al., 1991). A study of 23 pairs of monozygotic twins which were discordant for schizophrenia found that, compared to the unaffected co-twin, affected co-twins had significantly higher total scores of fourth and fifth month hand dysmorphologies (Bracha et al., 1991). This may reflect differential *in utero* exposure to, for example ischemic or toxic, insults which are known to affect ectodermal development during this developmental period.

As mentioned above, decreases have been observed in hippocampal volumes in a proportion of schizophrenics and lower hippocampal cell counts have been observed in a proportion of schizophrenic patients (Jones and Murray, 1991). This deficit could be caused either by neuronal degeneration or by a developmental abnormality. This can be determined by examining the brains of schizophrenics for gliosis. Gliosis is the term for the proliferation of glial cells and increase in glial-associated proteins which occurs in response to all types of disease processes which result in neuronal damage (Roberts, 1990). Gliosis has not been found in the majority of schizophrenic brains examined (Jones and Murray, 1993). This suggests

that the observed deficit in hippocampal volume in schizophrenia is caused by disturbances in brain development, rather than neurodegenerative processes or destructive lesions. Post-mortem studies indicate that the decreased cell volume may be due to a premature arrest of neuronal migration into the hippocampus and parahippocampal gyrus (Jakob and Beckmann, 1986; Falkai et al., 1988). This hypothesis is consistent with the observation that the lesions seem to predate the onset of symptoms and do not exhibit progression.

The results discussed above suggest that schizophrenia may be caused by defects in brain development, in particular in defects in the development of medial temporal lobe structures. Medial temporal lobe structures are thought to play a crucial role in the integration and processing of information from association cortex (Roberts, 1990). It is possible that defects in these areas could cause symptoms such as those which make up the core of those found in schizophrenia (Roberts, 1990) (although they could not account for the all abnormalities found in schizophrenia). Possible causes of defects in brain development are discussed in Section 1.4.

1.4 Aetiology Of Schizophrenia

1.4.1 Evidence for Genetic Predisposition

Family, adoption and twin studies have overwhelmingly established that there is a major genetic component to schizophrenia. The various types of studies indicate that the relatives of schizophrenics are at a higher risk of developing schizophrenia than the 1% risk which applies to the general population.

Family studies examine the family of a schizophrenic patient for psychiatric illness. Pooled Western European life-time risks for definite and probable schizophrenia in systematic family and twin studies, 1920-1987 (Gottesman and Bertelsen, 1991) indicate that the risk of schizophrenia in children of schizophrenics is 13 %, the risk to sibs is 9 % and the risk to parents is 6 %. If one parent is schizophrenic then the risk of schizophrenia in a sibling of a schizophrenic is 17 % and if both parents are schizophrenic then the risk of

schizophrenia to offspring is 46 %. These figures indicate that schizophrenia is familial, but they do not distinguish between genetic influences and within family environmental influences.

Twin studies control for some of the shared environmental component of schizophrenia. This is done by comparing concordance rates for schizophrenia in monozygotic and same-sex dizygotic twins. A genetic component of schizophrenia would manifest itself as a higher concordance in monozygotic twins than dizygotic twins. Pooled Western European life-time risks for definite and probable schizophrenia in systematic family and twin studies, 1920-1987 (Gottesman and Bertelsen, 1991) indicate that concordance in dizygotic twins is 17 %, while in monozygotic twins it is 48 %.

An extension to a twin study was described by Gottesman and Bertelsen (1989) who followed-up from, and confirmed the findings of, Fischer (1971). They studied the incidence of schizophrenia in the adult offspring of concordant and discordant monozygotic and dizygotic same-sex twin pairs. The offspring of pairs of monozygotic twins are related to both co-twins as first degree relatives while the offspring of dizygotic twins are related to the other co-twin as second degree relatives. Thus the risks to offspring of the same-sex dizygotic co-twins serve as controls for risks to first and second degree relatives under the study conditions. When the twin pair is discordant for schizophrenia, the offspring of the normal co-twin share little or none of the exposure to a schizophrenic parent, thus controlling for this within family environmental factor.

The study found that the risk of schizophrenia was 16.8 % in the offspring of the affected monozygotic co-twin and 17.4 % in the offspring of the unaffected monozygotic co-twin. The risk of schizophrenia was 17.4 % in the offspring of the affected dizygotic co-twin and 2.1 % in the offspring of the unaffected dizygotic co-twin. The difference in the risk for schizophrenia in the offspring of the unaffected monozygotic and dizygotic co-twins suggests that the genetic component to schizophrenia is high. The study also suggests that when discordance is observed for schizophrenia in monozygotic twins this is mainly due to a failure of expression of the genetic predisposition to schizophrenia (Gottesmann and Bertelsen, 1989). This study provides information about the possible mode of inheritance of

schizophrenia, which is unclear. It suggests that, in at least a proportion of cases, schizophrenia has a dominant mode of inheritance with reduced penetrance.

Adoption studies have also been used to investigate whether schizophrenia has a significant genetic component. The risk of schizophrenia in adopted away infants with schizophrenic biological parents was compared with the incidence in control adoptees who had unaffected biological parents (Heston, 1966). An age-corrected rate for schizophrenia of 16.6 % was found in the adoptees with a schizophrenic biological parent compared to a rate of <2 % in control adoptees. A study into the rates of schizophrenia-spectrum disorders in biological and adoptive family members of schizophrenic adoptees found that 8.7 % of biological relatives had some schizophrenic disorder, compared to 1.9 % of the adoptive family members (Kety et al., 1971). Adoption studies therefore also indicate a significant genetic component to schizophrenia.

1.4.1.1 Candidate Genes

There are many possible candidate genes for schizophrenia. As mentioned above, defects in normal brain development may be responsible for schizophrenia. Genes involved in the development of the brain are therefore candidate schizophrenia causing genes. Likely candidates are genes which are involved in development of the medial temporal lobe (in particular hippocampal formation) possibly those which are involved in control of neuronal proliferation and migration into the medial temporal lobe (Jones and Murray, 1991).

Genes involved in dopaminergic function have long been proposed as candidate schizophrenia genes. It was initially proposed that schizophrenia was caused by a hyperdopaminergic state. The evidence for this hypothesis is that the clinical effectiveness of the conventional neuroleptics is correlated with their ability to block dopamine receptors. Also, drugs which increase dopamine activity generally worsen the symptoms of schizophrenic patients (Davis et al., 1991). There are however a number of facts which are inconsistent with this hypothesis. Firstly, a substantial proportion of patients are resistant to neuroleptic treatment which suggests that other

neurochemical systems are involved in the disease in these patients (Davis et al., 1991). If drugs which increase dopaminergic activity are given to non-schizophrenics they do not develop schizophrenia-like symptoms (Davis et al., 1991). Neuroleptics are only partially successful in alleviating the negative symptoms of the disorder, suggesting that these symptoms can not be fully explained by an excess of dopaminergic function (Davis et al., 1991).

There is evidence for hyperdopaminergic functioning in mesolimbic neurones but post-mortem and cerebrospinal fluid studies of dopamine metabolites do not indicate a homogeneous excess in dopamine metabolism in schizophrenia and may be indicative of frontal lobe hypodopaminergia (Davis et al., 1991). This has been suggested as an explanation for the concurrent presence of positive and negative symptoms in schizophrenics. Dopaminergic hyperfunction of mesolimbic neurones has been proposed as a cause of the positive symptoms; and dopaminergic hypofunction of the frontal lobe has been proposed as a cause of the negative symptoms of the disorder (Davis et al., 1991; Waddington, 1993).

It is possible that the alterations in dopamine levels are a secondary effect of the primary schizophrenic causing lesion. The fact that drugs which increase dopaminergic activity do not induce schizophrenia-like symptoms in non-schizophrenics (Davis et al., 1991) would support this. If this is the case, then hypotheses as to the nature of the primary defect in schizophrenia must account for the difference between schizophrenics, who are sensitive to manipulation of dopaminergic neurotransmission, and non-schizophrenics who do not develop schizophrenia-like symptoms when administered drugs which augment dopamine activity (Davis et al., 1991).

A study of the dopamine D_4 gene, which has a high affinity for clozapine (Van Tol et al., 1991), an atypical neuroleptic which treats schizophrenic symptoms without causing Parkinsonian side effects, revealed that the receptor is polymorphic and that the different variants exhibit differences in pharmacological properties (Van Tol et al., 1992; Lichter et al., 1993). The authors suggest that such variation may result in differences in drug response and/or differences in the susceptibility to the development of psychiatric disorders. A recent study of post-mortem brains of schizophrenics (Seeman et al., 1993) found that the number of D_4 receptors

was six-fold higher in the brains of schizophrenics relative to the brains of controls.

The serotonin$_2$ receptor can be considered a candidate gene for schizophrenia, because of its affinity for the atypical neuroleptic clozapine (Hallmayer et al., 1992). The serotonin$_2$ receptor has been mapped to chromosome 13 (Hallmayer et al., 1992). Linkage analysis was carried out between the receptor, other chromosome 13 markers and schizophrenia in a Swedish family (Hallmayer et al., 1992). The study ruled out involvement of the serotonin$_2$ receptor gene in schizophrenia in this family (Hallmayer et al., 1992).

Polymorphisms at the porphobilinogen deaminase gene (PGBD) have been studied in two sets of schizophrenics (Sanders et al., 1991; Owen et al., 1992). One group of patients were found to have a higher than expected frequency of a particular allele, indicating an association of that allele, or of a closely linked gene with the disorder (Sanders et al., 1991). However this finding was not replicated in the second group of patients studied (Owen et al., 1992). The discrepancy in the outcome of the studies may be due to a difference in the patient groups under study.

Sobell et al. (1993) tested 200 unrelated schizophrenics for the presence of known mutations at the phenylalanine hydroxylase (PAH) locus (which cause the autosomal recessive disease phenylketonuria, PKU). PAH was considered as a candidate for a number of reasons (Sobell et al., 1993). Firstly, individuals suffering from PKU, and their first degree relatives, are reported to suffer from behavioural abnormalities, including psychosis. Schizophrenic individuals have been observed to have elevated levels of phenylethylamine (PEA) in their plasma and urine; excess PEA is a result of absence of PAH activity. The structure of PEA is also very similar to amphetamine, which can mimic some of the symptoms of schizophrenia, and PEA and amphetamine cause the same behavioural abnormalities in test animals (exaggerated motor activity and stereotypy). Finally, carriers of PKU have only 10% of wild type PAH levels. This level of reduction in other pathways, in the presence of environmental stress, often result in substantial morbidity.

When individuals suffering from schizophrenia were typed for these two particular mutant alleles the mutations were not found in levels above those expected. It is of course possible that other mutant alleles at this locus could be responsible for a proportion of cases of predisposition to schizophrenia.

Jones et al. (1992) reported a nucleotide substitution, which was predicted to cause an amino acid substitution, in the amyloid precursor protein (APP) gene in a schizophrenic individual who also had cognitive deficits.

Mutations in the APP gene have been shown to be responsible for a proportion of cases of familial Alzheimer's disease. Eighty-six schizophrenics were subsequently tested for this substitution but no positives were identified (Coon et al., 1993).

There have been reports of albinism and mental illness co-occurring in more than one individual in a family. Baron (1976) reported a pedigree where five individuals had oculocutaneous albinism (OCA) and schizophreniform psychosis. One non-albino member of the pedigree had schizophrenia and all others had neither albinism of mental illness. A family where two siblings out of three have both schizophrenia and tyrosinase negative OCA has also been reported (Clarke and Buckley, 1989). As tyrosinase mutations can be responsible for OCA, tyrosinase,or a linked gene, has been suggested as a candidate gene for schizophrenia.

Within the last two years expansions of trinucleotide repeats within genes have been found to be responsible for at least four different diseases (summarised in Mandel, 1993). These diseases exhibit anticipation, i.e. increase in disease severity, decrease in age of onset and increased frequency of affected individuals in successive generations, which largely correlates with successive increases in the number of repeats present in the mutated gene. The existence of anticipation in psychiatric disorders was suggested as early as 1911 (Mott, 1911) and evidence supporting the existence of anticipation in bipolar affective disorder has been published recently (McInnis et al., 1993). Genes which contain trinucleotide repeats which are susceptible to expansion may therefore be candidates for schizophrenia.

1.4.2 Evidence for Environmental Component

As described in section 1.4.1 there is clearly a significant genetic component to schizophrenia. However, every study has found that the genetic component cannot account for all of the variation observed between affected and unaffected individuals. For example, while monozygotic twins clearly exhibit a higher concordance for schizophrenia than dizygotic twins, this concordance is only approximately 50%. This level of discordance in genetically identical individuals is strong evidence for a non-genetic component to the disorder. A major part of the non-genetic component is likely to be environmental factors which affect individuals differentially and which will therefore alter the likelihood of a genetically predisposed individual becoming schizophrenic.

As mentioned previously, monozygotic twins discordant for schizophrenia have been shown to have differences in ventricular volumes (Jones and Murray, 1991). Two studies have found that in many cases the schizophrenic twin had larger cerebral ventricles and the second study also found that the volume of grey matter in the left temporal lobe was smaller in the schizophrenic twin than the unaffected twin. The frequency of minor physical abnormalities (MPAs) also differs between discordant monozygotic co-twins (schizophrenic co-twins exhibiting a higher number of MPAs). This may indicate that the co-twins differ in their exposure to environmental factors *in utero*. Co-twins may also differ in their exposure to environmental factors after birth. Possible examples of such environmental insults are discussed in section 1.4.2.1.

Further evidence for an environmental component to schizophrenia comes from the observation that schizophrenics, particularly those with no family history of psychiatric illness, are slightly more likely to be born in late winter and spring (Jones and Murray, 1991).

1.4.2.1 Candidate Environmental Factors

The environmental factors most consistently associated with schizophrenia are obstetric complications (Jones and Murray, 1991). It has been found that schizophrenics have had an excess of prenatal and perinatal problems

when they are compared with controls and with patients suffering from other psychiatric disorders. A number of reports have argued that obstetric complications can cause disturbances in early brain development which result in mental illness in adult life. However, an alternative hypothesis is that obstetric complications may be an indicator of an earlier insult to the nervous system, or even a secondary consequence of a genetic defect in neural development.

As the pyramidal cells of the hippocampus are one of the cell types most vulnerable to mild anoxia-ischemia this could cause damage preferentially to this region of the brain (Jones and Murray, 1991). Other prenatal insults which have been proposed as factors which may contribute to schizophrenia include anaemia, toxic exposure, twin transfusion syndrome and exposure to viral infections (Bracha et al., 1991).

As mentioned previously, it has been suggested that prenatal exposure, during mid-pregnancy, to viral infections increases the risk of schizophrenia in adult life (Wyatt et al., 1988). The mechanism by which viral infections could cause an alteration in normal brain development is unclear. One hypothesis is that neuraminidase-bearing viruses, e.g. influenza, which can interfere with the adhesive actions of neural cell adhesion molecules, may upset the migration of hippocampal cells (Conrad and Scheibel, 1987). Immunological mechanisms of schizophrenia predisposition have also been proposed. For example Wright et. al. (1993) have suggested that a mother's genotype may cause her to produce a vigorous antibody reaction to viral infection, and that during pregnancy, these antibodies cross the placenta and the immature foetal blood brain barrier and cause neurodevelopmental abnormalities in the foetal brain.

It has also been suggested that stress may cause schizophrenia in a predisposed individual. The particular types of stress which appear to have this effect have been studied (Leff, 1992). Life events, i.e. sudden discrete events which occur in a person's life and require them to adapt psychologically, such as death of a close relative or moving house, seem to be clustered before an episode of illness. However, this has been found to be true for a number of very different illnesses, for example heart attacks and depression. Another form of stress which has been quantified is the

emotional atmosphere in the home. This cannot, however, be used to judge whether this type of stress precipitates an initial episode of schizophrenia.

1.5 Treatment of Schizophrenia

As the causes of schizophrenia are unknown, rational treatment is not possible. The effectiveness of neuroleptic (or antipsychotic) drugs at relieving the symptoms of schizophrenia were discovered by chance and without any knowledge of their effects on brain chemistry.

Neuroleptic drugs block dopamine receptors and are effective, in many cases, in treating the positive symptoms of the disorder (Johnstone, 1993). However, about 30 % of patients show only limited improvement in acute treatment trials and approximately 7 % have no response to neuroleptics even when treated over a long period of time. Neuroleptics are partially successful in the prevention of relapse but are of limited effectiveness against the negative symptoms of the disorder. They also have the disadvantage of producing short and long-term extrapyramidial side effects, anticholinergic side effects, weight gain and tiredness (Johnstone, 1993). Clozapine, which differs from the other dopamine blocking agents in terms of its affinity for the different dopamine receptor subtypes (Van Tol et al., 1991), produces fewer extrapyramidial side effects (Johnstone, 1993). This use of this drug has however been curtailed because it causes agranulocytosis in a small proportion of patients (Johnstone, 1993). Other antipsychotic drugs which do not block dopamine receptors are occasionally used to treat schizophrenia. These however are generally not as effective as the neuroleptics (Johnstone, 1993).

Electroconvulsive therapy (ECT) has been used in controlled trials on schizophrenic patients (reviewed in Johnston, 1993). Those patients who were also being treated with neuroleptics responded to ECT treatment, but this improvement had disappeared on follow-up 12 weeks after treatment.

Various treatments based on psychology have been used in attempts to remove particular positive symptoms. These techniques seem to be only

partially effective; some symptoms are lessened in some patients (Johnstone, 1993).

In summary, schizophrenia is a relatively frequent, disabling disorder of adolescence or young adulthood. It appears likely, at least in a proportion of cases, that the disorder is caused by abnormal brain development. Schizophrenia has a large genetic component but the expression of the disorder is subject to environmental influence. Treatment of the disorder tends to be only partially effective and the majority of sufferers remain affected to some degree for the rest of their lives.

1.6 Positional Cloning of Monogenic Disorders

1.6.1 Introduction

The process of cloning genes solely by virtue of their position in the genome, previously known as "reverse genetics", is now generally referred to as "positional cloning". Most success in this field has been achieved for well defined, but relatively rare monogenic disorders. Positional cloning of the common polygenic disorders (for example psychiatric illness, cancer and cardiovascular disease) is much more difficult (see section 1.7.1). Many of the human polygenic disorders for which predisposing genes have been successfully isolated have been those cancers which involve chromosomal rearrangements (for example, isolation of the myeloid/lymphoid, or mixed lineage, leukaemia (MLL) gene, Ziemin-van der Poel et al., 1991).

1.6.2 Linkage Analysis and Mapping

The first step in a positional cloning strategy is establishing either physical or genetic linkage to the disease causing gene. This requires the collection of families where the disease gene is segregating. In some cases a chromosomal rearrangement, such as a translocation or a deletion, will be observed to segregate with the disease. Such rearrangements can be detected either by cytogenetics or Southern blotting. The detection of a cytogenetically visible chromosomal rearrangement is the quickest way of establishing such a linkage. The searches for most of the genes isolated by

positional cloning to date have exploited rearrangements found to be linked to the disease. Recently another type of rearrangement associated with human disease has been characterised. Expansions of intragenic trinucleotide repeats have been directly linked to X-linked spinal and bulbar muscular atrophy, fragile X syndrome, myotonic dystrophy and Huntington's disease. This results in an alteration of DNA structure which is readily detectable by Southern analysis.

In the absence of a detectable rearrangement, co-inheritance of the disease with arbitrary markers (usually of known chromosomal location) is studied. Restriction length fragment polymorphisms (RFLPs) were the first class of polymorphic DNA markers to be described. There are of two types of RFLP. In the first, alterations in the DNA sequence create or destroy a site for a particular restriction enzyme. Thus digestion with that enzyme, and hybridisation with an internal probe, results in different size bands in different chromosomes. This type of RFLP however, tends to have only two alleles and consequently, many individuals are not informative (i.e. are not heterozygous). The second type of RFLP is a consequence of minisatellite arrays or variable number of tandem repeat sequences (VNTRs) which are 11-60 bp units repeated in tandem arrays. As the number of repeats varies between alleles, digestion with a restriction enzyme which flanks the array, followed by hybridisation with an appropriate probe, results in different sized bands in different chromosomes. This RFLP system overcomes the problem of insufficient allele number, as the number of repeat units is highly variable. Similar to the minisatellites are the microsatellites. These loci are simple sequence tandem repeats which consist of 1 to 6 bp units typically repeated 10-50 times. Microsatellites have three advantages over the minisatellites. Whereas minisatellites are less common, are clustered near telomeres and tend to be over 1 kb in length, microsatellites are more abundant (at least one every 30 kb on average) and are more randomly distributed. Microsatellites are also small enough to be amenable to amplification by the polymerase chain reaction (PCR), removing the need for Southern analysis which is more time consuming and requires a far larger quantity of DNA. In PCR, oligonucleotide primers designed from unique sequence flanking the repeat are used to direct cycles of enzymatic amplification of that locus. This technique facilitates rapid allele characterisation, according to the size

of the amplified product (which is determined by the number of repeat units present).

Co-inheritance of the disease gene with particular alleles of a polymorphic marker within a family or families indicates that the marker is very close to, or linked to, the disease gene. The higher the proportion of affected individuals in the family showing such co-inheritance, the closer the locus is to the gene. This is because in general, the frequency with which recombination separates two loci decreases with decreased distance between them. The markers that show the lowest frequencies of recombination with the disease are those which flank the disease gene. The resolution of this type of data depends upon the number of meioses available for study. In practice this rarely exceeds 100, limiting the resolution to the equivalent of 1 % recombination. One percent recombination is defined as a genetic distance of 1 centimorgan (cM). In humans, 1 cM is approximately equivalent to 1 Mb, but this is not an exact measurement as the relationship between recombination frequency and distance varies between different regions of the genome and between the sexes.

In some diseases it may be possible to further narrow down the region to which linkage has been established. This is done by looking for the existence of an association between a particular disease allele and particular alleles at very tightly linked polymorphic loci. The particular combination of alleles in a region is known as the haplotype. Detection of a conserved haplotype allows increased mapping resolution as all the meiotic events that have taken place since the occurrence of the mutation are taken into consideration. Two approaches can be taken to detect such an association. Affected sib pair analysis (see section 1.7.3) examines the frequency of allele sharing between sibs, in order to detect that which occurs at a higher frequency than is expected by chance. This method is appropriate where many small families are available. Linkage disequilibrium mapping aims to detect common haplotypes in affected individuals. This method is more appropriate where a proportion of mutations have a common ancestory and multigenerational pedigrees are available. Recently, linkage disequillibrium mapping was successfully used to narrow down the chromosomal region which was most likely to contain the gene responsible

for Huntington's disease (Huntington's Disease Collaborative Research Group, 1993).

If a mouse model of the human disease exists then this can be used to identify potential locations of the human homologue. A mouse model of human disease is defined as having similarities to the human in criteria which relate both to the phenotype of the diseases (such as gross appearance; affected tissue; biochemical/metabolic abnormalities; behavioural defects and time of onset of the disease) and to genotypic characteristics (such as mode of inheritance; pleiotropic effects; variable penetrance; genetic heterogeneity and comparative mapping information) (Darling and Abbott, 1992).

Mapping of genes is much easier in the mouse than in humans for a number of reasons, which include the ability to set up a large number of specific crosses, and the existence of recombinant inbred strains and interspecific backcrosses in the mouse. Complex diseases, i.e. those diseases which are polygenic and which are subject to influence of environmental factors are very difficult to map in humans. However, genetic analysis of the mouse has lead to the identification of susceptibility genes for complex disorders. For example, ten murine genes which are involved in susceptibility to type I diabetes in the non-obese diabetic (NOD) mouse have been mapped (Ghosh et al., 1993).

Comparative maps of the mouse and human genomes can also be used to help assign markers. The maps are based on the deduced evolutionary relationships between the murine and human genomes. They indicate the regions of the human genome which are homologous to each murine chromosomal region. If a marker known to be linked to the disease gene has been mapped in the mouse, then the comparative map can indicate possible locations of the linked marker in the human genome. Conservation of linkage between the species is increasingly likely with decreased distance between the two loci. Loci which exhibit linkage in more than one species are said to show conservation of synteny.

Once linkage of a disease gene to a particular chromosomal region has been established then any known genes in that region can be considered as

candidates for the disease (see section 1.6.5). If this is unsuccessful then it is necessary to identify new candidate genes. Often, the first step towards this is the production of a physical map of the region. The estimated size of the identified region will determine the number of markers required to produce a comprehensive physical map. In many cases the generation of new markers is required. One good source of new markers are somatic cell hybrids.

Somatic cell hybrids are produced from the fusion of rodent and human cells. The hybrid cell lines segregate the human chromosomes, facilitating the assembly of a panel of hybrids each with different, stable human chromosome complements (Weiss and Green, 1967). These cell lines are one of the potential starting points for the production of lines which are further reduced in human genomic complexity. The reduction is achieved by exposing one cell line (the donor) to a lethal dose of ionising radiation followed by fusion of these cells with a recipient cell line (Goss and Harris, 1975). The resultant hybrids can express markers from both parents but most unselected fragments from the donor are eventually lost. Another method of producing fragmentation hybrids is by chromosome-mediated gene transfer (McBride and Ozer, 1973). A co-precipitate of donor chromosome fragments and calcium phosphate is applied to recipient cells. Independent clones, each bearing a chromosome fragment carrying a suitable selectable marker, can then be isolated.

Pools of probes generated from such hybrids, for example by Alu-PCR (Nelson, 1989) can be fluorescently-labelled and hybridised directly to metaphase chromosome spreads. This technique is known as fluorescent in-situ hybridisation (FISH). FISH can be used to determine the chromosomal origin of the pool of probes (known as a chromosome "paint") and to examine the pattern of integration of the human DNA in the rodent cell line.

Markers can be produced from somatic cell hybrids again, for example, by Alu-PCR amplification of the human component. Amplified fragments may be used directly as probes, or may be converted, by DNA sequencing, into sequence-tagged sites (STSs) which are short unique sequences of DNA which can be amplified by PCR (Olson et al., 1989).

Markers can also be produced from a precise chromosomal region by microdissection. In many cases the region of interest is dissected from banded metaphase chromosomes. This procedure can produce abundant, region specific probes but they tend to be short (for example, average size of 350 bp) (Hadano et al., 1991) and consequently, difficult to work with. We chose to follow protocols developed by Martinsson et al. (1989) to produce clones from unbanded chromosomes (Muir et al., manuscript in preparation). This procedure results in insert sizes which are approximately ten times larger than those produced by dissection of banded chromosomes. Although, in general, a smaller, more precise dissection can be made if banded chromosomes are used, the larger clones produced by dissection of unbanded chromosomes can be used simply and directly in a variety of mapping and probing experiments.

Once markers have been generated they must be chromosomally and regionally assigned. Somatic cell hybrids can be used for this. The DNA content of a series of reduced complexity hybrids can be analysed by FISH (see above) and by typing for markers of known chromosomal location. A set of hybrids which define a number of intervals on a particular chromosome can be assembled and used to regionally localise markers.

Complementary to the technique of hybrid mapping is pulsed field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984). This method allows the separation of very large DNA fragments (up to 12 Mb) (Orbach et al., 1988) and the construction of long range restriction maps. This allows probes to be linked and ordered, accurate measurements of physical distances to be made and chromosome breakpoints to be detected.

FISH is also important in the construction of physical maps. This technique allows determination of relative order of probes along the length of a chromosome. As mentioned previously, fluorescently labelled probes can be hybridised directly to metaphase chromosomes. This allows determination of the chromosomal origin of probes and reliable ordering of individual probes, which are separated by at least 1 Mb, with respect to each other (or with respect to a cytogenetic marker). When probes are closer together a variety of other FISH techniques can be used to determine relative order

(summarised in Buckle and Kearney, 1993). Probes can be hybridised to interphase nuclei where the chromatin is less condensed but each chromosome occupies a discrete region. This allows accurate ordering of probes which are separated by 50-1000 kb. Interphase nuclei can be used to prepare free chromatin in a two dimensional linear form. This type of substrate provides mapping information in the 200-350 kb range. Resolution can be achieved for probes separated by 10-200 kb by the preparation of a "halo" of DNA loops around the nuclear matrix. With this technique probes such as those derived from entire cosmids appear as a string of spots along the length of the chromatin fibre. The most recently described FISH technique is known as direct visual hybridisation (DIRVISH). The substrate for this technique is prepared by stretching duplex DNA across a slide, resulting in a stream of linear DNA strands. Like halo DNA, strings of signal are produced, but results are possible with this technique with a probe of 5 kb, allowing a very fine resolution for mapping. The upper limit of this technique has not been established, but a probe corresponding to a 700 kb YAC recombinant has been hybridised, resulting in a signal of the expected length (Parra and Windle, 1993).

1.6.3 Contig Mapping

Once markers have been generated and mapped they can be used to generate a clone map of the region of interest. Various vectors for such a map exist. Cosmids (Collins and Hohn, 1978) have the advantages of high cloning efficiency and the availability of simple methods for isolation of large amounts of purified insert DNA. However only ~40-45 kb of DNA can be cloned in cosmids. This was greatly improved upon with the development of yeast artificial chromosomes (YACs) (Burke et al., 1987). Several hundred kilobases of DNA could then be cloned as linear artificial chromosomes which were propagated in the budding yeast *Saccharomyces cereviseae.* This technology also provided a means for the stable cloning of chromosome regions which had proved impossible to obtain from cosmid libraries (Coulson et al., 1988). YAC recombinants with inserts of greater than 1 Mb can now be produced, resulting in another substantial increase in the rate at which a physical map can be produced. YACs however have some disadvantages. These include low cloning efficiency and the lack of a simple method for isolation of pure DNA. Another problem is that a proportion of

YAC inserts originate from more than one chromosomal location. This is due to co-cloning events and recombination between two YACs in one cell. Some YACs also show deletions of some chromosomal regions. The recent development of Bacterial Artificial Chromosomes (BACs), which appear to be capable of insert sizes approaching those of YACs, may be free of some of these problems (Shizuya et al., 1992). Cloning efficiency is increased, DNA isolation is easy and the BAC is maintained at a single copy per cell, reducing problems due to recombination between BACs. BACs appear to be relatively stable over a large number of generations and co-cloning events may occur less frequently than during YAC cloning.

Currently, the primary physical map of a particular region tends to be generated in YACs. YACs are isolated, using markers mapped to the region, and an overlapping series of clones, or contig is established from them. The pattern of STSs present in the YACs and "fingerprints" of the YACs (generated by hybridisation of a repetitive probe, such as an Alu element, to a restriction digest of the YAC) are used to order YACs and to detect overlaps between them. FISH and PFGE are also a important techniques in building contigs. As described above, they can be used to order clones with respect to each other and with respect to cytogenetic markers. Closing gaps in the contig may require the isolation of the ends of a particular clone and the use of these to select, or to "walk" to, the next clone. Smaller insert clones, especially cosmids, tend to be used to bridge gaps in YAC contigs and to sort out any ambiguities due to chimaeric or rearranged YACs.

Once a YAC contig has been established it is often used to isolate cosmids, with a view to forming a corresponding cosmid contig. Cosmids are often more practical than YACs for further experiments.

1.6.4 Gene Identification

Once a contig of the region of interest has been established it can then be examined for the presence of coding sequences. The size of the region to be searched will depend on how it was defined. For example, if a chromosome translocation is associated with the disease, then the smallest clone which spans this breakpoint is the starting point for the gene search. However, if the disease region is genetically defined, then a large contig may

have to be examined. In many cases cosmids are chosen as the starting material for gene isolation. However, the size of the search region may correspond to many YAC recombinants, making YACs a more practical resource. A number of different methods are used towards gene identification.

1. Clones can be examined for the presence of CpG islands. The dinucleotide CpG occurs at one fifth of its expected frequency in vertebrate DNA (Bird, 1986). This is because the cytosine in the majority of CpG dinucleotides is methylated, and over evolutionary time these 5-methyl cytosines are deaminated to a thymine. However, mammalian genomes also contain clusters of unmethylated CpGs dinucleotides. These are known as CpG islands and occur at the 5' ends of genes. Approximately 60 % of genes are estimated to have islands at their 5' end. This can be broken down into 40 % of tissue specific genes and most, if not all, housekeeping or widely expressed genes (Larson, 1992). CpG islands can be detected in genomic DNA by digestion with enzymes whose recognition sequences contain more than one CpG and which only cut non-methylated DNA. Such enzymes cut far more frequently in islands than in the majority of the genome and correspondingly a cluster of such enzyme sites is indicative of an island. Clones which are adjacent to CpG islands can be used as hybridisation probes to identify cDNAs but, as mentioned previously, only 60 % of genes have CpG islands. This technique has been used in the isolation of many genes, for example, four new genes encoded in the Class II region of the human major histocompatibility complex were detected in this way (Hanson et al., 1991).

2. As many genes are conserved between species, the cloned DNA can be examined for evolutionary conservation. The cloned material (cosmids or smaller) is checked for cross-hybridisation to DNA from a number of different species (using what is known as a Zoo Blot). This technique was used as a first step in the isolation of candidate genes for Duchenne muscular dystrophy (Monaco et. al., 1986).

3. Cloned material (cosmids or smaller) can be examined for expressed sequences by hybridisation to mRNA obtained from a tissue or tissues that are likely to express the disease gene. This technique, known as Northern

blot analysis, can in some cases determine whether the gene identified is that which is involved in the disease under study. If a probe picks up a signal from a normal individual but shows either no signal or an altered signal size in an individual suffering from the disease under study then it is likely to be involved in the disease. An absence or alteration in the length or stability of the transcript will occur when the disease-causing mutation abolishes transcription or produces a truncated, novel or unstable transcript. Limitations to this approach may be encountered if the gene under study is expressed either at low levels or is subject to temporal control. It may also be difficult to obtain samples of the appropriate tissue in which to study expression. It is sometimes possible to overcome this by amplification of the products of illegitimate transcription in more accessible tissues (reviewed in Kaplan et al., 1992).

4. A number of techniques for physical selection of cDNAs have been developed (Brookes and Porteous, 1991; Lovett et al., 1991; Parimoo et al., 1991 and Korn et al., 1992). These methods are all based on the isolation of sequences which are common to two different complex DNA sources, namely a contig of a genomic region and a cDNA library. Among the sequences which are coincident between the two resources will be the genes encoded by the genomic contig. The different approaches vary in technical details but all aim to isolate heteroduplexes, i.e. DNA duplexes where one strand comes from the genomic clone and the other comes from the cDNA source. Providing that the gene of interest is expressed in the cDNA source under study, these techniques potentially allow the isolation of transcripts which are expressed at low levels.

5. Isolation of transcribed sequences from particular genomic regions has been achieved through the use of somatic cell hybrids with reduced human DNA content, but containing DNA from the region of interest (Liu et al., 1989; Corbo et al., 1989). In one method heterogeneous nuclear RNA is isolated from a human-rodent cell line and a cDNA library is made from this material. The cDNA library is then screened for the presence of human clones (Liu et al., 1989). Another method uses human Alu repetitive sequences to direct construction of human specific cDNAs. Where Alu sequences are present in introns and in 3' untranslated regions of human genes, human cDNAs will be amplified (Corbo et al., 1989). Thus this version of the method avoids the

requirement for library construction and screening. These methods are most useful when somatic cell hybrids containing a very limited amount of human DNA are available. The techniques are limited to the isolation of genes which are constitutively expressed and, in the case of the second method described, to those genes which contain repeats in their introns.

6. Exon trapping and exon amplification are methods which identify genes by selection for functional splice sites from cloned DNA. The original method of exon trapping (Duyk et al., 1990) has largely been superseded by a second, more straightforward, method called exon amplification (Buckler et al., 1991) Further variation on the procedure has been described (Hamaguchi et al., 1992). In essence, the vector contains an entire intron flanked by 5' and 3' splice sites. Genomic DNA is shotgun-cloned into the intron, between the 5' splice donor site and the branch site. This material is then transformed into mammalian cells where it is transcribed. If the cloned DNA contains an entire exon and flanking intronic sequences in the sense orientation then splicing between these and the vector sites will occur. Mature mRNA can then be isolated from the cells and reverse transcription PCR is used to produce cDNA corresponding to the cloned sequences. The size of the product obtained indicates whether the inserted genomic material has been completely spliced out or whether an exon was present in the test DNA and has been trapped. The presence of cryptic splice sites in the inserted DNA may result in false positives and the small proportion of genes that do not contain introns will not be identified by this system. However, it has the advantage that detection is not dependent on the expression level of the gene. Recently, this technique was used in the isolation of the gene which is responsible for Huntington's disease (Huntington's Disease Collaborative Research Group, 1993).

7. Direct DNA sequencing can also be used in gene identification. The gene which causes Kallman's Syndrome was identified by one group (Legouis et. al., 1991) using this technique; 67 kb of genomic DNA which corresponded to an interval known to contain at least part of the gene was sequenced and examined for potential exons. Computer programs were then used to examine sequence for transcribed regions. These programs take account of features such as consensus splice sites, open reading frames and segmental similarities with known proteins and examine parameters such as

codon usage and differential nucleotide hexamer compositions in order to detect potential exons.

8. Many of the methods described above can be used to identify probes that can be used to screen cDNA libraries. Complex probes such as entire YACs have also been successfully used to directly screen cDNA libraries (Elvin et al., 1990; Wallace et al., 1990). Such schemes may have limitations as the larger the probe the less likely it is that a particular small region of it will pull out a cDNA, especially if the corresponding cDNA is small. Repetitive sequences may also cause a high false positive rate. Problems may also be caused by low expression levels or restricted expression patterns of the cDNA.

1.6.5 Testing Candidate Genes

Once a candidate gene has been identified by any of the above methods it must then be shown to cause the disease under study. If a chromosomal rearrangement segregates with the disease and interrupts the candidate gene then this is good evidence that the correct gene has been isolated. Other, less clear-cut evidence includes expression patterns that fit with the disorder such as expression from the correct tissues at the correct time in unaffected individuals along with alteration of expression in affected individuals. It may not, however, be easy to obtain the necessary material to carry out such analyses, as expression may be in a tissue which is difficult to sample, or expression may occur during embryonic development. Finally, screening affected individuals for consistent mutations (which are predicted to result in loss or alteration of function) in the candidate gene provides good evidence that the gene is responsible for the disease. There are a number of methods for detecting previously unknown mutations in genes (reviewed in Grompe et al., 1993). Large deletions and insertions (>1Mb) and inversions can be detected by cytogenetics. Southern blot analysis can be used to look for smaller deletions and inversions, and will also detect point mutations, if a restriction site is altered by the mutation. Microdeletions can also be detected by PCR. Multiplex PCR, of several sequences throughout the gene can be carried out with the result that a large area of the gene can be covered in a single analysis. Deletions are indicated by a failure of amplification of a particular product (or products). In addition to these

methods, a range of techniques exist for the examination of sequences for point mutations. These are summarised in Table 1.1 (adapted from Prosser, 1993).

1.7 Positional Cloning of Polygenic Disorders

1.7.1 Problems Associated with Positional Cloning of Schizophrenia

Positional cloning strategies can also be applied to polygenic disorders. The mapping of susceptibility genes for such disorders is however more difficult than the mapping of genes responsible for monogenic disorders. A major difficulty is in establishing the initial linkage.

The logarithm of the odds (LOD) score method is a commonly used method for testing for linkage of marker loci to a disease gene which is believed to segregate in a particular family or families. The frequency with which recombination occurs between the disease gene and a marker (the recombination fraction, θ) increases with increased distance between loci, until they are sufficiently far apart that the probability of parental and recombinant chromosomes is equal (as is the case when loci are on separate chromosomes). When testing for linkage between two loci an attempt is made to disprove the null hypothesis, that there is no linkage i.e. $\theta = 0.5$ (the alternative hypothesis is that linkage occurs at $\theta < 0.5$). This is done by examining the ratio of probability of observing a particular distribution of alleles under the hypothesis of linkage at θ to the probability under the hypotheses of no linkage. The decimal logarithm of the odds ratio (LOD) is calculated. When the distance between two markers is unknown then LOD scores are calculated at several values of θ in order to find the maximum value. A LOD score of three is equivalent to an odds ratio of 1000:1 that two loci are linked. This is the accepted minimum value required to show linkage in the case of a monogenic disorder. Such a high ratio is required because of the prior low probability that two loci are linked (for example the prior probability that two loci lie less than $\theta = 0.3$ apart is only ~2 %). This low prior probability means that, for $\theta = 0.3$, a LOD score of 3 indicates that the linkage detected has a 95 % probability of being true (Risch, 1992).

Table 1.1 Screening for Unknown Mutations

(adapted from Prosser, 1993)

	RNase A cleavage	CCM/HOT	Carbodimide	SSCP	Heteroduplex Analysis	DGGE	Sequencing
Basis for Detection	Enzyme cleavage	Chemical cleavage	Blockage of primer extension	Molecular shape in electrophoresis	Molecular shape in electrophoresis	Changing molecular shape in electrophoresis	DNA sequence
Molecules	Heteroduplex RNA/RNA; RNA/DNA	Heteroduplex DNA/DNA; (DNA/RNA)	Heteroduplex DNA/DNA; (DNA/RNA)	Single-stranded DNA (RNA)	Heteroduplex DNA/DNA (DNA/RNA?)	Heteroduplex DNA/DNA (homoduplex)	single or double stranded amplified DNA
PCR based	No, requires cloning for SP6 or T7 produced RNA	Yes (and No)	Yes	Yes	Yes	Yes	Yes (and No)
Size of fragments	1-2 kb	1-2 kb	1-2 kb	<200 bp	200-300 bp	<500 bp	<500bp
Efficiency of detection	60-70 %	100 %	100 %?	Up to 90%	80 %?	100 %?	100 %
Precise location determined	Yes	Yes	Yes	No	No	No	Yes
Advantages	Scans large fragments, precisely locates mutation, no hazardous chemicals	100 % detection, scans large fragments, precisely locates mutation, heterozygote detection	Probably 100% detection, precisely locates mutation, only one chemical, (less hazardous than CCM)	Rapid and apparently easy	Rapid and easy	Rapid and easy after initial setting up, probably 100 % detection, heterozygote detection	100 % detection, rapid and easy, no sequencing step to follow
Disadvantages	Low efficiency of detection, requires cloning, multistep	Multistep and labour intensive, uses hazardous chemicals	Multistep and labour intensive, relatively untried, does not detect multiple mutations	<100 % mutation detection, short fragments scanned, mutation not precisely localised, conditions require careful selection	<100 % mutation detection, short fragments scanned, mutation not precisely localised	Effort required to establish technique	Labour intensive if few mutations are expected
Comments	Used despite some disadvantages	Best for 100 % screening of large genes	Serious competitor for CCM/HOT	Frequently used because of great simplicity, especially across small regions of genes	Frequently used because of great simplicity, especially across small regions of genes	Easy and aims for 100 % detection, best for routine screening of small fragments	May replace scanning methods for short fragments, especially where many mutations are expected

Abbreviations: CCM- chemical cleavage of mismatch repair; HOT- hydroxylamine and osmium tetroxide; SSCP- single strand conformation polymorphism; DGGE- denaturing gradient-gel electrophoresis

The problems encountered when attempting to use this method of linkage analysis to detect a susceptibility gene for schizophrenia are discussed below.

Linkage analysis will only be possible if a gene of major effect is segregating in at least a proportion of the families under study. In the case of a Mendelian disorder the risk to relatives decreases by one half with each degree of relatedness. If the risk decreases more rapidly than this then a more complex genetic model is implicated. Schizophrenia falls into this category but the precise genetic model is unclear and probably varies between families (Levinson and Mowry, 1991).

Genetic heterogeneity will also greatly affect linkage analysis. Genetic heterogeneity is the situation where different mutations at different loci can give rise to the same disease phenotype. It seems likely that schizophrenia is genetically heterogeneous. Genetic heterogeneity within a study family or families will tend to obscure linkage rather than generate false linkages. This is because individuals who should be classified as unaffected will be classified as affected. If any of these individuals are recombinant at a locus linked to the disease then linkage to the locus will be weakened or missed completely.

Assortative mating may increase genetic heterogeneity by introducing another set of schizophrenia causing genes into a family. Assortative mating has been shown to occur in some psychiatric disorders, particularly in the affective disorders and possibly in schizophrenia (Baron et al., 1990) If assortative mating occurs without detection then the results of linkage analysis will be affected in the manner described above.

Phenocopies of the disease, for example disease caused solely by environmental factors, will tend to decrease LOD scores at linked loci. This is again because individuals who should be classified as unaffected are classified as affected. Conditions such as temporal lobe epilepsy, brain injuries, early-stage Huntington's disease, amphetamine overdoses, thyroid disease and porphyria can cause schizophrenia like symptoms (Lander, 1988).

A gene that can cause a predisposition to schizophrenia may show variable expressivity. In other words it may cause susceptibility to mental illnesses other than schizophrenia. A disorder which can be caused by a gene which predisposes to schizophrenia should occur at a higher frequency in close relatives of schizophrenics than it does in the general population. The possibility that a common environment may cause psychiatric illness in more than one family member must be ruled out. This is achieved by comparing the incidence and types of psychiatric illness in adopted relatives of schizophrenics to that seen in adopted relatives of controls. One such survey found that relatives of schizophrenics are subject to higher than expected levels of schizotypal personality disorder and paranoid personality disorder (Kety et al., 1971). The extent to which variable expressivity occurs and the diseases to which individuals are predisposed may vary from one disease causing gene to another. Hence defining affected status is very difficult. False positives will decrease positive LOD scores, as described above, while false negatives will be indistinguishable from non-penetrant carriers and therefore, in small numbers, may be less important.

As mentioned above, schizophrenia has a complex mode of inheritance, it exhibits incomplete penetrance or expressivity. When carrying out linkage analysis of a disease which shows reduced penetrance only ill individuals can be confidently counted as recombinants, as healthy individuals may be non penetrant disease gene carriers. The result of this limitation is that even very large pedigrees may not have enough recombination events to sufficiently narrow down the region containing the disease gene.

Linkage analysis requires that the penetrance of the gene under study be specified. The penetrance of the allele will depend on the genetic background of each individual as well as the environmental factors that each individual is exposed to. It is also likely that different genes which predispose to schizophrenia will exhibit different penetrance values. Thus determination of the penetrance of a particular gene is not possible and LOD scores are generally calculated for a range of penetrance values.

The gene frequency (in the general population) must also be specified in order to calculate LOD scores. Again, accurate determination of this value is

not possible and LOD scores tend to be calculated for a range of gene frequencies.

As mentioned above, schizophrenia exhibits a wide range of onset ages. This leads to difficulties when attempting to assess the penetrance of the disorder and when deciding which family members are affected and which are unaffected. The age of onset will vary between disease causing genes and between individuals with the same predisposing allele. Such variation is likely to be due to variation in environmental factors experienced by different individuals and by the different genetic background of each individual.

The result of such uncertainty is that different estimates of the various parameters (e.g. penetrance values and diagnostic boundaries of affected phenotype) are used to calculate a set of LOD scores. Because of this, it is considered necessary by some authors to have a higher minimum LOD than the score of 3 which is considered sufficient to define linkage of a monogenic disorder to a marker (Risch, 1992).

1.7.2 Failure of a Previous Attempt to Map Genes for Schizophrenia by Linkage Analysis

Following a report of an association between schizophrenia and a partial trisomy of chromosome 5 (Bassett et al., 1988) linkage of schizophrenia and other psychiatric illness to markers on the trisomic region of chromosome 5 was reported in seven British and Icelandic families (Sherrington et al., 1988). LOD scores were used to detect linkage; a LOD of 6.49 was achieved with the broad phenotype, which included disorders such as alcoholism as well as schizophrenia spectrum disorders.

This finding has however been followed by a series of studies which have failed to find evidence of linkage to that region of chromosome 5 (Kennedy et al., 1988; St.Clair et al., 1989; Detera-Wadleigh et al., 1989; Aschauer et al., 1990; McGuffin et al., 1990; Crowe et al., 1991 and Macciardi et al., 1992). The study was extended, by the use of polymorphic microsatellite markers which were more informative and increased the number of informative meioses (O'Donovan and Owen, 1992). Once this analysis had been carried out no evidence was found for linkage of chromosome 5 markers to the

broad phenotype. Although when the phenotype was restricted to schizophrenia and spectrum disorders it remained at approximately 3. The study was also extended by the addition of new families. Once this had been done no evidence for linkage remained (O'Donovan and Owen, 1992).

1.7.3 Possible Solutions to the Problems of Mapping Schizophrenia Genes

The problems of reduced penetrance, difficulty in determining the mode of transmission and definition of affected status, which are associated with the LOD score method of linkage analysis, could be lessened if subclinical characteristics (which were determined by a gene that predisposed to schizophrenia, but which showed higher penetrance than the disorder) could be used in the linkage analysis. Such characteristics are referred to as trait markers. Trait markers should satisfy the following criteria: genetic control; association with disease at the population level; state independence and co-segregation with illness in the families of index cases (Gershon et al., 1990). Of the proposed trait markers, smooth pursuit eye movement dysfunction and abnormal P300 event related potential appear to meet these criteria (Blackwood et al., 1991).

Alternatively, other methods of linkage analysis could be employed. The affected-sib-pair (ASP) method examines the frequency with which the members of affected sib pairs both inherit the same allele at a locus, with the frequency at which this would be expected if the locus was unlinked to the disease. As the prior probability that sibs will share an allele at a particular locus is high (even when both parents are heterozygous at a locus there is still a 50 % chance that the sibs will have a common allele at the locus) it is easier to exclude than to include loci.

This method has the advantage that it does not require the specification of genetic model of the disease (Nothen and Fimmers, 1993). However, one of its most important disadvantages, with respect to its usefulness in detecting linkage to schizophrenia, is that the power of the method is greatly affected by genetic heterogeneity.

Another method of linkage analysis is association studies. These examine individuals for an association between DNA variants and disease. Often, a candidate gene is selected and nearby polymorphic markers are used to compare patients and controls for an association of the disease and particular haplotypes (Nothen and Fimmers, 1993). An association will occur when there is strong linkage disequilibrium between the loci or when the marker contributes to the disease pathogenesis (Nothen and Fimmers, 1993). Linkage disequilibrium will only occur when there is either a low mutation rate, very tight linkage or a selective advantage of the haplotype which contains the disease gene (Nothen and Fimmers, 1993). Like the ASP method, the effectiveness of this method will be reduced by the presence of genetic heterogeneity. In addition there is the problem caused by population stratification. To avoid differences in allele frequencies patients and controls have to be carefully matched for ethnic, geographical and social background. A possible solution is to carry out association studies within families with the control sample corresponding to, for example, the parental alleles not inherited by the proband (Nothen and Fimmers, 1993).

The easiest way to identify the position of a disease gene is to identify a chromosomal rearrangement which segregates with the disorder. Chromosomal rearrangements include inversions, where breaks have occurred at two points on a chromosome and the segment has been inverted before being rejoined; deletions, where a segment of a chromosome has been lost and translocations, where breaks have occurred on two (or occasionally more chromosomes) and rejoining has happened between the wrong chromosome ends. Translocations can be balanced, where rearrangement has occurred without loss or duplication of any chromosomal material, or unbalanced, where duplications and/or deletions have occurred. Balanced translocations are the most common *de novo* structural chromosome rearrangements; estimates of their frequency range from 2.6-14/10,000 live births (Giaclone and Franke, 1992).

Chromosomal rearrangements as well as pointing to the location of the gene make it easy to determine when the appropriate region has been cloned. Deletions may cover a large chromosomal region making subsequent identification of the correct gene difficult. This may also be true in case of

translocations and inversions, the gene may not be at one of the breakpoints rather, the break may affect expression of a nearby gene or the rearrangement may have prevented crossing over in the region, fixing the rearranged chromosome as a marker for the disease gene.

Chromosomal rearrangements have a final advantage which is particularly important when carrying out linkage analysis in diseases with complex modes of inheritance, such as schizophrenia. The rearrangement allows all individuals who have inherited the proposed causative mutation to be identified. In the absence of a rearrangement this is not possible because of the reduced penetrance associated with such disorders. This means that when a rearrangement segregates with the disorder there is access to more recombination events for linkage analysis (although, as mentioned above, rearrangements may reduce the number of recombination events in that region).

1.8 A Family Segregating A Balanced Translocation t(1;11)(q42.2;q21)

As mentioned previously, a large Scottish pedigree has been reported where a balanced translocation t(1;11)(q42.2;q21) appears to co-segregate with a predisposition to schizophrenia and other major mental illnesses (St.Clair et al., 1990). The family was identified by a survey carried out on the MRC Cytogenetics Registry in Edinburgh. The Registry holds up to date clinical data on 282 pedigrees, identified over a 20 year period, which have familial autosomal abnormalities. These pedigrees were examined for any co-segregation of mental illness with the chromosomal abnormalities. This search resulted in the identification of pedigree K26. No phenotypic abnormalities were reported in the original description of the family but cumulative annual follow-up data from general practitioners indicated that many members of the family had been treated for psychiatric illness. The family was reapproached and the mental health of each family member was re-evaluated. Seventy-seven individuals were included in the study, 58 living and 19 deceased. Twenty-three individuals from this family were diagnosed as suffering from mental illness, as defined by the Research Diagnostic Criteria for mental and/or behavioural disorders. These diagnoses are three cases of schizophrenia, two of schizoaffective disorder,

six of major depressive disorder, six of generalised anxiety disorder, one of minor depressive disorder and three of alcoholism. Five of the family members had received or were receiving treatment for adolescent psychiatric disorders, three of whom now have a subsequent diagnosis. Of the 23 cases, three individuals had attempted suicide and a further two had achieved suicide. Twenty-one of these individuals could therefore be karyotyped, along with 56 other family members. It was found that 34 of the 77 individuals carry the t(1;11)(q42.2;q21) translocation. Sixteen of these had been diagnosed as suffering from a mental illness while, of the 43 without the translocation, only five were diagnosed as suffering from a psychiatric disorder. None of these diagnoses was for a major mental illness; there was one case of minor depressive disorder, three cases of alcoholism and one of generalised anxiety.

LOD scores against chance linkage of the translocation to mental illness were calculated. It was not known which mental disorders could potentially be accounted for by the chromosomal rearrangement. LOD ratios were therefore calculated for five definitions of affected phenotype. As gene frequency and gene penetrance were also unknown, the LOD score for each phenotypic model was calculated for a range of gene frequency and penetrance values.

When the affected phenotype was defined as schizophrenia and schizoaffective disorder the maximum LOD score was 2.19. Recurrent major depression was included and the maximum LOD rose to 3.33. The third model produced the highest maximum LOD score, 4.34, when the definition of the affected phenotype was expanded to include adolescent conduct and emotional disorder. The maximum LOD score fell to 1.37 and 1.73 when generalised anxiety, minor depression and chronic alcoholism were added to the second and third models respectively.

The psychiatric diagnoses that made up model II are commonly found together in families with many cases of mental disorders. The adolescent conduct and emotional disorders that were added to model II to form model III are often found in people who develop recurrent mental illness in adult life, as has been observed in this pedigree. They also tend to be found at a higher than expected frequency among the offspring of adults with major

mental illness. It is not surprising therefore that inclusion of these diagnoses in the definition of affected phenotype causes the maximum LOD score to rise. It is also unsurprising that the addition of generalised anxiety, minor depression and chronic alcoholism should cause LOD scores to fall. Such diagnoses lack specificity and occur at relatively high frequencies in the general population. These results therefore indicate a strong likelihood of linkage of the translocation to mental illness in this family.

Mapping and cloning the regions of the translocation breakpoints would therefore facilitate a search for a gene or genes (that may be located at or near one or both of the translocation breakpoints) which may predispose to schizophrenia and other mental illnesses.

The identification of a gene which predisposes to schizophrenia has many implications. The same gene may be involved in mental illness in other patients. This could be tested firstly by linkage analysis in families and by mutational analysis of the gene in individuals. This would provide a validating factor for diagnosis of mental illnesses and would provide information on the genetic relationship between different diagnoses.

The advantages of identifying the gene predisposing to mental illness in this family would not however depend on its involvement in other cases of mental illness. Study of the gene and its product would provide information which may give clues to the aetiology of the disorder and correspondingly to possible treatments for the disorder. This information may also implicate other proteins and genes (for example proteins involved in the same or in a related pathway or homologous genes) in involvement in other cases of mental illness.

The discovery and study of a gene which, if mutated, can cause a predisposition to mental illness may increase our knowledge of normal brain development. It may also provide clues as to the nature and the mode of action of the environmental factors which are important in schizophrenia.

1.9 Objectives of this Thesis

The objectives of this thesis are as follows:

1. To refine the position of both translocation breakpoints.

2. To establish relative marker order with respect to the chromosome 11 breakpoint.

3. To identify and map new markers in the region of the chromosome 11 breakpoint.

4. To isolate YACs from the region immediately distal to the translocation breakpoint.

5. To identify a clone which crosses the breakpoint on chromosome 11.

To this end a number of resources were assembled and exploited. Of these the following were key to the execution of my studies:

1. Somatic cell hybrids which segregate the translocation chromosomes.

2. Reduced chromosome hybrids which contain complimentary fragments of human chromosome 11 in rodent backgrounds.

3. A microdissection library corresponding to a 30 Mb region surrounding the translocation breakpoint of the derived chromosome 1.

4. Gridded YAC libraries.

5. Fingerprint analysis of YAC recombinants.

6. Marker segregation analysis of YAC recombinants.

7. Pulsed field gel electrophoretic analysis of normal and translocation chromosomes with microdissection clones.

CHAPTER 2

2. Materials and Methods

2.1 Mammalian Cell Culture

2.1.1 Cell Lines

The following cell lines were analysed. Somatic cell hybrid lines which segregate the reciprocal translocation chromosomes are MIS 7.4 (murine, X63, background) and MAR 12 (murine, RAG, background), which carry the derived 1 translocation chromosome, and MIS 39.8 and MAR 1 which carry the derived 11 translocation chromosome (each in the absence of any normal chromosome 1 or 11 material). Segregation of the translocation chromosomes was achieved by cell-surface marker selection strategies (Fletcher et al., 1993). Four X irradiation hybrids, WJX 3.4, WJX 5.4, WJX 7.4 and WJX 11.2, which contain fragments of chromosome 11 in the background of a Chinese hamster ovary cell line, WG3H, were also analysed. These fragmentation hybrids were produced by X irradiation of a chromosome 11-only hybrid J1Cl4 (Jones et al., 1984), followed by rescue of the fragments produced, by fusion with WG3H cells and finally, cell surface marker selection to isolate those cell lines which retained fragments of chromosome 11 (Fletcher et al., 1993). Two additional chromosome 11 fragmentation hybrids, E67.1 and E67.4, were studied. These were derived by transfer of metaphase chromosomes from a human EJ bladder carcinoma cell line to the murine C127 cell line. This was followed by selection for HRAS 1 mediated transformed growth, in addition to selection for the HRAS locus and other markers on 11p (Porteous et al., 1986). E67.1 and E67.4, had been analysed previously for 11p markers (Porteous et al., 1987) and a limited number of 11q markers (Spurr et al., 1988). Two additional translocation cell lines were analysed. PG48 has a translocated chromosome 11 which lacks 11q22-11qter (and a number of other human chromosomes) in a, a23, hamster background (McConville et al., 1993). CF52 has a well characterised t(11;16)(q13;p11) translocation chromosome as its sole human component in the background of a murine cell line, A9 (Koeffler et al., 1981).

MAFLI, which is a permanent B lymphoblastoid cell line established from a patient with schizophrenia and bearing the t(1;11)(q42.2;q21) translocation (Fletcher et al., 1993), and J1CL4, see above, (Jones et al., 1984), were positive controls. Cell lines WG3H, X63 and RAG (see above) were negative controls.

In a few assays, a number of other cell lines were studied: MIS 39.5 is a hybrid cell line which contains a rearranged chromosome 11, in addition to the derived chromosome 11 translocation chromosome (Veronica van Heyningen, personal communication). CF37 (Mohandras et al., 1980) is a hybrid cell line which contains a translocated chromosome 11, whose chromosome 11 breakpoint is at a similar position to that of the translocated chromosome 11 present in CF52. M11X is a hybrid which has a t(X;11)(q26;q23.2) as its sole human component (Chiang et. al., 1984).

MIS 7.4, MIS 39.8, X63, MAR 1, MAR 12, CF52, PG48, CF37, MIS 39.5, M11X and RAG were all grown in Veronica van Heyningen's laboratory. They were obtained in the form of frozen cell pellets, from which DNA was isolated (see section 2.2.1). Similarly, E67.1 and E67.4 were obtained as frozen cell pellets from David Porteous. J1CI4, WG3H, WJX 3.4, WJX 5.4, WJX 7.4, WJX 11.2, and MAFLI were obtained from Veronica van Heyningen in the form of liquid nitrogen stocks and were cultured as described below.

2.1.2 Maintenance of Cells in Culture

All cell culture was carried out under sterile conditions in a Lamina flow hood. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Flow Laboratories) supplemented with heat inactivated 10 % foetal calf serum (FCS) (Gibco). Penicillin and streptomycin (Gibco) were added to cultures at 100 units/ml and 100 μg/ml respectively. Cultures were grown at 37°C in 25 cm^2, 75 cm^2 (Nunclon) and 175 cm^2 (Falcon) tissue culture flasks in a 10 % CO_2 atmosphere.

2.1.3 Reviving Frozen Cells

Cells which had been previously frozen and stored under liquid nitrogen were rapidly thawed by placing the vial in a beaker of water warmed to 37°C. Once the cell suspension had completely liquefied, the storage medium was diluted with 5 ml culture medium and the cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was then resuspended in a small volume of culture medium and the cells were transferred to a 25 cm^2 culture flask, which contained 10 ml culture medium.

2.1.4 Harvesting Cells

The culture medium was removed by aspiration, and the cell monolayer rinsed in a small volume of Dulbecco's phosphate buffered saline (PBS) (approximately 2 ml/25 cm^2 flask, 5 ml/75 cm^2 flask, 8 ml 175 cm^2 flask) to remove traces of serum. The PBS was then removed and the same quantity of 10 % trypsin in versene was added (versene is 12 ml 0.2 % phenol red (Flow laboratories); 10 Dulbecco tablets (Oxoid Ltd.) and 0.4 g EDTA). The flasks were briefly incubated at 37°C, to activate the trypsin, facilitating loosening of the cells. The cells were then harvested into a 50 ml centrifuge tube (Corning) and the flask was rinsed out with a small amount of medium (to maximise the harvest). The tube was filled with medium, to dilute and inactivate the trypsin, and the cells were pelleted by centrifugation at 1000 rpm for 5 minutes.

2.1.4.1 Harvesting Cells for the Expansion of Clones

The cells were harvested as described in section 2.1.4. The cell pellet was resuspended in the appropriate volume of medium and added to new flasks.

2.1.4.2 Harvesting Cells for Storage at -70°C

Cells were harvested as described in section 2.1.4. The cell pellet was resuspended in 2 ml of ice cold freezing mix (FCS containing dimethyl sulphoxide (DMSO) at a final concentration of 10 %). Approximately 1 ml of this cell suspension was stored in each of two freezing vials (Nunc) which had been previously chilled on ice. The vials were initially placed at -70°C in

a cotton wool padded box (to slow the freezing process). After a minimum of 16 hours the vials were transferred to liquid nitrogen storage.

2.1.4.3 Harvesting Cells for Preparation of DNA

The cells were harvested as described in section 2.1.4. The cells were then washed, by resuspension in 20 ml PBS, followed by centrifugation at 1000 rpm for 5 minutes. This procedure was repeated twice to remove all traces of medium. Pellets were stored at -70°C, prior to DNA preparation (see section 2.2.1).

2.1.4.4. Harvesting Cells for Preparation of Agarose Plugs

The cells were harvested as described in section 2.1.4. The cells were then washed by resuspension in 20 ml PBS and centrifugation at 1000 rpm for 5 minutes. This was repeated twice in order to remove all traces of medium. The pellet was then resuspended in approximately 5 ml PBS, prior to agarose plug preparation (see section 2.2.2).

2.2 Preparation of DNA from Cultured Cells

2.2.1 Isolation of Genomic DNA from Cultured Cells

This method was adapted from van Heyningen (in press).

Frozen cell pellets were allowed to thaw before being resuspended in 0.5 ml TNE (10 mM Tris, pH 8.0; 1 mM ethylenediaminetetra-acetic acid (EDTA); 150 mM NaCl). The cells were maintained in suspension and lysed by the addition of 2.5 ml lysis buffer (0.5 % sodium dodecyl sulphate (SDS); 150 mM NaCl; 100 mM Tris.HCl, pH 8; 100 mM EDTA). RNA was digested by the addition of 300 μg RNase A (Sigma) and incubation at 37°C for 15 minutes. Proteins were digested by the addition of Proteinase K (Boehringer Mannheim) at 0.5 mg/ml with incubation overnight at 50°C. An equal volume of water saturated phenol was then added to the extracts, they were mixed thoroughly and centrifuged at 3000 rpm for 5 minutes. The aqueous layer was removed and extracted, as described above, with an equal volume of firstly 1:1 phenol/chloroform and secondly chloroform. The DNA was

precipitated from the aqueous layer with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol. The DNA was spooled out of the solution onto a glass rod. It was allowed to air dry, was washed in 75 % ethanol and again allowed to air dry. The DNA was then dissolved in 200 µl TE (10 mM Tris.HCl pH7.5; 1 mM EDTA).

2.2.2 Preparation of Plugs for Pulsed Field Gel Electrophoresis

This method was adapted from van Heyningen (in press).

The cells were harvested and washed as described in section 2.1.4. The pellet was then resuspended in approximately 5 ml PBS. A drop of the suspension was taken and the number of cells per ml estimated using a haemocytometer. The cells were centrifuged at 1000 rpm for 5 minutes and resuspended in equal volumes of PBS and 1 % low melting temperature agarose (Gibco BRL) such that the final concentration of cells was ~10^7 /ml. After thorough mixing the solution was quickly dispensed in 100 µl aliquots into plug moulds and allowed to set on ice. Once solidified the plugs were ejected into 20 ml 1 % NDS solution (0.45 M EDTA; 10 mM Tris.HCl pH 9; 1 % SDS) containing proteinase K at a final concentration of 1 mg/ml. The plugs were then incubated at 50°C for 24 hours. The solution was then replaced with fresh 1 % NDS/Proteinase K solution and a second 24 hour incubation carried out. The plugs were then stored in 20 ml 1 % NDS, without Proteinase K, at 4°C.

2.3 Yeast Cell Culture

2.3.1 Media and Additives

All media was sterilised by autoclaving.

AHC Broth and Agar

AHC is a rich, selective medium which lacks uracil and tryptophan. It was used for selective growth of YAC recombinants prior to production of plugs and isolation of DNA.

1.7 g yeast nitrogen base (without amino acids and ammonium sulphate) (Difco), 5 g ammonium sulphate, 10 g casein hydrolysate-acid (low salt) were added to 1 litre distilled water. The pH was adjusted to 5.8. 17-20 g of Bacto agar (Difco) per litre broth was added at this stage for AHC agar. After autoclaving and allowing to cool, 50 ml filter sterilised 40 % glucose and 10 ml 2 mg/ml sterile adenine sulphate solution were added to the broth (or agar).

YPD Broth And Agar

YPD medium was used for growing yeast cultures.

20 g bactopeptone (Difco), 5 g yeast extract (Difco), 5 g sodium chloride were added to 1 litre distilled water. 1.5 % agar was added at this stage for YPD agar. After autoclaving and allowing to cool, 50 ml sterile 40 % glucose was added to the broth or agar.

2.3.2 The ICI YAC Library

The ICI YAC library (Anand et al., 1990) was screened by Southern hybridisation (Southern, 1975) (see section 2.11.4). The library consists of approximately 35,000 YAC recombinants gridded at a high density on 23 filters of 8 cm x 12 cm. The average insert size is approximately 350 kb and the theoretical complexity of the library is therefore >3.5 genome equivalents. The percentage of chimaerism of the library is estimated at approximately 15 %. The ICI YAC library was obtained through the Human Genome Mapping Project (HGMP) Resource Centre, London.

2.3.3 Yeast Strains

Saccharomyces cereviseae strains YP148 (Jones et al., 1989) and AB972 (Link and Olson, 1991) were used as size markers for pulsed field gel electrophoresis. Table 2.1 lists the sizes of the chromosomes of the two strains.

TABLE 2.1 SIZES OF YEAST CHROMOSOMES (kb)	
YP148	AB972
90	240
220	280
280	350
360	440
445	590
555	680
610	755
690	810
760	840
800	950
830	980
920	1095
960	1120
1010	1130
1100	1640
1600	
2500	

2.4 Manipulation of YAC DNA

2.4.1 Yeast DNA Preparation

This method was adapted from Hoffman and Winston (1987).

10 ml yeast cultures were grown to saturation and the cells collected by centrifugation at 3000 rpm. The cells were resuspended in 500 µl dH_2O, transferred to an eppendorf tube and collected by a brief centrifugation at 13000 rpm. Most of the supernatant was removed and the pellet was briefly vortexed. 200 µl lysis buffer (2 % Triton X-100 (Sigma); 1 % SDS; 100 mM NaCl; 10 mM Tris.HCl, pH 8; 1 mM EDTA, pH 8), 400 µl phenol/chloroform and 0.3 g 0.4 mm glass beads (BDH) were added to the cells, which were then vortexed for 4 minutes. This process results in cell lysis and separation

of the nucleic acids, which remain in the aqueous layer, from the cell debris which remains in the organic layer. After vortexing 200 µl TE (10 mM Tris.HCl pH7.5; 1 mM EDTA) was added and the tubes were centrifuged at 13000 rpm for five minutes. The supernatant was decanted to a fresh tube and 1 ml cold ethanol was added to the supernatant (in order to precipitate the DNA). The tubes were inverted and centrifuged at 13000 rpm for 2 minutes. The pellet obtained was dried and resuspended in 40 µl TE. RNA was digested by addition of 30 µg RNaseA (Sigma) followed by a 5 minute incubation at 37°C. The DNA was precipitated by addition of 10 ml 4 M Ammonium Acetate and 1 ml ethanol (at -20°C). The tube was inverted and centrifuged at 13000 rpm for 2 minutes. Once dry the pellet was resuspended in 50 µl TE.

2.4.2 Preparation of Agarose Plugs for Pulsed Field Gel Electrophoresis

This method allows the extraction of intact yeast chromosomes, including yeast artificial chromosomes (YACs), from cells. It was adapted from Maule (in press).

A 100 ml yeast culture was grown, with shaking, at 200 rpm, from a single colony, at 30°C for 24 hours, or until the number of cells per ml was ~10^8. The culture was chilled on ice for 15 minutes. The cells were then harvested, by centrifugation at 4000 rpm, for 10 minutes at 4°C. The supernatant was discarded and the pellet dispersed in 50 ml chilled 50 mM EDTA pH 7.5. The cells were harvested, resuspended in fresh EDTA and harvested again, as described above. The pellet was resuspended in 3 ml chilled 50 mM EDTA, pH 7.5 and warmed to 37°C. Cell walls were disrupted by addition of 1.2 ml freshly prepared cell wall digestion solution (2 ml SCE, pH 8 (1 M sorbitol; 0.1 M sodium citrate; 60 mM EDTA); 100 µl 2-mercaptoethanol (Sigma); 2 mg zymolyase (ICN Biomedicals). 6 ml 1 % low melting temperature agarose (Gibco), in 0.125 M EDTA pH 7.5, at 50°C was added to this. After thorough mixing 100 µl aliquots of the solution were quickly dispensed into plug moulds and allowed to set on ice. Once solidified, the plugs were ejected into 25 ml ETM solution (O.45 M EDTA; 10 mM Tris.HCl pH 8; 7.5 % 2-mercaptoethanol) and placed in a 37°C waterbath for 24 hours. The ETM was replaced with 20 ml 1 % NDS solution (0.45 M EDTA; 10 mM Tris.HCl pH 9; 1 % SDS) containing proteinase K

(Boehringer Mannheim) at 1 mg/ml and the plugs incubated at 50°C for 24 hours. This step was repeated with fresh 1 % NDS solution. Finally, the plugs were stored in 20 ml ETM at 4°C.

2.5 Bacterial Cell Culture

2.5.1 Media and Additives

All media was sterilised by autoclaving.

L-Broth and Agar

2.46 g $MgSO_4$, 10 g tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl were added per litre of distilled water. 15 g agar (Oxoid Ltd) was added per litre broth for L-agar.

Terrific Broth

12 g tryptone, 24 g yeast extract, 4 g glycerol were added per 900 ml of distilled water. After autoclaving, 100 ml autoclaved phosphate solution (0.1M KH_2PO_4 and 0.72M K_2HPO_4) was added.

Ampicillin

Ampicillin was added to agar and broth in order to select for bacteria carrying plasmids which confer resistance to this antibiotic. A stock solution of ampicillin (Sigma) was made up at a concentration of 50 mg/ml. This solution was filter sterilised and stored at -20°C. It was added to broth and agar to give a final concentration of 50 μg/ml.

Kanamycin

Kanamycin selection was required for growth of 021, the cosmid containing the human OMP gene. A stock solution of kanamycin (Sigma) was made up at a concentration of 10 mg/ml. This solution was filter sterilised and stored at -20°C. Kanamycin was added to broth and agar to give a final concentration of 10 μg/ml.

5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-gal)

X-gal (Sigma) acts as a substrate for β-galactosidase. It was added to agar for the growth of plasmids carrying blue/white colour selection (see section 2.5.3). A stock solution of X-gal was made up at a concentration of 20 mg/ml in dimethyl sulphoxide (DMSO) (Sigma) and stored, protected from light at -20°C. It was added to agar to give a final concentration of 40 μg/ml.

Isopropyl β-D-Thiogalactopyranoside (IPTG)

IPTG is an derepressor of the Lac operon. It was added to agar for the growth of plasmids carrying blue/white colour selection (see section 2.5.3). A stock solution of IPTG (Sigma) was made up at a concentration of 100 mM and stored at -20°C. It was added to agar to give a final concentration of 0.5 mM.

2.5.2 Bacterial Strains

DH10B genotype: F-, araD139, Δ(ara, leu)7697, ΔlacX74, galU, galK, mcrA Δ(mrr- hsdRMS- mcrBC) rpsL, dor, ϕ80dlacZΔM15, endA1, nupG, recA1.

This bacterial host strain was used for the growth of Bluescribe (pBS) plasmid (see section 2.5.3).

2.5.3 Bacterial Vectors

Bluescribe (pBS) (Stratagene) is a plasmid vector based on pUC19 (Yanisch-Perron et al., 1985). The plasmid contains an ampicillin resistance gene which allows selection of transformed bacterial cells and the bacterial origin of replication allows attainment of high plasmid copy number. The vector has a blue/white colour selection system which allows colonies containing recombinant plasmids to be distinguished from those containing non-recombinant plasmids. The polylinker is inserted into the β-galactosidase (lac Z) gene. Correspondingly, plasmids without inserts have an active lac Z gene and produce a produce a blue indolyl derivative when grown on plates containing X-gal and IPTG. Colonies containing

recombinant plasmids have an interrupted, non-functional lac Z gene and are white.

2.5.4 Preparation of Competent Cells for Electro-Transformation

A sterile loop was used to streak out a bacterial host strain (DH10B) from a frozen glycerol stock onto an agar plate. The plate was grown at 37°C overnight. A single colony was then inoculated into 5 ml L-broth and incubated overnight at 37°C with shaking at 250 rpm. The following day, 500 ml of prewarmed L-broth was inoculated with the overnight culture and incubated overnight at 37°C with shaking at 250 rpm. The cells were grown to an absorbance (measured at 600 nm) of 0.5-1.0, transferred to a chilled flask and incubated on ice for 15-30 minutes. The suspension was then divided between ten chilled 50 ml centrifuge tubes and centrifuged at 4°C for 15 minutes at 3500 rpm. Each pellet was resuspended in 50 ml of treated water at 4°C. The water had previously been de-ionised, UV-sterilised, and autoclaved in bottles with no trace of detergent. This treatment was carried out as is very important that the water does not contain any impurities (which will alter its pH from 7.0), or any detergents. The suspension was then centrifuged, as described above, and the supernatant was carefully removed. Each pellet was resuspended in 25 ml of treated water, containing glycerol at a final concentration of 10 %. The cells were pelleted as described above and the supernantant carefully removed leaving the pellet in as small a volume as was possible. The pellets were pooled and resuspended in 10 % glycerol to give a final volume of 1 ml, resulting in a final cell concentration of approximately 3 X 10^{10}. 40 µl aliquots of the suspension were dispensed into Eppendorf tubes, which had previously been placed in a dry ice/ethanol bath. Finally, the pellets were stored at -70°C. The competence of the cells produced by this method ranged from 10^7 to 10^9 colony forming units/µg pUC19.

2.5.5 Electro-transformation of Competent Cells

40 µl aliquots of competent cells (~10^8 colony forming units/µg pUC19) were thawed at room temperature and placed on ice. 1-2 µl of the ligation reaction (see sections 2.7.5 and 2.7.6) was added to the cells. The cell suspension was mixed and incubated on ice for approximately 1 minute. It

was then transferred to a chilled electroporation cuvette (Flowgen) and subject to a 2.5 kV pulse, using a Bio Rad Gene Pulser. 1 ml L-broth was quickly added to the cell suspension. The cells were transferred to an Eppendorf tube and incubated at 37°C for approximately 45 minutes. After this time, a fraction of the cells were spread on an agar plate containing the appropriate antibiotic selection and incubated at 37°C overnight.

2.6 Preparation of plasmid DNA

2.6.1 Small Scale Plasmid DNA Preparation

This method is adapted from Jones and Schofield (1990).

A single bacterial colony was picked into 4 ml of "terrific broth" with appropriate antibiotic selection and incubated overnight at 37°C, with shaking at 250 rpm. 1.5 ml of the culture was decanted into an Eppendorf tube and the cells pelleted by centrifugation at 13000 rpm for 1 minute. The supernatant was decanted from the pellet and the tube allowed to drain for several minutes to remove any remaining fluid. The pellet was resuspended in 150 µl of GTE (50 mM glucose; 10 mM EDTA; 25 mM Tris.HCl pH 8.0, which had been filter sterilised) by vortexing. 300 µl of alkali solution (0.2 M sodium hydroxide, 1% SDS) was prepared and added. The tube was inverted several times to mix the contents and placed on ice for 5 minutes to allow cell lysis to occur. 225 µl of ice cold, high salt solution (3 M potassium acetate pH 4.8, which had been sterilised by autoclaving and stored at 4°C) was added, in order to precipitate the, unwanted, bacterial DNA. The solution was then mixed, by inversion of the tube, and placed on ice for 5 minutes. After this time the tube was then centrifuged at 13000 rpm for 5 minutes. The supernatant was decanted to a fresh tube and one volume of ethanol was added. The tube was immediately centrifuged at 13000 rpm for 5 minutes. The pellet obtained was drained, washed in 70 % ethanol and dried under vacuum for 5 minutes. It was then resuspended in 40 µl TE(10 mM Tris.HCl pH7.5; 1 mM EDTA).

The concentration of the DNA obtained was determined by measurement of the optical density. The absorbance at 260 nm (ABS_{260})= 1 at 20 µg/ml double stranded DNA.

DNA obtained in this way was used in restriction endonuclease digests (see section 2.7.1) and as template in double-stranded sequencing (see section 2.13.1)

2.6.2 Large Scale Plasmid DNA Preparation

This method was adapted from Sambrook et.al. (1989).

A single bacterial colony was picked into 4 ml of "terrific broth" with appropriate antibiotic selection and incubated overnight at 37°C, with shaking at 250 rpm. This culture was then used to inoculate 400 ml of "terrific broth," containing the appropriate antibiotic selection, in a 1 litre flask. This was incubated overnight at 37°C, with continuous shaking at 250 rpm. Cells were harvested, in two 200 ml Sorvall bottles, by centrifugation at 6000 rpm for 10 minutes at room temperature. The supernatants were decanted and both pellets were resuspended in 9 ml GTE (50 mM glucose; 25 mM Tris pH 8.0; 10 mM EDTA pH 8.0, which had been filter-sterilised). The two suspensions were then combined. 100 mg lysozyme was dissolved in 2 ml GTE and added to the suspension which was mixed and incubated at room temperature for 10 minutes (in order to initiate cell lysis). 40 ml alkali solution (0.2 M sodium hydroxide, 1% SDS) was prepared and added. The bottle was then swirled, to mix the contents, and incubated on ice for 5 minutes (to allow cell lysis to go to completion). 20 ml of ice-cold high-salt solution (3 M potassium acetate, pH 4.8, which had been sterilised by autoclaving) was then added to the bottle (to precipitate the, unwanted, bacterial chromosomal DNA). After gentle but thorough mixing, the solution was incubated on ice for 15 minutes. The cell lysate was then centrifuged at 4000 rpm for 15 minutes at 4°C; the rotor was allowed to stop without the brake to minimise dislodging of the pellets. The supernatant was filtered through muslin, into a sterile measuring cylinder and 0.6 volumes of isopropanol were added. The solution was mixed thoroughly by shaking and incubated at room temperature for 10 minutes. The precipitated plasmid DNA was recovered by centrifugation at 5000 rpm for 15 minutes at room temperature. The pellet was drained, rinsed in 70 % ethanol and dried under vacuum. The pellet was then resuspended 7.7 ml TE(10 mM Tris.HCl pH7.5; 1 mM EDTA). To this was added 400 µl ethidium bromide (10 mg/ml) and 9.2 g caesium chloride. The resultant solution was

carefully pipetted into quickseal ultra-centrifuge tubes (Beckmann) and centrifuged in a fixed-angle rotor at 100,000 rpm for 16-18 hours at room temperature. The covalently-closed-circular plasmid DNA was visible as a tight band about half way up the centrifuge tube. Bands were extracted from the tube using a sterile 0.8 gauge hypodermic needle attached to a syringe. The ethidium bromide was removed from the DNA by repeated extraction with water-saturated butan-1-ol (extractions were carried out until no pink colour remained in the organic phase). The DNA was then precipitated from the caesium chloride-containing solution by addition of 2.5 volumes 70 % ethanol. The solution was mixed and incubated at -70°C for 20 minutes. It was then centrifuged for 10 minutes at 10,000 rpm. The pellet obtained was drained, vacuum-dried and resuspended in 200 µl TE.

The ABS_{260} was measured in order to estimate the DNA concentration (see section 2.6.1.

2.7 Enzymatic Manipulation of DNA

2.7.1 Restriction Endonuclease Digestion of DNA

Digestions with restriction endonucleases (Boehringer Mannheim) were carried out in the appropriate buffer (supplied by the manufacturer) at the recommended temperature. 1 µg DNA was digested in 5-10 µl, containing 1/20th volume of 0.1 M spermidine (Sigma), with 1-2 units of restriction enzyme, for 1 hour. Reactions were scaled up accordingly, except that the amount of enzyme was reduced when longer incubation times were used. When two different enzymes, both of which required the same buffer, were used digests were carried out simultaneously. Otherwise, digestion with the enzyme requiring the lower buffer salt concentration was carried out first. The salt concentration was then altered by addition of the appropriate amount of sodium chloride solution and the second digestion carried out. Reactions were terminated by heating to 68°C or 80°C for 15 minutes, according to the heat sensitivity of the enzyme (New England Biolabs catalogue 1992), or alternatively by phenol/chloroform extraction of the reaction. Restriction digests which were run on agarose gels were terminated by the addition of 1/10th of their volume of "stop mix" (100 mM EDTA, pH 8; 20 % Ficoll and orange G)

2.7.2 Restriction Enzyme Digestion of Agarose Plugs

This method was adapted from Maule (in press).
Agarose plugs were soaked overnight on a shaker at room temperature in a large excess of sterile TE (10 mM Tris.HCl pH7.5; 1 mM EDTA). The following day they were soaked for 10 minutes in fresh TE before being transferred to TE containing phenylmethylsulfonylfluoride (PMSF) (Sigma) at 40 μg/ml (PMSF was dissolved in isopropanol at 20 mg/ml, 2 μl of this was added to each ml TE). The plugs were then incubated at 50°C for 30 minutes. They were transferred to fresh TE/PMSF and incubated at 50°C for a further 30 minutes. The TE/PMSF was then replaced with 10 volumes of the appropriate restriction buffer (Boehringer Mannheim) and shaken at room temperature for 2 hours. The plugs were then digested in a 100 μl volume which contained 1 X restriction buffer, 0.1 % Triton-X 100 (Sigma), 200 μg/ml bovine serum albumin (BSA) (Boehringer Mannheim), and 20 units of restriction enzyme. Incubation, at the appropriate temperature, was carried out overnight. The following day the plugs were cooled on ice for 15 minutes and the liquid was removed. They were then rinsed in 1 ml ice cold TE. The TE was removed, 200 μl "plug stop mix" (9.4 ml dH_2O; 0.4 ml EDTA, pH 8; orange G (Sigma); 0.25 ml TAE, see section 2.8.1) was added and they were left on ice for 20 minutes. The plugs were then ready for electrophoresis (see section 2.8.4).

2.7.3 Dephosphorylation of Linear Plasmid DNA

Dephosphorylation reactions were carried out in a 50 μl volume which contained 7 mg of linear plasmid DNA, 0.1 unit of calf intestinal phosphatase (CIP) (Boehringer Mannheim) and 5 μl 10 X CIP buffer (Boehringer Mannheim). The reaction was incubated at 56°C for 30 minutes, another 0.1 unit CIP was added and incubation was continued for a further 30 minutes. The reaction was stopped by the addition of 1 μl 0.5 M EDTA (giving a final concentration of 10 mM). The volume was increased to 150 μl by the addition of TE (10 mM Tris.HCl pH7.5; 1 mM EDTA). The solution was extracted twice with an equal volume of phenol:chloroform:isoamylalcohol. This was followed by addition of an equal volume of chloroform:isoamylalcohol. The DNA was then precipitated, by the addition of 1/10th volume 3 M sodium acetate and an equal volume of ice-

cold ethanol. The solution was then incubated at -20°C for 30 minutes followed by centrifugation at 15000 rpm for 15 minutes at 4°C. The pellet was washed in 70 % ethanol, dried and resuspended in 10 µl TE.

2.7.4 Conversion of 5' Overhangs to Blunt-ended Molecules

The Klenow fragment of *E.coli* DNA polymerase I has a 5' to 3' polymerase activity, and a 3' to 5' exonuclease activity (Sambrook et al., 1989). It was utilised to 'fill in' recessed 3' ends of DNA molecules, to generate blunt-ended molecules.

Approximately 5 µg of plasmid DNA, which had been previously linearised generating a 5' overhang, was diluted in sterile water to give a final volume of 28.5 µl. To this 1.5 µl 50 x dGTP, dCTP, dATP, dTTP mix (to give a final concentration of 400 µM of each dNTP) and 3 units Klenow fragment (Boehringer Mannheim, sequencing grade) were added. The reaction was incubated at 37°C for 60 minutes, after which the enzyme was inactivated by incubation at 68°C for 20 minutes.

2.7.5 Ligation of Blunt-ended Molecules

The optimal ratio of ends for such a ligation event is 3:1 vector:insert (Cobinachi and Wilson, 1987). Typically, between 10 and 100 ng of vector was used. Reactions were carried out in a volume of 10 µl which contained 1 µl 10 X T4 DNA ligase buffer (to give a final concentration of 20 mM Tris.HCl pH 7.6; 5 mM magnesium chloride; 5 mM DTT; 50 mg/ml BSA) (Sambrook et al., 1989), 30 mM potassium chloride, 10 mM ATP and 0.1 unit T4 DNA ligase. Reactions were incubated overnight at 4°C. The enzyme was then heat inactivated by incubation at 68 °C for 20 minutes.

2.7.6 Ligation of Sticky-ended Molecules

The optimal ratio of ends for such a reaction is 1:2 vector:insert (Cobinachi and Wilson, 1987). Typically, between 10 and 100 ng of vector was used. Reactions were carried out in a volume of 10 µl which contained 1 µl 10 X ligase buffer (Boehringer Mannheim) (to give a final concentration of 50 mM Tris.HCl pH 7.4; 10 mM magnesium chloride; 10 mM DTT; 1 mM spermidine;

1 mM ATP; 100μg/ml BSA) and 0.1 unit T4 DNA ligase (Boehringer Mannheim). The reaction was incubated overnight at 16°C. The enzyme was then heat inactivated by incubation at 68 °C for 20 minutes.

2.8 DNA Electrophoresis

2.8.1 Electrophoresis Solutions

20 X TBE

1 M Tris, 20 mM EDTA and 1 M boric acid pH 8.3.

20 X TAE

0.8 M Tris, 20 mM EDTA, pH 8 and 0.4 M acetic acid.

10 X DNA Loading buffer ("stop mix")

20 % ficoll, 100 mM EDTA, orange G.

6 % Denaturing Polyacrylamide

57 g of acrylamide, 3 g of bis-acrylamide and 460 g of urea were dissolved in 1 litre of 1 X TBE. This was stored at 4°C in foil covered bottles. 60 ml of this 6 % acrylamide solution was used for each polyacrylamide gel. 75μl N,N,N,N'-tetramethylethylenediamine (TEMED) (Sigma) and 0.5 ml 10 % ammonium persulphate were added to this.

2.8.2 Agarose Gel Electrophoresis

DNA molecules were separated, according to size, in horizontal agarose gels by electrophoresis. The percentage of agarose (Type II medium EEO) (Sigma) in the gel was varied according to the range of size separation required. Restricted genomic DNA was run in a 0.8 % or 1 % agarose gel. Smaller DNA fragments, such as plasmids or PCR products (see section 2.14) were run on 1-2 % gels, with

fragments smaller than 200 bp being run on either 2 % low melting point agarose (Ultrapure LMP agarose, Gibco BRL) or 4 % 3:1 Nusieve agarose (FMC Bioproducts). All agarose gels were made from and run in 0.5 X TBE. Ethidium bromide was added to all agarose gels and buffer at a concentration of 1 μg/ml buffer. 1/10th of the sample volume of 10 X "stop mix" was added to DNA samples prior to loading on the gel. The size marker used was 1kb ladder (Gibco BRL). Gels were run in Electro-4 gel boxes (Hybaid) generally at 75-120 volts or at 15-30 volts overnight (genomic digests only).

DNA fragments were visualised on a UV transilluminator at 305 nm and photographed using a video copy processor (Mitsubishi).

2.8.3 Preparative Agarose Gel Electrophoresis

DNA fragments were run in low melting point agarose gels (Ultrapure LMP agarose) (Gibco BRL) in 0.5 X TBE. Gels were viewed on a UV transilluminator at 305 nm and the required fragment was cut out of the gel using a sterile scalpel blade. Care was taken to insure that the minimum size of gel slice was excised.

DNA was isolated from the gel slice using agarase (Boehringer Mannheim), according to the manufacturers instructions.

DNA fragments which were to be used only as hybridisation probes were not isolated from the gel but prepared as follows. The gel slice was weighed and 1-3 X the weight of water was added (such that 11 μl of diluted gel slice contained 25-50 ng DNA).

2.8.4 Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gels were used to visualise DNA sequencing reactions.

The glass plates were prepared by washing thoroughly with detergent and rinsing with water and then 100 % ethanol. The front plate was then coated in dimethyldichlorosilane solution and the back plate was

coated in a solution of 10 ml 100 % ethanol; 30 µl acetic acid; 30 µl methacryoxypropyltrimethoxysilane. Spacers were placed on either side of the back plate and the front plate was placed on top. The plates were then taped together at the sides and the bottom. The freshly prepared polyacrylamide was then poured between the plates, a sharkstooth comb was inserted and clamps were applied. The gel was allowed to set for at least 40 minutes at room temperature.

Gels were run in 1x TBE on vertical slab gel apparatus (Scotlab) at 30 volts for 1.5-4.5 hours.

After electrophoresis the tape and the front plate were removed. The gel was placed in fix solution (10 % methanol; 10 % acetic acid) for 15 minutes. It was then rinsed in dH_2O for 15 minutes and placed at 68°C until dry. The gel was then exposed to film (see section 2.11.4).

2.8.5 Pulsed Field Gel Electrophoresis

The agarose plugs were loaded into the wells of a 1 % agarose, 0.5 X TAE gel and the wells were sealed with 0.5 % LMP agarose. The gel was placed into the apparatus (made by John Maule) (Maule and Green, 1990) which contained sufficient buffer such that the gel was submerged 3 mm below the buffer. The buffer had previously been cooled and was kept at a constant temperature of 10°C (by circulation of the buffer through a cooling unit).

Pulsed field times depended on the size of the DNA fragments which were being separated.

After electrophoresis, the DNA was visualised by staining in buffer containing ethidium bromide at 5 µg/ml for 40 minutes.

2.9 Transfer of DNA to Membranes

2.9.1 Southern Transfer Protocols

DNA which had been run on agarose gels was transferred onto nylon membranes by capillary blotting. This method was adapted from Southern (1975).

Gels were photographed next to a ruler to allow future sizing of DNA fragments.

An additional step was included when blotting pulsed field gels. To aid the transfer of the large yeast chromosomes and YACs, pulsed field gels were UV irradiated on a transilluminator (at 305 nm) for 5 minutes, after being photographed.

The DNA was denatured by gentle shaking of the gel in denature solution (0.5 M NaOH, 1.5 M NaCl) for 45 minutes. The gel was then neutralised by gentle shaking in neutralising solution (1 M Tris.HCl; 2 M NaCl, pH 5.5) for 45 minutes. A large strip of 3 MM filter paper (Whatman) was soaked in 20 X SSC (3 M NaCl, 0.3 M $Na_3C_6H_5O_7.2H_2O$, pH 7) and placed on a board. The ends of the paper were placed into a reservoir of 20 X SSC, generating a wick. The gel was placed on top of the wick and a piece of nylon membrane (Hybond-N, Amersham), which had been cut to the size of the gel and prewetted in 2 X SSC, was placed directly onto the gel, avoiding bubbles. On top of this was placed two pieces of 3 MM blotting paper, also cut to size and prewetted in 2 X SSC, followed by two dry pieces of 3 MM paper. Any exposed wick was covered in Saran-wrap (Dow Chemical Company) and a 12 cm stack of paper towels and a weight (approximately 1 kg) were placed on top of the gel.

The length of blotting time varied according to the size of fragments which were being transferred. Pulsed field gels were blotted for two days, genomic digests for 4-16 hours and smaller fragments for 4 hours.

The membranes were then rinsed briefly in 2 X SSC and baked at 80°C. The DNA was then cross-linked to the filter by exposure to UV on a

transilluminator (at 305 nm) for 3.5 minutes. Nylon membranes were stored in Saran-wrap (Dow Chemical Company) at room temperature.

2.9.2 Transfer of Plasmid DNA from Bacterial Colonies to Membranes

This method was used to screen large numbers of bacterial colonies for recombinant plasmid.

A bacterial culture was spread onto a gridded nitrocellulose filter which had been placed on a L-agar plate. The plate was incubated at 37°C until colonies were visible. A second filter was then briefly placed on top of the first, before being transferred to another plate (colony side uppermost). Both plates were incubated at 37°C to allow colony growth. One plate, designated the master plate, was stored at 4°C. The filter was carefully removed from the second plate and floated, colony side up, on a solution of 0.5 M NaOH; 1.5 M NaCl. After 10 minutes the filter was briefly transferred to a piece of Whatman 3MM paper to remove excess solution. The filter was then floated on a solution of 0.5 M Tris.HCl pH 7.4; 1.5 M NaCl for 5 minutes. It was then transferred to a solution of 0.5 M Tris.HCl pH 7.4; 1.5 M NaCl; 100 μg/ml Proteinase K for 30 minutes. The filter was then rolled between two 3MM sheets in order to remove bacterial matter and excess moisture. The filter was floated on chloroform for 30 seconds before being allowed to air dry. It was then baked at 80°C for 1.5-2 hours under vacuum.

2.10 Radiolabelling of DNA

2.10.1 Random Priming of DNA Probes

Probes were labelled with [α-^{32}P] dCTP by random priming from hexadeoxyribonucleotides with the Klenow fragment of *E. coli* DNA polymerase I (Feinberg and Vogelstein, 1983 and 1984).

25-50 ng DNA (either in the form of a gel slice or in solution), in a volume of 11 μl was denatured by incubation at 100°C for 10 minutes. If the DNA was in solution the reaction was cooled rapidly on ice or, if a gel derived probe was used, the reaction was cooled to 37°C. A Boehringer Mannheim Random Priming kit was used to label the

probe. 2 µl of 10 X reaction buffer, 1 µl each of dATP, dGTP and dTTP (0.5 mM), 2 units of Klenow and 30 µCi [α-^{32}P] dCTP were added to the probe which was then incubated at 37°C for a minimum of 45 minutes. After this time the level of [α-^{32}P] dCTP incorporation was measured by trichloroacetic acid precipitation (Sambrook et al., 1989). Once a sufficiently high level of incorporation (at least 50 %) had been achieved unincorporated nucleotides were removed from the probe. The nucleotides were removed by running the probe through a Nick column (Pharmacia). The storage buffer was removed from the column which was then equilibrated with 1 ml TNE (10 mM Tris.HCl, pH 8; 1 mM EDTA, pH 8; 0.2 M NaCl). The probe was then added to the column in a volume of 100 µl. 300 µl TNE was added and the probe was eluted in a further 400 µl. 500 µg denatured sonicated salmon sperm DNA was added to the eluate and this mixture was denatured at 100°C for 10 minutes. After this time the probe was chilled on ice, to prevent reannealing, before being added to the hybridisation mix.

2.10.2 Preannealing of Repetitive Sequences

Probes which contained repetitive sequences were annealed, after labelling, to repeat containing DNA. This was carried out in order to prevent the repetitive element of the probe taking part in the hybridisation reaction. This procedure was adapted from Sealey et al. (1985).

The probe was labelled and incorporation tested, as described in 2.10.1, but the probe was not put down a Nick column. After incorporation had been checked the probe was incubated at 100°C, with 1 mg sonicated human DNA, for 10 minutes. The probe was then incubated, in a prewarmed lead pot, at 68°C for 30-45 minutes. After this time the probe was added to the hybridisation mix.

2.10.3 End Labelling of DNA Oligonucleotides

Oligonucleotides were labelled by the transfer of the ^{32}P-labelled γ-phosphate from [γ-^{32}P] ATP onto the terminal 5'-OH group.

30 ng oligonucleotide was labelled in a 20 µl volume which contained 1 X polynucleotide kinase (PNK) buffer (5 mM Tris.HCl, pH 8; 1 mM $MgCl_2$; 0.5 mM DTT) (Boehringer Mannheim), 10 units of polynucleotide kinase (PNK) (Boehringer Mannheim) and 30 µCi of [γ-^{32}P] ATP (Amersham). Reactions were incubated at 37°C for 30-40 minutes and were then added to the hybridisation mix.

2.11 Hybridisation Protocols

2.11.1 Hybridisation Solutions

Hybridisation Mix

6 X SSC, 10 % dextran sulphate, 0.1 % sodium pyrophosphate, 0.5 % SDS; 4 X Denhardts solution (100 X Denhardts solution is 2 % BSA; 2 % polyvinylpyrrolidone; 2 % ficoll; 1 mM EDTA, stored at 4°C).

Oligo Hybridisation Mix

5 X SSC, 0.05 % BSA, 0.05 % ficoll, 0.1 % sodium dodecyl sulphate, 0.05 % polyvinylpyrrolidone, 0.1% sodium pyrophosphate.

2.11.2 Pre-hybridisation Protocols

Pre-hybridisation of filters was carried out in oligo hybridisation mix at 5°C below the T_m of the oligonucleotide (see section 2.14.1) (end labelled oligonucleotides) and in hybridisation mix at 68°C (random primed probes). Filters were placed between two gauze sheets in a hybridisation bottle (Hybaid). 10 ml of hybridisation mix was used with the small bottles and 20 ml with the larger bottles. Bottles were placed in a Hybaid hybridisation oven with a rotating spindle for at least 1 hour (end labelled oligonucleotides) and at least 4 hours (random primed probes). Denatured sonicated salmon sperm was added to the hybridisation mix at a concentration of 100 µg/ml.

2.11.3 Hybridisation and Washing Protocols

End labelled oligonucleotide hybridisations were carried out for 4-16 hours at 5°C below the T_m of the oligonucleotide. Filters were then removed from the bottles and washed three times for 5 minutes each at room temperature in 4 X SSC, 0.1 % SDS, heated to the same temperature as the hybridisation reaction.

Random primed hybridisations were incubated for 16 hours at 68°C. Filters were then washed in the hybridisation bottles with 2 X SSC, 0.1 % SDS for 20 minutes at 68°C. They were then removed from the bottles and washed with increasing stringency to 1 X SSC, 0.1% SDS; 0.5 X SSC, 0.1% SDS or 0.1 X SSC, 0.1% SDS at 68°C.

2.11.4 Removal of Hybridisation Signal

Probes were removed from Southern blots according to the manufacturer's instructions. A solution of 0.1 % SDS was prepared and boiled. This solution was then poured onto the membrane and allowed to cool to room temperature.

2.12 Detection of Hybridisation

2.12.1 Autoradiography

Filters were exposed to Kodak X-OMAT film in cassettes with intensifying screens. Filters hybridised to ^{32}P labelled probes were exposed at -70°C and ^{35}S reactions at room temperature. Films were exposed for a period of 30 minutes to 2 weeks (depending on the signal strength) before being developed, using an automatic x-ray film processor RGII (Fuji).

2.12.2 Phosphorimaging

A Molecular Dynamics PhosphorImager was also used for detecting and quantifying hybridisation. Filters were exposed on a phosphor screen for periods of 30 minutes to 48 hours. They were then

scanned on the PhosphorImager, where a laser beam converts the radioactive signal into a digital image, with variations in the pixel values proportional to the amount of radioactivity present. The image was displayed on a grey scale, the upper and lower limits of which were adjusted to give good image visualisation. The image was then printed out on a grey scale laser printer.

2.13 Sequencing of DNA

2.13.1 Sequencing of Double Stranded DNA

Adapted from Sanger et al. (1977).

Template plasmid DNA was prepared according to section 2.6.1. RNA was digested by the addition of 1 µl of RNase A (50 mg/ml) to 7 µl of the template DNA and incubation at room temperature for 15 minutes. 1 µl of sequencing primer (see section 2.15.2) (at 10 ng/ml) and 1 µl of 1 M sodium hydroxide were then added, and the mixture was incubated at 68°C for 10 minutes (this is to allow denaturation of the template DNA in the presence of the primer). The primer was then annealed to the template by addition of 4 µl of TDMN (0.3 M TES (Sigma); 0.05 M DTT; 80 mM magnesium chloride; 0.2 M sodium chloride; 1 % chloroform; pH 1.6, stored at 4°C) and incubation at room temperature for 10 minutes. The reaction was then placed on ice until required. To this reaction 1µl 0.1 M DTT, 0.4 µl 5 X Sequenase labelling mix (7.5 mM each dGTP; dCTP; dTTP) (USB) , 2.1 µl LoTE (3 mM Tris.HCl, pH 7.5; 0.2 mM EDTA), 0.5 µl [α^{35}S] dATP (Amersham) and 2 µl diluted T7 DNA polymerase (Sequenase) (USB) (enzyme had previously been diluted 1:7 with enzyme dilution buffer) were added. This extension reaction was incubated at room temperature for 3 minutes. The extension reactions were then terminated by addition of 4 µl of the reaction to 2.5 µl of the four dideoxynucleotide termination mixes (containing either ddATP, ddGTP, ddTTP or ddCTP, USB), which had previously been warmed at 37°C for 5 minutes. The termination reactions were incubated at 37°C for 10 minutes after which time 5 µl STOP mix (USB) was added. The reactions were then stored at -20°C prior to polyacrylamide electrophoresis.

2.14 Amplification of DNA by the Polymerase Chain Reaction (PCR)

PCR (Saiki et al., 1985; Mullis and Faloona, 1987) is an enzymatic amplification technique which permits the cyclic amplification of a specific DNA sequence. The sequence specificity is provided by a pair of oligonucleotide primers which direct amplification. The primers are complimentary in sequence to the 5' ends of sequence to be amplified and are annealed to the template DNA under conditions where they hybridise only to their exact compliment. The PCR reaction is a series of cycles, each of which consists of three steps. The first step is a high temperature step, which causes denaturation of the template. This is followed by a reduction in temperature (to that which is optimum for precise annealing of the primers) and finally an extension step at 72°C (which is the optimum temperature of the thermostable polymerase).

2.14.1 PCR from Genomic DNA

Reactions were carried out in a 50 µl volume which contained 5 µl 10 X buffer (50 mM KCl; 100 mM Tris.HCl pH 8.4; 1 % Triton X-100) (Promega), 3 µl 25 mM $MgCl_2$ (Promega), 1 µl of each primer at 250 ng/µl, 1 µl of 50 X dNTP stock where each dNTP at 10 mM (Pharmacia), 100 ng of template DNA and 1 unit of thermostable DNA polymerase (Promega or Perkin Elmer Cetus). Reactions were overlaid with mineral oil (Sigma).

PCRs were carried out on various machines; a Perkin Elmer Cetus DNA Thermal Cycler, Hybaid Thermal Reactors and a Hybaid Omnigene. The temperature and length of the different steps were varied according to the primer pair, the machine and the length of the fragment to be amplified. When published PCR conditions were available these were used as the basis of the initial reaction. If no information was available, as was the case for example for primers designed from published sequence information, then initial conditions were determined as follows. Each set of conditions always had an initial cycle with a longer denaturation step at a higher temperature (generally, 94°C for 3 minutes- Perkin Elmer Cetus DNA Thermal Cycler, 93°C for 2 minutes- Hybaid machines). This cycle was followed by a series of cycles, the number of which was dependent on the efficiency of

amplification (generally 30 cycles were performed initially). This series of cycles consisted of a denaturation step (generally, 92°C for 45 seconds- Perkin Elmer Cetus DNA Thermal Cycler, 91°C for 30 seconds- Hybaid machines) followed by an annealing step. The annealing temperature used was that which was 5°C lower than the melting temperature (T_m) of the primers. This was determined by the equation T_m, at 1M Na^+ concentration, = 4(G+C) + 2(A+T). Annealing was generally carried out for 1 minute- Perkin Elmer Cetus DNA Thermal Cycler and for 30 seconds- Hybaid machines. Annealing was followed by extension at 72°C for approximately 1 minute/kb to be amplified- both machines. The final cycle had an increased extension step of 5 or 10 minutes depending on the size of the amplified fragment. In a number of cases the initial conditions required modification, in order to promote optimum amplification. Changes were made to the annealing temperature and the number of cycles of amplification. Formamide has been reported to enhance the specificity of PCR reactions (Sarkar et al., 1990). Formamide (BDH) was added to PCR reactions to a final concentration of 5 %. "Touchdown" PCR (Don et al., 1991) has also been reported to enhance the specificity of PCR reactions. In "touchdown" PCR the annealing temperature of the first cycle (or the first two cycles) is approximately 10°C higher than the theoretical annealing temperature. Over subsequent cycles the temperature is lowered by 1 or 2°C every one or two cycles until the theoretical temperature is reached. The cycles which are carried out at the stringent conditions provided by higher temperatures should promote amplification of the correct template molecules, rather than aberrant amplification from partially mismatched template. Thus, by the time the temperature is at that which allows mispriming, the correct product has an advantage over aberrant products.

10 µl of the PCR product was visualised on an ethidium bromide-stained agarose gel (see 2.8.1).

2.14.2 PCR from Bacterial Colonies

This method was adapted from Taylor (1991).

The reaction was set up essentially as described in 2.14.1, except that a sterile toothpick was used to add a small amount of a bacterial colony to the

reaction (in the place of the genomic template). Reaction conditions were determined as described in 2.14.1 except that the initial cycle denaturation time was increased (to 5 minutes- Perkin Elmer Cetus DNA Thermal Cycler and to 3 minutes- Hybaid machines).

2.14.3 Primer Design

Where published primers were not available primers were designed from published sequence or, in the case of D11S388 from sequencing of an appropriate clone (see section 2.16.2). Primers were designed either by eye, or by the Oligo4 programme (Hybaid).

2.15 Oligonucleotides

2.15.1 Oligonucleotide Synthesis

Oligonucleotides were synthesised, in the form of ammonium stocks, on an Applied Biosystems 381A oligonucleotide synthesizer.

Oligonucleotides were precipitated from stocks by the addition of 1/10th volume 3 M sodium acetate and 2.5 volumes ethanol followed by a 1 hour incubation at -20°C. They were pelleted by a 15 minute centrifugation at 1300 rpm, washed twice in 80 % ethanol and dried under vacuum. The pellet was resuspended in 200 µl TE. The concentration of the DNA obtained was determined by measurement of the optical density; where the absorbance at 260 nm (ABS_{260}) =1 at 25 µg/ml single stranded DNA.

2.15.2 Sequence of oligonucleotides

The oligonucleotides which were used in PCR analysis of chromosome 1 and chromosome 11 markers are described in section 2.16.1.

The following oligonucleotides are complementary to sequences in the cloning vector pBS (Stratagene) (see section 2.5.3).

5' CAG GAA ACA GCT ATG AC 3'

5' GTA AAA CGA CGG CCA GT 3'

These oligonucleotides were used individually to sequence (see section 2.13.1) inserts contained in pBS. They were used together to promote PCR amplification (see section 2.14.2) of inserts contained in pBS. The PCR conditions used with these primers are described bellow. One cycle of 94°C, 5 minutes; 52°C, 30 seconds; 72°C, 1 minute. Thirty cycles of 91°C, 30 seconds; 52°C, 30 seconds; 72°C, 1 minute. One cycle of 91°C, 30 seconds; 52°C, 30 seconds; 72°C, 5 minutes.

2.16 Details of Marker Assays

This section describes the details of the assays for published chromosome 1 and chromosome 11 markers.

2.16.1 Details of PCR primers

The primer sequences, conditions of amplification, the size of the fragment amplified and source references are described in Table 2.2. In the case of the TYRLI assay the PCR primers amplified from both the TYRLI locus and the TYR gene (Giebel et al., 1991). A restriction digestion step was necessary after the PCR reaction, in order to distinguish between the products of amplification of the two loci (Giebel et al., 1991). The products were digested with MspI, giving rise to fragments of 120 bp and 140 bp from the TYR locus and fragments of 29 bp, 91 bp and 140 bp from the TYRLI locus.

2.16.2 Development of a PCR assay for D11S388

The cosmid CJ52.4, which corresponds to the D11S388 locus (Julier et al., 1990) was subcloned in a two step procedure. The first step was the identification and cloning of subfragment which contained a $(CA)_n$ repeat. This clone was then itself subcloned, in order to identify a fragment which both contained the $(CA)_n$ repeat and which was small enough to allow sequencing of the entire insert (using primers complementary to the vector sequence).

Table 2.2 PCR Primers for Chromosome 11 and Chromosome 1 Markers

LOCUS	PRIMER SEQUENCES	AMPLIFICATION CONDITIONS		FRAGMENT SIZE	REFERENCES
HBB	5' ATG GTG CAC CTG ACT CCT GAG G 3' 5' GCC ATC ACT AAA GGC ACC GAG C 3'	94^0: 3'; 65^0: 1'; 72^0: 1' 92^0: 45", 65^0: 30"; 72^0: 30" 92^0: 45"; 65^0: 30"; 72^0: 10'	1x 30x 1x	355 bp	S-L Thien, pers. comm. Glaser et al., 1989; Gaensler et al., 1991
D11S87	5' CCC TGG AAA CAC TTT CTG CC 3' 5' GGC TGG GTT GGA GGC AAG G 3'	94^0: 3'; 65^0: 30"; 72^0: 30" 92^0: 45"; 65^0: 30"; 72^0: 30" 92^0: 45"; 65^0: 1'; 72^0: 10'	1x 30x 1x	120 bp	In-house sequence and design Lewis et al., 1988
TYRL1	5' ACA ATA TGT TTC TTA GTC TG 3' 5' TGG TAA CAC TAG ATT CAG C 3'	94^0: 3'; 51^0: 45"; 72^0: 1' 92^0: 45"; 51^0: 45"; 72^0: 1' 92^0: 45"; 51^0: 45"; 72^0: 10'	1x 35x 1x	260 bp	Giebel et al., 1991 Oligos P1 & P2 (MspI digest to distinguish from TYR)
PGA	5' GCA TCT CTG ACA CCA ATC AG 3' 5' TGG AGA AGA GAC AGA TGG AG 3'	94^0: 3'; 54^0: 1'; 72^0: 1' 92^0: 45", 54^0: 30"; 72^0: 45" 92^0: 45", 54^0: 30"; 72^0: 5'	1x 27x 1x	188 bp	W Cookson, pers. comm. Evers et al., 1989
ROM1	5' AGG CCT GGA GCT TGG GGT GA 3' 5' CCA TCC AAA AAC TTT ATT GAG TCT 3'	94^0: 3'; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 5'	1x 30x 1x	275 bp	Primers designed from Bascom et al., 1992
MDU1	5' TCT TCA AAG CCT CTG CAG TAC C 3' 5' CTC ATC TCC AAC CTG TCT AAC C 3'	94^0: 3'; 57^0: 1'; 72^0: 2' 92^0: 45"; 57^0: 1'; 72^0: 2' 92^0: 45"; 57^0: 1'; 72^0: 10'	1x 35x 1x	859 bp	Primers designed from Gottesdiener et al., 1988
GSTPI (was GST3)	5' GGG AGG GAT GAG AGT AGG ATG 3' 5' GGA GGT TCA CGT ACT CAG GGG 3'	93^0: 2'; 66^0: 15"; 72^0: 1' 91^0: 30"; 66^0: 15"; 72^0: 1' 91^0: 30"; 64^0: 15"; 72^0: 1' 91^0: 30"; 62^0: 15"; 72^0: 1' 91^0: 30"; 60^0: 15"; 72^0: 1' 91^0: 30"; 60^0: 15"; 72^0: 5'	1x 1x 2x 2x 30x 1x	450 bp	Primers designed from Cowell et al., 1988
FGF4 (was HSTF1)	5' GAT GAG TGC ACG TTC AAG GAG 3' 5' CAG AGA TGC TCC ACG CCA TAC 3'	94^0: 3'; 62^0: 1'; 72^0: 1' 92^0: 45"; 62^0: 1'; 72^0: 1' 92^0: 45"; 62^0: 1'; 72^0: 10'	1x 30x 1x	542 bp	Primers designed from Yoshida et al., 1987
D11S527	5' ATG CGC CTC CAA GAC AAG TTC 3' 5'GCC CCT CTA CTT GTC TGG AG 3'	94^0: 3'; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 5'	1x 30x 1x	142-146 bp	Brown et al., 1991

continued overleaf

Table 2.2 PCR Primers for Chromosome 11 and Chromosome 1 Markers (continued)

LOCUS	PRIMER SEQUENCES	AMPLIFICATION CONDITIONS		FRAGMENT SIZE	REFERENCES
D11S533	5' CTC TGC CTA GTC CCT GGG TG 3' 5' TGG GGG TCT GGG AAC ATG 3'	93^0: 2'; 68^0: 15"; 72^0: 90" 91^0: 30"; 68^0: 15"; 72^0: 90" 91^0: 30"; 66^0: 15"; 72^0: 90" 91^0: 30"; 64^0: 15"; 72^0: 90" 91^0: 30"; 62^0: 15"; 72^0: 90" 91^0: 30"; 62^0: 15"; 72^0: 5'	1x 1x 2x 2x 32x 1x	300-900 bp	Eubanks et al., 1991
D11S901	5' CCC ACA TAG ATT ACT GGC CTC 3' 5' TCC TAC ATT AGC AGT TGG CA 3'	93^0: 2'; 58^0: 30"; 72^0: 30" 91^0: 30"; 58^0: 30"; 72^0: 30" 91^0: 30"; 58^0: 30"; 72^0: 5'	1x 30x 1x	160-176 bp	Weissenbach et al., 1992
TYR	5' GCA AGT TTG GCT TTT GGG GA 3' 5' CTG CCA AGA GGA GAA GAA TG 3'	94^0: 3'; 55^0: 35"; 72^0: 1' 92^0: 45"; 55^0: 35"; 72^0: 1' 92^0: 45"; 55^0: 35"; 72^0: 10'	1x 28x 1x	512 bp	Giebel and Spritz, 1990 (exon 1 oligos)
D11S931	5' GAT TGC TTG AGC CCA G 3' 5' GAG AAA TAG TAT GTG TTT GCC 3'	93^0: 2'; 67^0: 30"; 72^0: 45" 91^0: 30"; 67^0: 30"; 72^0: 45" 91^0: 30"; 65^0: 30"; 72^0: 45" 91^0: 30"; 63^0: 30"; 72^0: 45" 91^0: 30"; 61^0: 30"; 72^0: 45" 91^0: 30"; 59^0: 30"; 72^0: 45" 91^0: 30"; 59^0: 30"; 72^0: 5'	1x 1x 2x 2x 2x 30x 1x	251-276 bp	Weissenbach et al., 1992
D11S873	5' CCT GGT TTA GAA TAA TAC CT 3' 5' ATA ATG TAC TGT GAT AAA TGC T 3'	93^0: 2'; 54^0: 30"; 72^0: 30" 91^0: 30"; 54^0: 30"; 72^0: 30" 91^0: 30"; 54^0: 30"; 72^0: 5'	1x 35x 1x	176-206 bp	Litt et al., 1993
EST16	5' GTC TTT GGA TTC TAC GTA GA 3' 5' CGA TAA TGA CAT TTC TTC TGG 3'	94^0: 3'; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 45" 92^0: 45"; 55^0: 30"; 72^0: 5'	1x 35x 1x	73 bp	Polymeropoulos et al., 1992
D11S388	5' CAG TAA GAA CCA AAG AAA GGT TAC 3' 5' CAT CCA CGC TGT TGG TCT GC 3'	93^0: 2'; 51^0: 30"; 72^0: 30" 91^0: 30"; 51^0: 30"; 72^0: 30" 91^0: 30"; 51^0: 30"; 72^0: 5'	1x 27x 1x	120 bp	In house sequence and design Julier et al., 1990

continued overleaf

Table 2.2 PCR Primers for Chromosome 11 and Chromosome 1 Markers (continued)

LOCUS	PRIMER SEQUENCES	AMPLIFICATION CONDITIONS	FRAGMENT SIZE	REFERENCES
CLG	5' AGT CAG TAC AGG AGC CGA ACA G 3' 5' GGA GAA AAG CTG TGC ATA CTG G 3'	94^0: 3'; 59^0: 1'; 72^0: 1' 1x 92^0: 45"; 59^0: 30"; 72^0: 1' 28x 92^0: 45"; 59^0: 30"; 72^0: 10' 1x	517 bp	Primers designed from Angel et al., 1987
STMY1	5' CAG TTT TCT CCT CTA CCA AGA C 3' 5' ACT GGC TTT ACT TAG CTC TAT G 3'	94^0: 3'; 50^0: 45"; 72^0: 45" 1x 92^0: 45"; 50^0: 30"; 72^0: 30" 30x 92^0: 45"; 50^0: 30"; 72^0: 10' 1x	300 bp	Primers designed from Sirum and Brinkenhoff, 1989
D11S385	5' TTT TAT AGG GAC AGG ATC TTG C 3' 5' GGC TGT ATA ATC TTG TGT TCT C 3'	94^0: 3'; 54^0: 1'; 72^0: 30" 1x 92^0: 45"; 54^0: 30"; 72^0: 30" 30x 92^0: 45"; 54^0: 30"; 72^0: 10' 1x	286 bp	In-house sequence and design Julier et al., 1990
NCAM	5' TGG AAA TCT CTT CCA AAC ATC GGA G 3' 5' AAT TAG AAC TTT GGA GAG GGA TGG G 3'	94^0: 3'; 55^0: 1'; 72^0: 30" 1x 92^0: 45"; 55^0: 30"; 72^0: 30" 30x 92^0: 45"; 55^0: 30"; 72^0: 5' 1x	179 bp	G Gillett, pers. comm. Barton et. al., 1990
DRD2	5' GAG GCC CTC TCA CTG ACA C 3' 5' AGT GCA GGG CCC TGC TGG A 3'	94^0: 3'; 55^0: 1'; 72^0: 30" 1x 92^0: 45"; 55^0: 30"; 72^0: 30" 35x 92^0: 45"; 55^0: 30"; 72^0: 5' 1x	390 bp	R Todd, pers. comm. Grandy et al., 1989
D11S351	5' CTT GGG TAG CTG GTA CTA CAG G 3' 5' AGG TCA CTA CAC ATC AAA ACA GC 3'	94^0: 3'; 59^0: 1'; 72^0: 30" 1x 92^0: 1'; 59^0: 30"; 72^0: 30" 30x 92^0: 1'; 59^0: 30"; 72^0: 5' 1x	209 bp	In house sequence and design Julier et al., 1990
THY1	5' CAG AAG GTG ACC AGC CTA ACG 3' 5' CTG AGC ACT GTG ACG TTC TGG 3'	94^0: 3'; 40^0: 1'; 72^0: 1' 1x 92^0: 45"; 40^0: 1'; 72^0: 1' 30x 92^0: 45"; 40^0: 1'; 72^0: 5' 1x	324 bp	Cotter et al., 1989
EST111	5' GGA AAT TAG GCT TAG CTC AC 3' 5' GTG CAG AAT ACT TAG AGT CC 3'	94^0: 3'; 57^0: 30"; 72^0: 30" 1x 92^0: 45"; 57^0: 30"; 72^0: 30" 30x 92^0: 45"; 57^0: 30"; 72^0: 5' 1x	93 bp	Polymeropoulos et al., 1992
D1S251	5' GTC TCC AGC CTG CCA C 3' GAC CAA GCA ACT TCA CTC C 3'	93^0: 2'; 57^0: 30"; 72^0: 30" 1x 91^0: 30"; 57^0: 30"; 72^0: 30" 35x 91^0: 30"; 57^0: 30"; 72^0: 5' 1x	250-270 bp	Weissenbach et al., 1992
D1S179	5' GAT CCT TTT ATA GAA GAT AGT 3' 5' TGT ACG ATG CAT AGG TTT GC 3'	94^0: 3'; 55^0: 30"; 72^0: 30" 1x 92^0: 45"; 55^0: 30"; 72^0: 30" 35x 92^0: 45"; 55^0: 30"; 72^0: 5' 1x	180 bp	Engelstein et al., 1993

continued overleaf

Table 2.2 PCR Primers for Chromosome 11 and Chromosome 1 Markers (continued)

LOCUS	PRIMER SEQUENCES	AMPLIFICATION CONDITIONS		FRAGMENT SIZE	REFERENCES
D1S163	5' TCT TCG TGT GTG GAA CCG TA 3' 5' GCG AGA AAT GAA CTT GGC TC 3'	94^0: 3'; 55^0: 30"; 72^0: 30" 92^0: 45"; 55^0: 30"; 72^0: 30" 92^0: 45"; 55^0: 30"; 72^0: 5'	1x 30x 1x	200 bp	Hudson et al., 1992
ACTN2	5' AGA ATG TAA AAC AGG GGT CGA ACC AG 3 5' TTA TAG AAG GGA CAA GAG TGT ATT TG 3'	94^0: 3'; 54^0: 30"; 72^0: 30" 92^0: 45"; 54^0: 30"; 72^0: 30" 92^0: 45"; 54^0: 30"; 72^0: 5'	1x 35x 1x	91-107 bp	Beggs et al., 1992

The cosmid was digested with EcoRI and BamHI (see section 2.7.1). The digested cosmid was run on a 0.8% gel (see section 2.8.2) and the DNA transferred to a nylon membrane by Southern blotting (Southern, 1975) (see section 2.9.1). The blot was hybridised with an end-labelled $(CA)_{15}$ oligonucleotide (see section 2.10.3 and 2.11). DNA corresponding to the band which hybridised most strongly to this oligonucleotide was obtained by preparative agarose gel electrophoresis (see section 2.8.3). Vector DNA pBS (Stratagene) (see section 2.5.3) was prepared in parallel and was also digested with EcoRI and BamHI. Digested plasmid DNA was purified by preparative agarose gel electrophoresis (see section 2.8.3). An equal volume of water saturated phenol was then added to both DNAs. They were mixed thoroughly and centrifuged at 13000 rpm for 5 minutes. The aqueous layer was removed and extracted, as described above, with an equal volume of firstly 1:1 phenol/chloroform and secondly chloroform. The DNA was precipitated from the aqueous layer with 1/20th volumes of 5 M NaCl and three volumes of cold ethanol. The DNAs were then placed at -20°C for one hour and then centrifuged at 13000 rpm for 15 minutes. The pellets were then washed in 70 % ethanol, dried and resuspended in a minimal volume of sterile distilled water. A ligation reaction was then set up (see section 2.7.6). The ligation reaction product was used to transform an aliquot of competent DH10B cells (see sections 2.5.4 and 2.5.5) which were plated out on L-agar plates containing ampicillin, X-gal and IPTG (see section 2.5.1). DNA from the resultant colonies was transferred to nitrocellulose filters (see section 2.9.2). The filters were hybridised with the same end-labelled $(CA)_{15}$ oligonucleotide. Plasmid DNA was prepared from a subset of the hybridising colonies (see section 2.6.1). This DNA was digested with EcoRI and BamHI and run on an agarose gel, in order to check that the correct size band was obtained. A Southern blot was also prepared from this gel. This blot was hybridised with the end-labelled $(CA)_{15}$ oligonucleotide, in order to check that the correct fragment had been cloned. This fragment was too large (4 kb) for sequence analysis. It was therefore digested with HinfI, the digest was run on a 1 % gel and the DNA was transferred to a nylon membrane. The blot was hybridised with the end-labelled $(CA)_{15}$ oligonucleotide, identifying a ~500 bp fragment. DNA corresponding to this band was obtained by preparative agarose gel electrophoresis. The recessed 3' ends left by this enzyme were converted to blunt-ended molecules (see section 2.7.4). Vector DNA was prepared in

parallel; pBS was digested with SmaI and subject to phenol/chloroform extraction and precipitation (see above). The DNA was then dephosphorylated (see section 2.7.3). Both vector and insert DNA were then subject to phenol/chloroform extraction and precipitation (see above) and a ligation reaction was set up (see 2.7.5). The ligation reaction product was transformed, colonies were obtained and screened as described above resulting in the identification of clones which contained the appropriate insert. Plasmid DNA from these clones was sequenced, using primers complementary to vector sequence (see section 2.13.1). The sequence obtained was used to design PCR primers (see section 2.14.3) which promoted amplification, of an appropriate sized band, from human genomic DNA, as well as for the cosmid CJ52.4.

2.16.3 Details of Assays carried out by Southern Blotting

2.16.3.1 Assaying D1S8

The lambda probe corresponding to the D1S8 locus (Royle et al., 1988) was obtained from ICI Diagnostics, Cheshire . The 5.9 kb insert was released by digestion with Sau3A (see section 2.7.1). The appropriate band was excised from a 1 % LMP gel and used directly as a hybridisation probe (see section 2.8.3).

2.16.3.2 Assaying ADROA2

PCR primers corresponding to plasmid sequence were used to amplify a ~550 kb insert from a plasmid containing an insert corresponding to part of the ADROA2 locus (Libert et al., 1989 and 1991) (the plasmid was obtained from Gilbert Vassart, Universite de Bruxelles, Brussels). The DNA was excised from a 1 % LMP gel and used directly as a hybridisation probe (see section 2.8.3).

2.16.3.3 Assaying OMP

PCR primers derived from the sequence of the rat OMP gene were used to amplify an exonic fragment from the equivalent human locus. This DNA was excised from a 1 % LMP gel for use as a hybridisation probe (see section

2.8.3). This fragment was initially used to probe a Southern blot of digested somatic cell hybrid DNA, in order to localise the locus on a hybrid mapping panel. This probe, however hybridised to both human and rodent sequences. The human specific signal was insufficiently intense to allow unambiguous scoring. The human exonic fragment was therefore used as a probe to screen a human cosmid library (the library screening was carried out by Craig Simpson). This cosmid was digested with PstI (see section 2.7.1). The digested cosmid was run on a 1 % gel (see section 2.8.2) and the DNA transferred to a nylon membrane by Southern blotting (Southern, 1975) (see section 2.9.1). The blot was hybridised (see section 2.11) with the exonic fragment described above. DNA corresponding to the band which hybridised to this probe was obtained by preparative agarose gel electrophoresis (see section 2.8.3). Vector DNA pBS (Stratagene) (see section 2.5.3) was prepared in parallel. It was digested with PstI and subject to phenol/chloroform extraction and precipitation (as described in section 2.16.2). The DNA was then dephosphorylated (see section 2.7.3). Both vector and insert DNA were then subject to phenol/chloroform extraction and precipitation and a ligation reaction was set up (see section 2.7.6). This ligation reaction product was used to transform an aliquot of competent cells (see sections 2.5.4 and 2.5.5) which were plated out on L-agar plates containing ampicillin, X-gal and IPTG (see section 2.5.1). The inserts in white colonies were examined by PCR amplification (see section 2.14.2) which was directed by primers corresponding to vector sequences (see section 2.15.2). This indicated inserts of the appropriate size. A Southern blot of the PCR products was then hybridised with the human exonic fragment described above, confirming the identity of clones which contained the appropriate insert. DNA prepared from clones identified in this way was digested with PstI. The digested DNA run on a 1 % LMP gel, the insert was excised from the gel and used directly as a hybridisation probe (see section 2.8.3).

CHAPTER 3

3. Marker Analysis of Hybrid Cell lines

3.1 Introduction

It was decided to focus primarily on the cloning of the chromosome 11 translocation breakpoint. The chromosome 11 translocation breakpoint was chosen, because of the established chromosome 11 resources available in the laboratory. These included chromosome 11 derived somatic cell hybrids and assays for markers of known location on this chromosome. The existence of two other balanced translocations which appeared to segregate with schizophrenia, and which involved chromosome 11q (Smith et al., 1989; Holland and Gosden, 1990) (the breakpoints were not accurately defined) also prompted this focus.

A set of hybrid cell lines was analysed for chromosome 1 and chromosome 11 markers, by PCR and Southern blot analysis. This was carried out firstly, to produce a chromosome 11 mapping panel, which could be used to regionally localise markers on chromosome 11, thus defining the location of the chromosome 11 breakpoint at high resolution. Secondly, this analysis was carried out to determine the location of chromosome 1 markers with respect to the t(1;11)(q42.2;q21) translocation breakpoint. Details of the marker assays and source references are described in section 2.16.

The hybrid cell lines which constituted the mapping panel were as follows. Somatic cell hybrid lines which segregate the reciprocal translocation chromosomes are MIS 7.4 (murine, X63, background) and MAR 12 (murine, RAG, background) which carry the derived 1 translocation chromosome and MIS 39.8 and MAR 1, which carry the derived 11 translocation chromosome (each in the absence of any normal chromosome 1 or 11 material) (Fletcher et al., 1993). Four X irradiation hybrids, WJX 3.4, WJX 5.4, WJX 7.4 and WJX 11.2, contain fragments of chromosome 11 in the background of a Chinese hamster ovary cell line, WG3H (Fletcher et al., 1993). Two independent HRAS 1-selected chromosome mediated gene transformants (CMGT), E67.1 and E67.4, which carry single fragments of chromosome 11 material, in the background of a murine, C127, cell line were also analysed (Porteous et al., 1986). PG48 has a translocated chromosome 11, which lacks 11q22-11qter, as well as a number of other human chromosomes in a,

a23, hamster background (McConville et al., 1991). CF52 has a well characterised t(11;16) (q13;p11) translocation chromosome as its sole human component in the background of a murine cell line, A9 (Koeffler et al., 1981).

MAFLI, which is a permanent B lymphoblastoid cell line established from a patient with schizophrenia and bearing the t(1;11)(q42.2;q21) translocation (Fletcher et al., 1993), and J1CL4, which is a hybrid cell line which contains an intact chromosome 11 as its sole human component (Jones et al., 1984), were positive controls. Cell lines WG3H, X63 and RAG (see above) were negative controls.

Cytogenetic analysis of the translocation chromosomes had suggested that the translocation breakpoints occurred at 11q21 and 1q42.2 (St.Clair et al., 1990). The chromosome 11 markers which were analysed were largely chosen on this basis. A few short arm markers and markers spaced down the length of the long arm were included, but the emphasis was on those markers from the 11q13-11q23 region. Chromosome 1 markers could only be mapped relative to the translocation breakpoint. Markers thought to map in the region of 1q41-43 were analysed in order to determine whether they were proximal or distal to the chromosome 1 translocation breakpoint.

A few markers were assayed by Southern blot analysis, but the majority were PCR assays. In the case of the tyrosinase like gene (TYR LI), the PCR primers amplify from both the TYR LI locus and the tyrosinase (TYR) gene. A restriction digestion step was necessary after the PCR reaction in order to distinguish between the products of amplification from the two loci (Giebel et al., 1991) (see section 2.16.1).

Most of the loci which were localised using the hybrid mapping panel were already known to map to chromosome 11. The exception to this was olfactory marker protein (OMP). OMP is of unknown function. It is found in a wide variety of vertebrates and is expressed predominantly in mature, functioning olfactory receptor neurones where it is present as a highly abundant cytoplasmic protein (Farbman and Margolis, 1980; Rogers et al., 1987 and Danciger et al., 1989). In the mouse, OMP maps to a region of mouse chromosome 7 which exhibits conservation of synteny with several human chromosomal regions, including 11q and 11p (Rinchik et al., 1992).

OMP was assayed by Southern hybridisation, using a subclone of a cosmid corresponding to this locus. The development of the OMP assay is described in section 2.16.3.3.

A number of chromosome 11 brain expressed sequence tags (ESTs) (Polymeropoulos et al., 1992; Durkin et al., 1992 and Polymeropoulos et al., 1993) were regionally localised on chromosome 11. The ESTs had been derived from partial sequencing of brain cDNAs and were formatted for PCR typing.

3.2 Development of PCR Assays

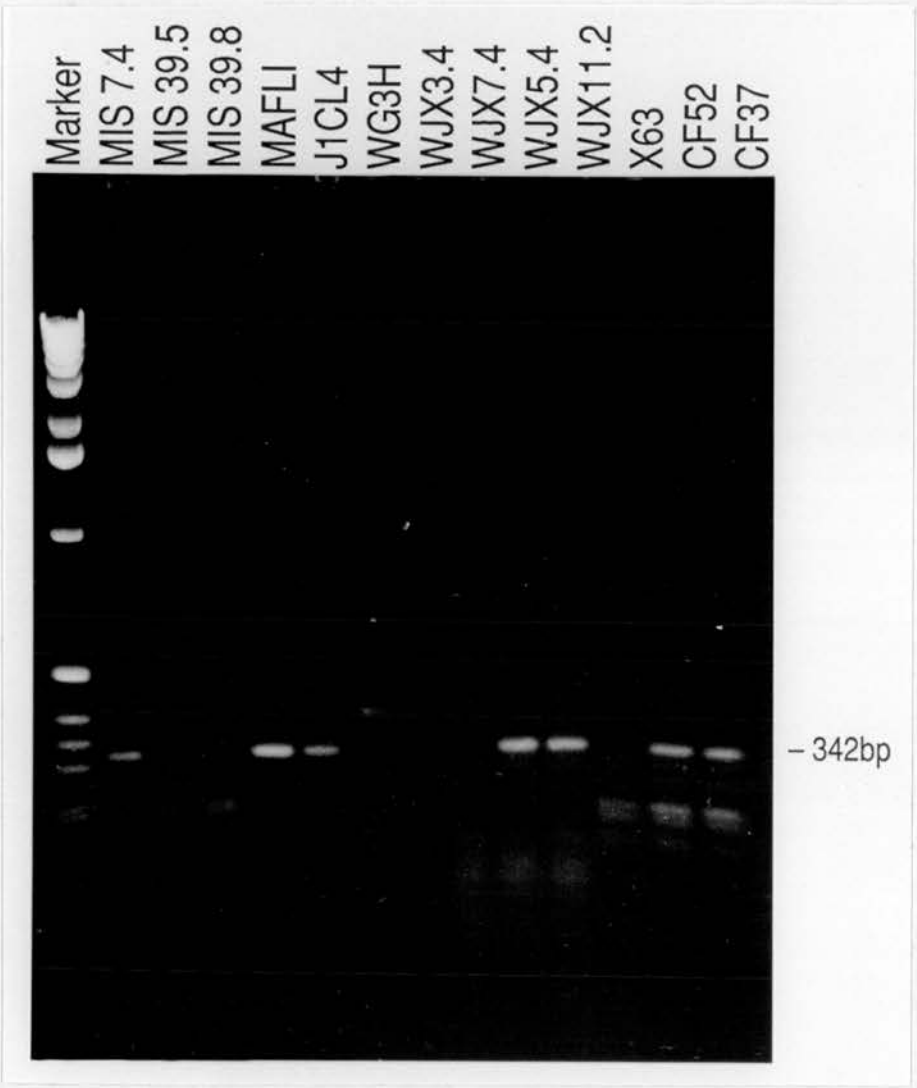
3.2.1 Preliminary PCR Assays

Initial PCR conditions were devised as explained in section 2.14.1. Selected examples of the results of preliminary PCR reactions are shown in Figure 3.1 (the cell surface marker thy 1, THY1), Figure 3.2a (fibroblast growth factor, FGF4) and Figure 3.3a (D11S533). The initial conditions of the THY1 assay resulted in amplification of extra bands, as well as the target band. However, scoring of the correct band was still possible. The initial conditions of the FGF4 assay resulted in unreliable amplification of the predicted band and amplification of many spurious bands. The initial conditions of the D11S533 assay did not promote amplification.

3.2.2 Modification of PCR Assays

It can be seen in Figure 3.3a, which shows D11S533 amplified under published conditions, that the conditions which result in optimum amplification vary from laboratory to laboratory. It is likely that this is due to variation between PCR machines, variation in buffer constituents and variation in the type of enzyme used. Often, when primers were designed from published sequence (as described in section 2.14.1) the initial amplification conditions also required modification (see Figure 3.2a). In assays where specific amplification was not initially obtained the PCR conditions were modified in the following ways. The annealing temperature was raised, the number of cycles of amplification were altered, "touchdown" PCR (Don et al., 1991) was carried out and formamide (BDH) was added to a final concentration of 5%

Figure 3.1: PCR Analysis of Segregation of THY1 in the Hybrid Panel



The cell lines are described in section 2.1.1. The size marker is the 1 kb ladder (Gibco BRL). Aliquots of somatic cell hybrid DNA were assayed by PCR (see section 2.14.1) for the presence of the THY1 locus. Positive signal is marked, at 342 bp. The results demonstrate that THY1 maps distal to the t(1;11)(q42.2;q21) translocation breakpoint (as MIS 7.4 is positive). This result, together with the segregation pattern in the fragmentation hybrids means that THY1 can be placed close to D11S351, consistent with the data of Richard et al., (1993) who place this marker distal to D11S351 (see Table 3.2a). PCR products, other than the predicted fragment of 342 bp are present. It is likely that these products are caused by mispriming. The aberrant amplification does not, however, affect the ability to score the locus specific band.

Figure 3.2: PCR Analysis of Segregation of FGF4 in the Hybrid Panel

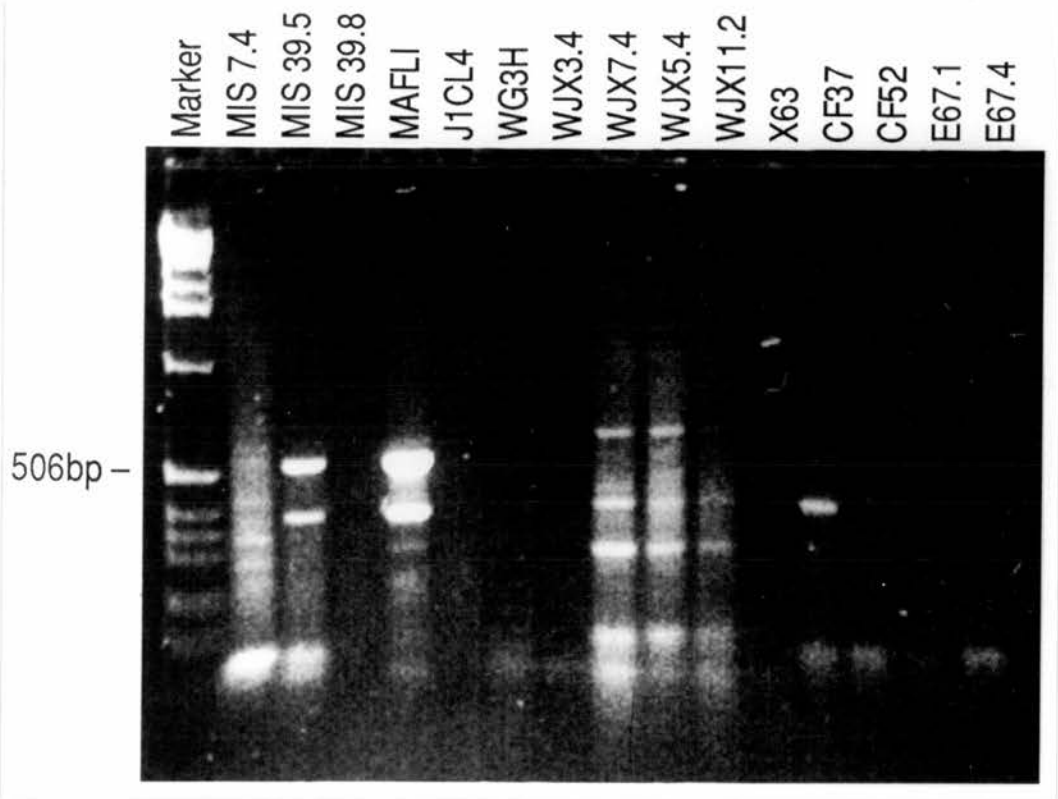


Figure 3.2a

The cell lines are described in section 2.2.1. The size marker is the 1 kb ladder (Gibco BRL).

Aliquots of somatic cell hybrid DNA were assayed by PCR (see section 2.14.1) for the presence of the FGF4 locus. The conditions of amplification were designed as described in section 2.14.1. A positive result for the FGF4 assay is indicated by a PCR product of 542 bp. Signal was not present in the positive control hybrid J1CL4 (which contains an intact human chromosome 11 as its sole human component) and, in many of the tracks, PCR products other than the predicted band of 542 bp, are present. It is likely that these products are caused by mispriming.

Figure 3.2: PCR Analysis of Segregation of FGF4 in the Hybrid Panel

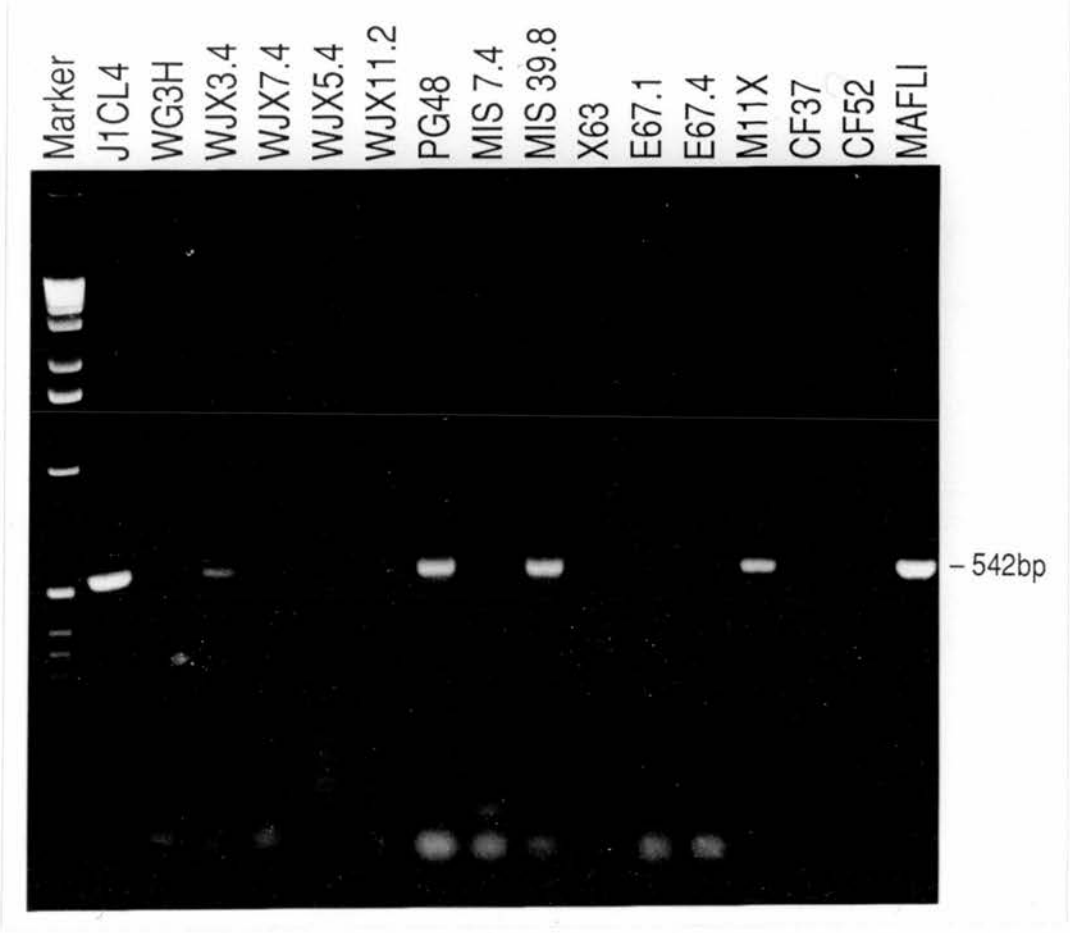


Figure 3.2b

The cell lines are described in section 2.1.1. The size marker is the 1 kb ladder (Gibco BRL). The hybrids were re-assayed for FGF4, following sequential modification of the amplification conditions, as described in Table 3.1. Positive signal is now present at the expected size. The results of this segregation analysis place this marker in a new interval, which is proximal to the translocation breakpoint of CF52, at 11q13 (Koeffler et al., 1981). This result, in conjunction with the results obtained in the fragmentation hybrids, and with published information (FGF4 is distal to Bcl1 (Petty et al., 1993) and Bcl1 is distal to ADROA2, Szepetowski et al., 1993), can be used to localise this marker (see Table 3.2a).

Figure 3.3: PCR Analysis of Segregation of D11S533 in the Hybrid Panel

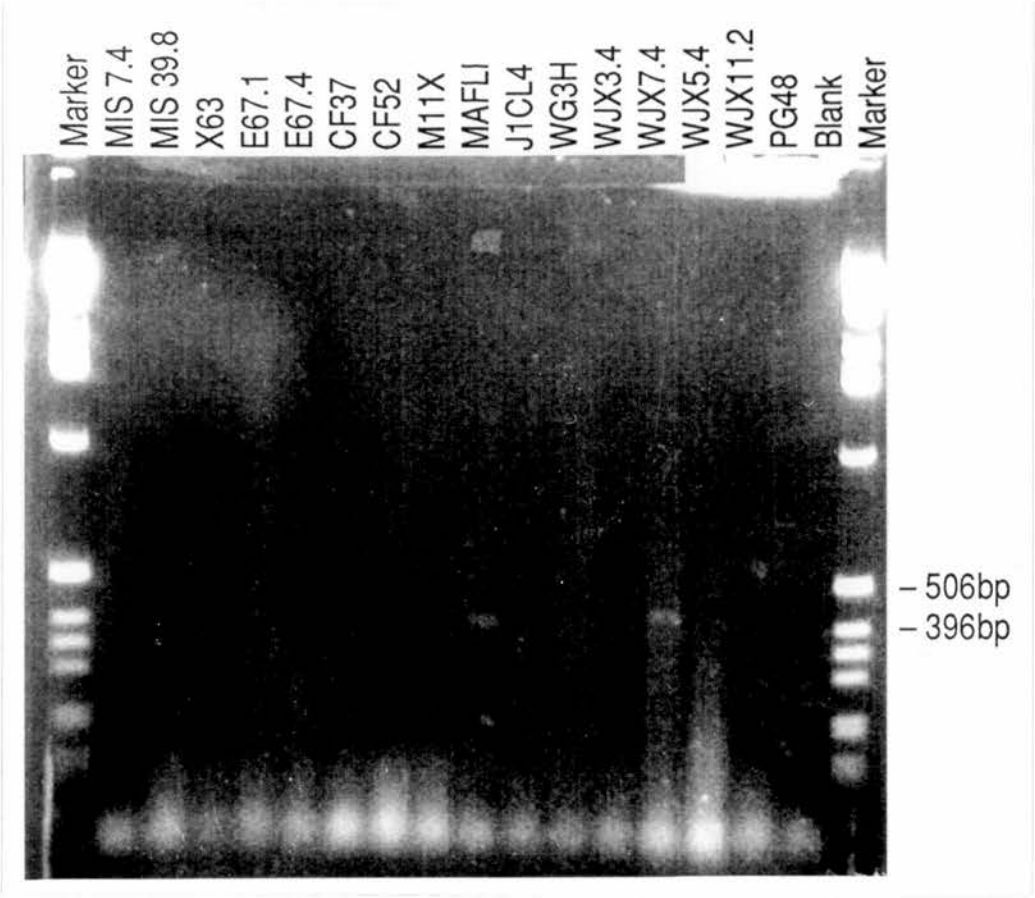


Figure 3.3a

The cell lines are described in section 2.1.1. The size marker is the 1 kb ladder (Gibco BRL).

Aliquots of somatic cell hybrid DNA were assayed by PCR (see section 2.14.1), using published conditions, for the presence of the polymorphic marker D11S533. Positive signal was expected within the range of 300-900 bp. There are a few faint bands in some of the tracks but the conditions of amplification are clearly suboptimal.

Figure 3.3: PCR Analysis of Segregation of D11S533 in the Hybrid Panel

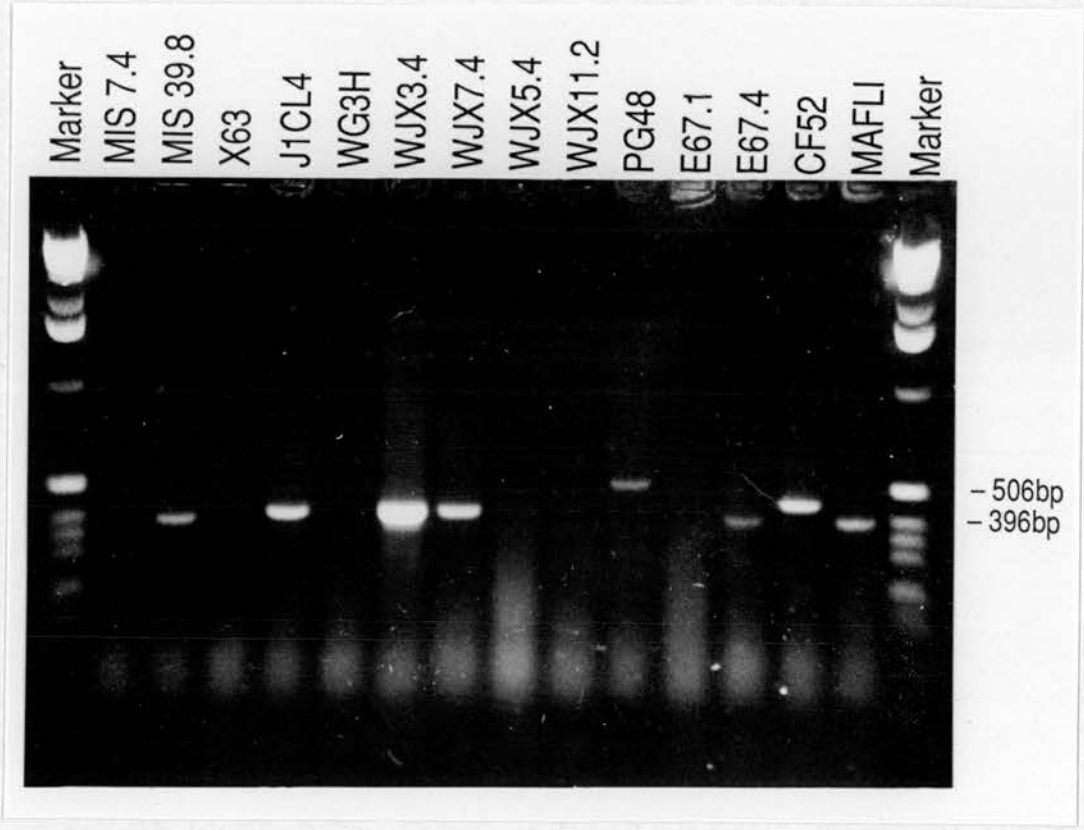


Figure 3.3b

The cell lines are described in section 2.1.1. The size marker is the 1 kb ladder (Gibco BRL).

The hybrids were re-assayed for D11S533, following sequential modification of the amplification conditions, as described in Table 3.1. Positive signal is now present in the expected size range. The results of this segregation analysis place this marker in an interval which is proximal to the t(1;11)(q42.2;q21) translocation breakpoint, but distal to the translocation breakpoint of CF52, at 11q13 (Koeffler et al., 1981). This result, in combination with the results obtained in the fragmentation hybrids and published information (D11S533 maps to 11q13.5 (Eubanks et al., 1991) and is very closely linked to D11S527, Li et al., 1992) can be used to localise this marker (see Table 3.2a).

(see section 2.14.1). Successful modification of the initial conditions was often possible and examples of this are shown in Figures 3.2b and 3.3b. Figure 3.2b shows the results of sequential modifications to the FGF4 assay. The addition of formamide to this assay resulted in specific amplification. Figure 3.3b shows the results of sequential modification of the D11S533 assay. In this case a lower annealing temperature, "touchdown" PCR and increased number of cycles were necessary to promote specific amplification. Table 3.1 describes the modifications which were made to the PCR assays of all the initial markers.

3.3 Scoring of PCR assays

Where possible, markers were ordered relative to each other taking published information into account and assuming the least number of independent blocks of human DNA in the various hybrids.

3.4 Marker Analysis of the Hybrid Cell Lines

Tables 3.2a and 3.2b describe the results of initial marker analysis. This analysis resulted in the determination of flanking markers of the translocation breakpoints. Tyrosinase (TYR) was found to be proximal and fibroblast collagenase (CLG) was found to be distal to the translocation breakpoint on chromosome 11 (Table 3.2a). D1S8 was found to be distal to the translocation breakpoint on chromosome 1 (Table 3.2b). D1S8 (Royle et al., 1988) was mapped by Southern blotting. This result is shown in Figure 3.4. Susan Maguire determined that D1S103 (Weber et al., 1990) maps proximal to the translocation breakpoint on chromosome 1. This result is also described in Table 3.2b.

Genetic analysis of members of pedigree K26 was carried out prior to this work (Fletcher et al., 1993). RFLP and microsatellite analysis with chromosome 11 markers found recombination events amongst the ten translocation individuals with the distal marker D11S97 (Jeffreys et al., 1988) and the proximal marker D11S35 (Litt et al., 1990). Two intervening (by genetic analysis in CEPH pedigrees) markers, TYR and D11S388, did not detect any recombinants. On chromosome 1, no recombinants were found

Table 3.1 Modification of Initial Chromosome 11 PCR Assays

MARKER	Results Of Primary Amplification	Modifications Carried Out
HBB	Sub-optimal, but scoring possible	None
D11S87	Sub-optimal, but scoring possible	None
TYR LI	Faint bands, of correct size, in a few tracks	Increased cycle number
PGA	Spurious bands and strong band, correct size	Raised Ta Reduced cycle number
MDU1	Faint bands, of correct size, in a few tracks	Increased cycle number
GST PI	Many spurious bands	Raised Ta Touchdown PCR
FGF 4	Many spurious bands	Formamide added
D11S527	Worked	None
D11S533	Few faint bands	Lowered Ta Touchdown PCR Increased cycle number
TYR	Many spurious bands	Raised Ta
CLG	Spurious bands and strong band, correct size	Raised Ta Reduced cycle number
STMY1	Many spurious bands	Raised Ta Formamide added
D11S385	Many spurious bands	Raised Ta Formamide added
NCAM	Many spurious bands	Raised Ta
DRD2	Many spurious bands	Raised Ta Increased cycle number
D11S351	Many spurious bands	Raised Ta Formamide added
THY1	Sub-optimal, but scoring possible	None

Table 3.1: Ta= annealing temperature (of PCR reaction).

Table 3.2a Initial Chromosome 11 Marker Analysis of Somatic Cell Hybrids										
LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
HBB	-	+	ND	ND	-	-	+	-	+	-
D11S87	-	+	ND	ND	-	-	-	-	ND	ND
TYR LI	-	+	-	ND	+	-	+	-	-	+
PGA	-	+	-	ND	+	+	-	-	+	-
ROM 1	-	+	-	+	+	+	-	-	-	-
MDU 1	-	+	-	ND	+	+	-	-	-	-
GST PI	-	+	-	ND	+	-	-	-	+	+
ADROA2	-	+	ND	ND	+	-	-	-	+	-
FGF 4	-	+	-	+	+	-	-	-	-	-
D11S527	-	+	+	+	+	-	-	-	-	-
D11S533	-	+	+	+	+	+	-	-	-	+
OMP	-	+	+	+	+	+	-	-	-	+
TYR	-	+	+	ND	-	+	-	+	-	+
CLG	+	-	+	-	-	-	+	+	-	+
STMY1	+	-	+	-	-	-	+	+	-	+
D11S385	+	-	+	-	-	-	+	+	-	+
NCAM	+	-	+	-	-	+	-	+	-	+
DRD2	+	-	+	-	-	+	-	+	-	-
D11S351	+	-	+	ND	-	-	+	+	-	-
THY1	+	-	+	ND	-	-	+	+	ND	ND

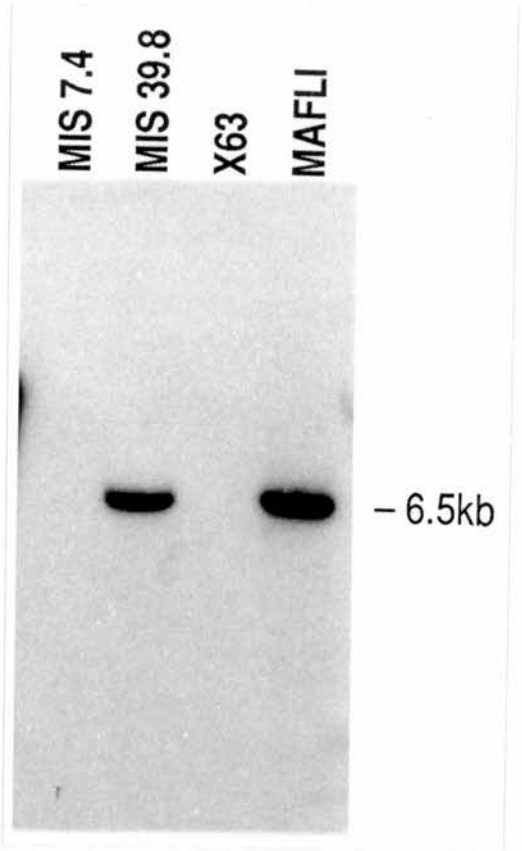
Table 3.2a: The cell lines are described in section 2.1.1. Where possible, the loci are arranged in their most likely order, from 11pter to 11qter, taking account of published information and assuming the least number of independent blocks of human DNA in the various hybrids. The double horizontal line marks the position of the centromere. + indicates the presence of the marker in the hybrid cell line, - indicates its absence. ND indicates not determined.

Table 3.2b Initial Chromosome 1 Marker Analysis Of Somatic Cell Hybrids

LOCUS	MIS 7.4	MIS 39.8
D1S103	+	-
D1S8	-	+

Table 3.2b: The cell lines are described in section 2.1.1. + indicates the presence of the marker in the hybrid cell line, - indicates its absence. The D1S103 assay was carried out by Susan Maguire.

Figure 3.4: Southern Analysis of Segregation of D1S8 in the Hybrid Panel



The cell lines are described in section 2.1.1. DNA (~5 µg/track) was digested with AluI and separated in a 0.8 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a radio-labelled probe corresponding to D1S8 (see sections 2.9.1, 2.11. 2.10.1 and 2.16.3.1). The blot was washed to 0.5 X SSC and, after exposure for 2 days, hybridisation was detected by autoradiography (see sections 2.11.3 and 2.12.1).

Human specific hybridisation is at ~6.5 kb. The results of this segregation analysis place this marker distal to the t(1;11)(q42.2;q21) translocation breakpoint, on chromosome 1 (see Table 3.2b).

with the proximal marker D1S103, but several were found with the distal marker D1S8.

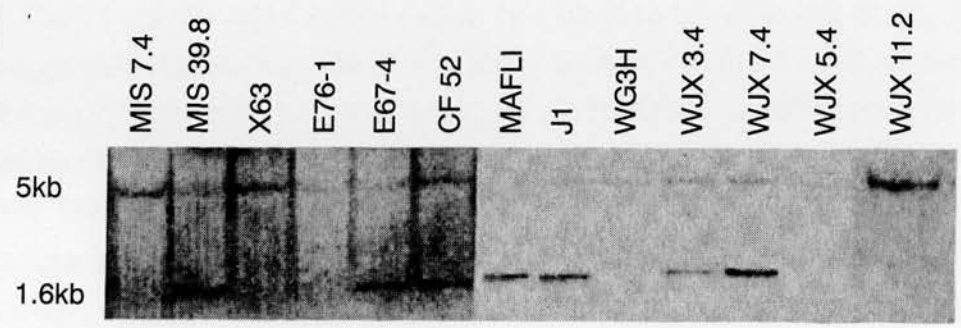
FISH analysis , also carried out prior to this work, identified TYR as being proximal and D11S388 as being distal to the translocation breakpoint on chromosome 11 (Fletcher et al., 1993).

The results obtained from the mapping panel, genetic analysis and FISH analysis are in agreement. However, D11S388 had been defined as a flanking marker by FISH analysis, but satisfactory Southern hybridisation mapping results could not be obtained with the corresponding cosmid, even after suppression hybridisation. The RFLP detected by this probe was also relatively uninformative. It was therefore decided to clone and sequence a band, which hybridised to a $(CA)_{15}$ oligonucleotide, from the cosmid, in an attempt to design a PCR assay which could be used to map D11S388 on the hybrid panel and which was more informative than the available RFLP assay (for use in linkage analysis). This procedure (described in section 2.16.2) resulted in the production of a PCR assay, although the CA repeat amplified was too short to be sufficiently polymorphic for use in linkage analysis. The analysis of the hybrid cell lines for D11S388 places it closer to the translocation breakpoint than the previous flanking marker, CLG.

The marker analysis of the hybrid cell lines suggests that the panel has sufficient resolution to separate quite closely linked markers such as the neural cell adhesion molecule (NCAM) and the dopamine receptor D_2 (DRD2) (which hybridise to the same 500 kb pulsed field gel fragment) (McConville et al., 1990). Also, glutathione S-transferase π (GSTPI) and the adenosine receptor 2 (ADROA2), which are within 290-360 kb (Szepetowski et al., 1993), are separated by the panel. However, markers which are known to be very close together, such as CLG, stromelysin 1 (STMY1) and D11S385 (which are known to be on the same 135 kb pulsed field fragment) (Formstone et al., 1993), are not separated by the mapping panel.

Figure 3.5 shows the results of hybridisation of a subclone of a human OMP cosmid to the hybrid mapping panel (this result is described in Table 3.2a). The localisation of OMP to 11q was somewhat surprising when its chromosomal location in the mouse is taken into consideration. Comparative

Figure 3.5: Southern Analysis of Segregation of OMP in the Hybrid Panel



The cell lines are described in section 2.1.1. DNA (~5 µg/track) was digested with BglII and separated in a 0.8 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a radio-labelled probe corresponding to the locus OMP (see sections 2.9.1, 2.11, 2.10.1 and 2.16.3.3). The blot was washed to 1 X SSC and, after exposure for 24 hours, hybridisation was detected by a Molecular Dynamics PhosphorImager (see sections 2.11.3 and 2.12.2).

Human specific hybridisation is at ~1.6 kb, while rodent specific hybridisation is at ~5 kb. The results of this segregation analysis place this marker in an interval which is proximal to the t(1;11)(q42.2;q21) translocation breakpoint, but distal to the translocation breakpoint of CF52, at 11q13 (Koeffler et al., 1981). This, in conjuction with the results obtained in the fragmentation hybrids, indicate that this marker lies between TYR and D11S533 (see Table 3.2a).

studies with genes on mouse chromosome 7 have established extensive homology to human chromosome 19q, 11p and 11q and to a lesser extent 15q (Darling and Abbott, 1992). The murine gene *Mod-2* is exceptional in that the human homologue, ME2, maps to 6p (Kompf, 1985). Genetic linkage studies indicate that *Omp* lies on chromosome 7, 5.36 cM distal to *Tyr* and 1.2 cM proximal to *Hbb*. The mouse map of this region with the location of the human homologue in parenthesis can be summarised as follows: *Fes* (15q) - *Tyr* (11q21) - *Mod-2* (6p) - *Calc I / Hbb* (11p15) (Rinchik et al., 1992). This suggested that human OMP was most likely to map to 11p15, or possibly 6p. However, the results of this analysis place OMP on chromosome 11q, flanked by TYR and D11S533 (Evans et al., 1993). Correspondingly, FISH analysis (carried out by Judith Fantes) with a human OMP cosmid to metaphase spreads, indicated that OMP maps to 11q13.5, centromeric to tyrosinase. Genetic linkage to this region has recently been established for Usher Syndrome Type I (Kimberling et al., 1992). Usher Syndrome Type I is an autosomal recessive disorder characterised by profound congenital deafness, vestibular areflexia and progressive retinitis pigmentosa. In the mouse, *Omp* maps within 200 kb of the mouse mutant *shaker I* (Brown et al., 1992). *Shaker I* is an autosomal recessive deafness mutant which shows associated circling and head-tossing behaviour and hyperactivity (Green, 1989). It is possible that Usher Syndrome I and the *shaker I* defect are caused by mutations in the same gene and that OMP causes both disorders. This hypothesis is now under test by others in the group and elsewhere.

Five ESTs which had been assigned previously to chromosome 11 (Polymeropoulos et al., 1992 and Durkin et al., 1992) were regionally mapped using the hybrid mapping panel. Each of the five ESTs define a new interval on the hybrid mapping panel. ESTs 109, 501, 532 have been assigned to 11p. Further analysis, using cell lines which define more intervals on 11p, will be required for more specific localisation of these markers. EST 111 and EST 16 have been localised to chromosome 11q. This is described in Table 3.3. EST 16 maps to an interval either directly proximal or directly distal to the previous translocation breakpoint flanking marker D11S388.

The mapping of another set of ESTs which have also been assigned previously to chromosome 11 (Polymeropoulos et al., 1993) was extended by Euan Slorach (Slorach et al., manuscript in preparation). Table 3.4 describes

Table 3.3 Initial Results of Mapping of ESTs to Somatic Cell Hybrids

LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
PGA	-	+	-	ND	+	+	-	-	+	-
ROM 1	-	+	-	+	+	+	-	-	-	-
MDU 1	-	+	-	ND	+	+	-	-	-	-
GST PI	-	+	-	ND	+	-	-	-	+	+
ADROA2	-	+	ND	ND	+	-	-	-	+	-
FGF 4	-	+	-	+	+	-	-	-	-	-
D11S527	-	+	+	+	+	-	-	-	-	-
D11S533	-	+	+	+	+	+	-	-	-	+
OMP	-	+	+	+	+	+	-	-	-	+
TYR	-	+	+	ND	-	+	-	+	-	+
EST 16	**+**	**-**	**+**	**+**	**-**	**-**	**-**	**+**	**-**	**+**
D11S388	+	-	+	+	-	-	-	+	-	-
CLG	+	-	+	-	-	-	+	+	-	+
STMY1	+	-	+	-	-	-	+	+	-	+
D11S385	+	-	+	-	-	-	+	+	-	+
NCAM	+	-	+	-	-	+	-	+	-	+
DRD2	+	-	+	-	-	+	-	+	-	-
D11S351	+	-	+	ND	-	-	+	+	-	-
THY1	+	-	+	ND	-	-	+	+	ND	ND
EST 111	**+**	**-**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

Table 3.3: The ESTs which were found to map to chromosome 11q are shown in bold. EST 16 co-segregates with D11S388 in all but the E67.4 result. This places EST16 either directly proximal or directly distal to this marker. EST 111 also creates a new interval in the hybrid mapping panel. The most likely position of this marker is distal to THY1. See legend to Table 3.2a for other details.

Table 3.4 Final Results of Mapping of ESTs to Somatic Cell Hybrids

LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
PGA	-	+	-	ND	+	+	-	-	+	-
EST 220	-	+	-	+	+	+	-	-	+	-
ROM 1	-	+	-	+	+	+	-	-	-	-
MDU 1	-	+	-	ND	+	+	-	-	-	-
EST 218	-	+	-	+	+	-	-	-	-	+
EST1878	-	+	-	+	+	-	-	-	-	+
EST2002	-	+	-	+	+	-	-	-	-	+
EST 518	-	+	-	+	+	-	-	-	-	+
GST PI	-	+	-	ND	+	-	-	-	+	+
ADROA2	-	+	ND	ND	+	-	-	-	+	-
FGF 4	-	+	-	+	+	-	-	-	-	-
D11S527	-	+	+	+	+	-	-	-	-	-
EST 294	-	+	+	+	+	+	-	-	-	-
D11S533	-	+	+	+	+	+	-	-	-	+
OMP	-	+	+	+	+	+	-	-	-	+
EST2145	-	+	+	+	-	+	-	-	-	+
TYR	-	+	+	ND	-	+	-	+	-	+

continued overleaf

Table 3.4: The cell lines are described in section 2.1.1. Where possible, the loci on 11q are arranged in their most likely order, taking account of published information and assuming the least number of independent blocks of human DNA in the various hybrids. The page break corresponds to the position of the t(1;11)(q42.2;q21) translocation breakpoint. + indicates the presence of the marker in the hybrid cell line, - indicates its absence. ND indicates not determined.

The ESTs which were found to map to chromosome 11q are shown in bold. The nine additonal ESTs were mapped by Euan Slorach. The EST data defines three new intervals on chromosome 11q.

Table 3.4 Final Results of Mapping of Ests to Somatic Cell Hybrids (continued)

LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
EST 16	+	-	+	+	-	-	-	+	-	+
D11S388	+	-	+	+	-	-	-	+	-	-
CLG	+	-	+	-	-	-	+	+	-	+
STMY1	+	-	+	-	-	-	+	+	-	+
D11S385	+	-	+	-	-	-	+	+	-	+
NCAM	+	-	+	-	-	+	-	+	-	+
DRD2	+	-	+	-	-	+	-	+	-	-
D11S351	+	-	+	ND	-	-	+	+	-	-
EST 116	+	-	+	-	-	-	+	+	-	-
EST 652	+	-	+	-	-	-	+	+	-	-
THY1	+	-	+	ND	-	-	+	+	ND	ND
EST 111	+	-	+	-	-	-	-	-	-	-

For legend, see previous page

the results of those mapped to chromosome 11q using the hybrid mapping panel. This analysis has resulted in the localisation of an additional nine brain ESTs on chromosome 11q and has resulted in the definition of three new intervals on the hybrid mapping panel.

A number of new markers became available during the course of this work and were mapped using the hybrid panel. For the sake of completeness this analysis is described in this section, but it should be emphasised that it was carried out subsequent to the mapping of the microdissection clones described in Chapter 4.

The results obtained are described in Table 3.5a. Firstly D11S873 and subsequently, D11S931 were found to map to an interval directly distal to the chromosome 11 translocation breakpoint. D11S901 was placed between TYR and OMP. Genetic analysis indicates that the distance between D11S901 and D11S931 is ~8 cM (Weissenbach et al., 1992) and that D11S901 is ~8.5 cM from D11S873 (Litt et al., 1993). This tentatively suggests that D11S931 is 0.5 cM proximal to D11S873, but this has yet to be confirmed by mapping both markers on the same set of individuals.

Several chromosome 1 markers have been mapped relative to the translocation breakpoint on chromosome 1 (described in Table 3.5b). The closest flanking marker below the breakpoint is D1S179, as it is the most proximal of the three new markers, D1S169; D1S179 and α-actinin 2 (ACTN2), which this analysis places distal to the translocation breakpoint. D1S103, which is 6 cM from D1S179 (Engelstein et al., 1993), and D1S251 are both proximal to the translocation breakpoint. The relative order of these two markers is not known.

Having established the power of the mapping panel, but exhausted the available markers, the next step was to generate many more markers in a region close to the translocation breakpoint.

Table 3.5a Results of Mapping of New Chromosome 11 Markers to Somatic Cell Hybrids

LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
PGA	-	+	-	ND	+	+	-	-	+	-
EST 220	-	+	-	+	+	+	-	-	+	-
ROM 1	-	+	-	+	+	+	-	-	-	-
MDU 1	-	+	-	ND	+	+	-	-	-	-
EST 218	-	+	-	+	+	-	-	-	-	+
EST1878	-	+	-	+	+	-	-	-	-	+
EST2002	-	+	-	+	+	-	-	-	-	+
EST 518	-	+	-	+	+	-	-	-	-	+
GST PI	-	+	-	ND	+	-	-	-	+	+
ADROA2	-	+	ND	ND	+	-	-	-	+	-
FGF 4	-	+	-	+	+	-	-	-	-	-
D11S527	-	+	+	+	+	-	-	-	-	-
EST 294	-	+	+	+	+	+	-	-	-	-
D11S533	-	+	+	+	+	+	-	-	-	+
OMP	-	+	+	+	+	+	-	-	-	+
EST2145	-	+	+	+	-	+	-	-	-	+
D11S901	**-**	**+**	**+**	**+**	**-**	**+**	**-**	**-**	**-**	**+**
TYR	-	+	+	ND	-	+	-	+	-	+

continued overleaf

Table 3.5: The markers which have been added to Table 3.4 are shown in bold. For other details see legend to Table 3.4.

Table 3.5a Results of Mapping of New Chromosome 11 Markers to Somatic Cell Hybrids (continued)

LOCUS	MIS 7.4	MIS 39.8	CF 52	PG48	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
D11S931	+	-	+	+	-	+	-	+	-	+
D11S873	+	-	+	+	-	+	-	+	-	+
EST 16	+	-	+	+	-	-	-	+	-	+
D11S388	+	-	+	+	-	-	-	+	-	-
CLG	+	-	+	-	-	-	+	+	-	+
STMY1	+	-	+	-	-	-	+	+	-	+
D11S385	+	-	+	-	-	-	+	+	-	+
NCAM	+	-	+	-	-	+	-	+	-	+
DRD2	+	-	+	-	-	+	-	+	-	-
D11S351	+	-	+	ND	-	-	+	+	-	-
EST 116	+	-	+	-	-	-	+	+	-	-
EST 652	+	-	+	-	-	-	+	+	-	-
THY1	+	-	+	ND	-	-	+	+	ND	ND
EST 111	+	-	+	-	-	-	-	-	-	-

For legend, see prevous page.

Table 3.5b Results of Mapping of New Chromosome 1 Markers to Somatic Cell Hybrids

LOCUS	MIS 7.4	MIS 39.8
D1S103	+	-
D1S251	+	-
D1S179	-	+
D1S163	-	+
ACTN2	-	+
D1S8	-	+

Table 3.5b: The cell lines are described in section 2.1.1. + indicates the presence of the marker in the hybrid cell line, - indicates its absence.

The markers which have been added to Table 3.2b are shown in bold. The relative order of D1S103 and D1S251 is not known.

CHAPTER 4

4. Mapping of Microdissection Clones to the Hybrid Mapping Panel

4.1 Introduction

New markers in the region of the breakpoint were produced by microdissection of a ~30 Mb region surrounding the derived chromosome 1 translocation breakpoint (Muir et al., manuscript in preparation). The dissected DNA was digested and cloned, and each clone was individually amplified by PCR (this was done by Susan Maguire and Tony Brookes).

4.2 Mapping of Microdissection Clones

PCR amplified inserts were run out on agarose gels, transferred to nylon membranes and hybridised with Cot I human DNA (this was done by Susan Maguire). The microdissection clones which were negative on Cot I hybridisation and which were 0.5-2 kb in size were selected first for somatic cell hybrid mapping. Initially microdissection clones were hybridised to Southern blots of restriction digested total human DNA, in order to determine whether they were suitable probes. Examples of this are shown in Figure 4.1. Panels a-e represent mapping of five microdissection clones to human DNA. In each case, a single band is observed, indicating that all of these clones are suitable probes. Of eleven probes examined in this way nine were found to be suitable. Due to this high success rate, this practise was abandoned and probes were hybridised directly to Southern blots of restriction digested hybrid DNA. A subset of the hybrid cell lines described in section 3.1 were used to map the microdissection clones. The hybrids were MIS 7.4, E67.4, CF52, WJX 5.4, WJX 11.2 and PG48. These hybrids were chosen as they constituted the minimum set which defined the maximum number of intervals distal to the translocation breakpoint on chromosome 11.

4.3 Scoring of Results

Hybridisation to MIS 7.4 was expected for every clone, as this cell line corresponds to the chromosome which was dissected to produce the microdissection library. Hybridisation to CF52 was expected for every clone which originated from chromosome 11, as this hybrid contains chromosome

Figure 4.1: Mapping of Microdissection Clones to Human DNA

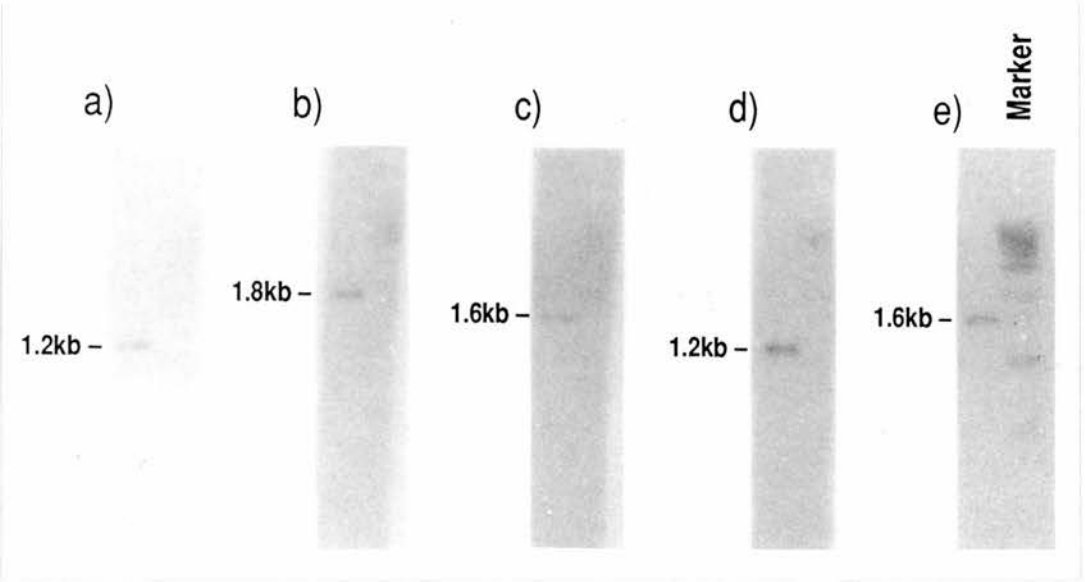


Figure 4.1 shows representative examples of the results of mapping individual microdissection clones to human DNA by Southern blotting (Southern, 1975). MAFLI DNA (~5 μg/track) was digested with EcoRI and separated in a 1 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with microdissection clone probes (see sections 2.9.1 and 2.11). Probes were produced by PCR amplification of inserts of individual microdissection clones, using primers corresponding to vector sequence (by Susan Maguire and Tony Brookes). This material was digested, to remove vector sequence and run on a 1 % LMP gel. The band corresponding to the insert was cut out of the gel, labelled by random priming and used directly as a hybridisation probe (see sections 2.8.3 and 2.10.1). Blots were washed to 0.1 X SSC and, after exposure for 7 days, hybridisation was detected by autoradiography (see sections 2.11.3 and 2.12.1).

The labels on the panels indicate the probe used in each case: a) = MD 459, b) = MD 437, c) = MD 431, d) = MD 429, e) = MD 273. The marker is the 1 kb ladder (Gibco BRL). The size of each of the signals obtained corresponds to that of the particular insert, as the same enzyme was used both to release the microdissection clone insert and to digest the target DNA. These results demonstrated that all of these microdissection clones are suitable for somatic cell hybrid mapping.

11q13-qter material. Clones which hybridised to MIS 7.4 and not CF52 (or any other hybrid cell line) were therefore provisionally assigned to chromosome 1. Panel a of Figure 4.2 shows an example of such a result; microdissection clone 459 (MD 459) hybridises only to MIS 7.4. Where probes mapped to chromosome 11, the pattern of segregation in the hybrids was used to localise them to a particular region of chromosome 11. Panel b shows the results obtained with MD 283. This clone hybridises to MIS 7.4, CF52, PG48, WJX 7.4 and E67.4, but not WJX 5.4. This localises MD 283 to the closest interval to the translocation breakpoint. Panel c shows an example of a microdissection clone which maps to the next interval down. MD 128 hybridises to MIS 7.4, CF52. PG48 and E67.4, but not WJX 5.4 and WJX 7.4. In the majority of cases, microdissection clones were placed in a previously defined interval, but occasionally the pattern of segregation introduced a new interval. In this situation markers were arranged in the order which assumed the least number of independent blocks of human DNA in the various hybrids. An example of a result which defined a new interval is shown in panel d, MD 21 hybridises to MIS 7.4, CF52, PG48, WJX 5.4 and E67.4, but not WJX 7.4.

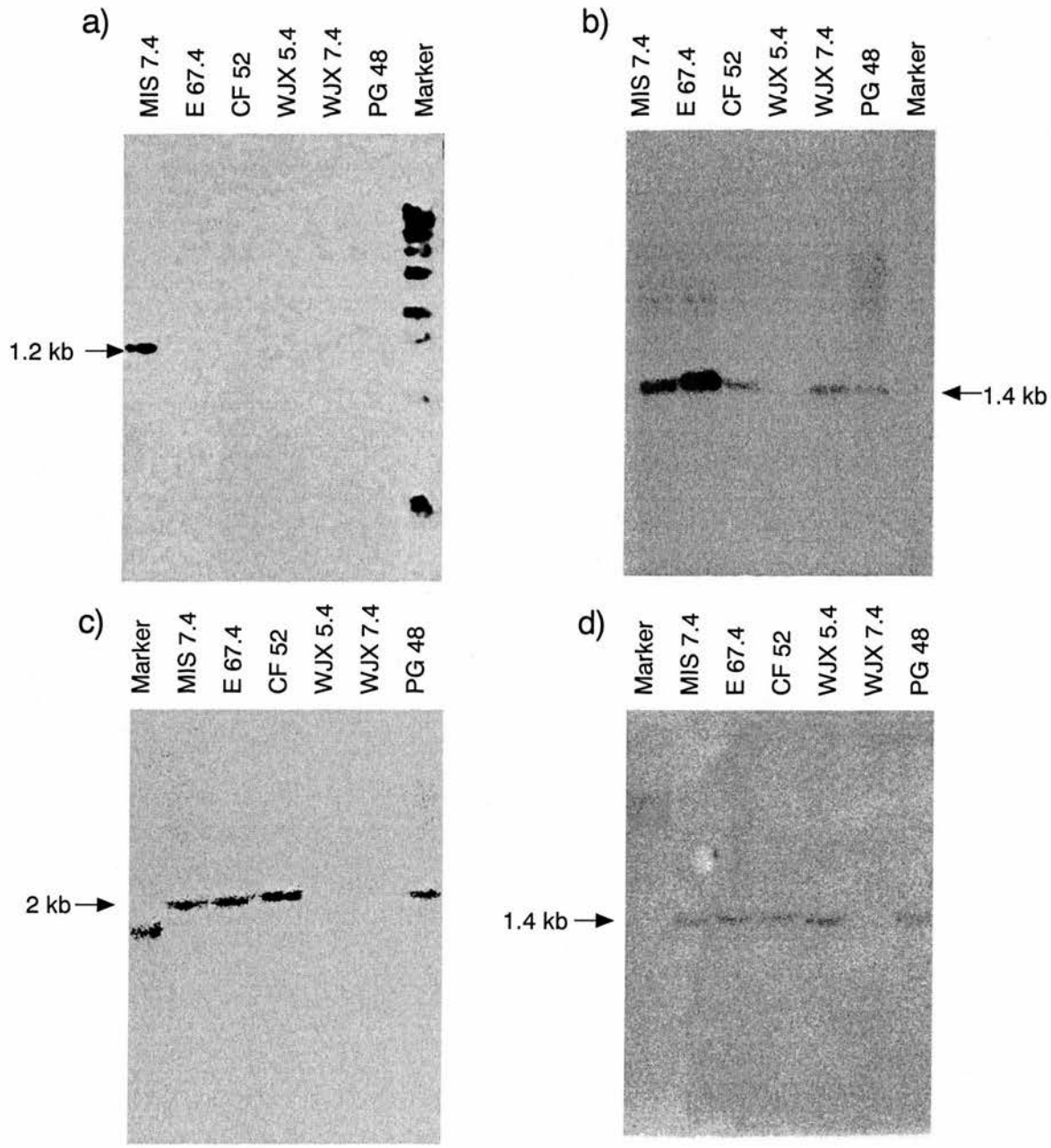
4.4 Results of Microdissection Clone Mapping

The results of mapping the first set of microdissection clones to the hybrid panel are shown, in part, in Table 4.1. Table 4.1 describes the results obtained with the 18 probes which originate from chromosome 11. Two new intervals on chromosome 11q were identified in the process. A further 10 probes were found to originate from chromosome 1. This work was extended by Sheila Christie, Euan Slorach and Susan Maguire. Table 4.2 describes the accumulated data for those markers which mapped to chromosome 11. In total 86 microdissection clones have been successfully mapped. Forty-nine of these clones originate from chromosome 11 and 37 originate from chromosome 1.

4.5 Discussion

The microdissection clones have greatly increased the number of markers in the regions distal to the translocation breakpoint on chromosome 11. Although the intervals on chromosome 11 are not necessarily of a standard

Figure 4.2: Mapping of Microdissection Clones to the Hybrid Panel



For legend, see overleaf

Figure 4.2: Mapping of Microdissection Clones to the Hybrid Panel (continued)

Legend:

The cell lines are described in section 2.1.1. DNA (~5 μg/track) was digested with EcoRI and separated in a 1 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a microdissection clone (see sections 2.9.1, 2.11 and the legend to Figure 4.1). Blots were washed to 0.1 X SSC and, after exposure for 24 hours, hybridisation was detected by a Molecular Dynamics PhosphorImager (see sections 2.11.3 and 2.12.2).

The marker is the 1 kb ladder (Gibco BRL). The size of each of the signals obtained corresponds to that of the particular insert, as the same enzyme was used both to release the microdissection clone insert and to digest the target DNA.

The labels on the panels indicate the probe used in each case: a) = MD 459, b) = MD 283, c) = MD 128, d) = MD 21. All clones should hybridise to MIS 7.4, as this hybrid represents the microdissected chromosome. If no other signal was obtained, clones were provisionally assigned to chromosome 1. The pattern of hybridisation observed in the other hybrids was used to assign each clone to an interval distal to the t(1;11)(q42.2;q21) translocation breakpoint (see Table 4.1). For example, in panel b) MD 283 is positive in MIS 7.4, CF 52, PG48, WJX 7.4 and E67.4 and negative in WJX 5.4. This localises MD 283 to the interval which is closest to the translocation breakpoint.

TABLE 4.1 Results of Initial Mapping of Microdissection Clones

LOCUS	MIS 7.4	CF52	PG48	WJX 7.4	WJX 5.4	E67.4
D11S931	+	+	+	+	-	+
D11S873	+	+	+	+	-	+
MD 104	+	+	+	+	-	+
MD 283	+	+	+	+	-	+
MD 220	+	+	+	+	-	+
MD 128	+	+	+	-	-	+
MD 421	+	+	+	-	-	+
MD281	+	+	+	-	-	+
EST 16	+	+	+	-	-	+
MD 174	+	+	+	-	-	-
D11S388	+	+	+	-	-	-
MD 558	+	+	+	-	+	+
MD 38	+	+	+	-	+	+
MD 286	+	+	+	-	+	+
MD 21	+	+	+	-	+	+
CLG	+	+	-	-	+	+
STMY1	+	+	-	-	+	+
D11S385	+	+	-	-	+	+
MD 211	+	+	-	-	+	+
MD 596	+	+	-	-	+	+
MD 75	+	+	-	-	+	+
MD 399	+	+	-	-	+	+
MD 214	+	+	-	-	-	+
NCAM	+	+	-	+	-	+
DRD2	+	+	-	+	-	-
MD 54	+	+	-	-	+	-
MD44	+	+	-	-	+	-
D11S351	+	+	ND	-	+	-
EST116	+	+	-	-	+	-
EST652	+	+	-	-	+	-
THY1	+	+	ND	-	+	ND
EST 111	+	+	-	-	-	-

Table 4.1: The cell lines are described in section 2.1.1. Where possible, the loci on 11q distal to the t(1;11)(q42.2;q21) translocation breakpoint are arranged in their most likely order, taking account of published information and assuming the least number of independent blocks of human DNA in the various hybrids. + indicates the presence of the marker in the hybrid cell line, - indicates its absence. ND indicates not determined.

(continued overleaf)

Legend to Table 4.1 (continued)

The results obtained with the microdissection clones are shown in bold. Eighteen microdissection clones were found to map to chromosome 11q, defining two new intervals. A further ten probes hybridised to MIS 7.4, but to no other chromosome 11 hybrid. These were, therefore, provisionally assigned to chromosome 1.

Table 4.2 Results of Analysis of Complete Set of Microdissection Clones

LOCUS	MIS 7.4	CF52	PG48	WJX 7.4	WJX 5.4	E67.4
D11S931	+	+	+	+	-	+
D11S873	+	+	+	+	-	+
MD 104	+	+	+	+	-	+
MD 122	+	+	+	+	-	+
MD 176	+	+	+	+	-	+
MD 412	+	+	+	+	-	+
MD 471	+	+	+	+	-	+
MD 437	+	+	+	+	-	+
MD 282	+	+	+	+	-	+
MD 283	+	+	+	+	-	+
MD 220	+	+	+	+	-	+
MD 543	+	+	+	+	-	+
MD 128	+	+	+	-	-	+
MD 118	+	+	+	-	-	+
MD 310	+	+	+	-	-	+
MD 74	+	+	+	-	-	+
MD 130	+	+	+	-	-	+
MD 40	+	+	+	-	-	+
MD159	+	+	+	-	-	+
MD129	+	+	+	-	-	+
MD 377	+	+	+	-	-	+
MD 421	+	+	+	-	-	+
MD281	+	+	+	-	-	+
EST 16	+	+	+	-	-	+

Continued Overleaf

Table 4.2: The cell lines are described in section 2.1.1. Where possible, the loci on 11q distal to the t(1;11)(q42.2;q21) translocation breakpoint are arranged in their most likely order, taking account of published information and assuming the least number of independent blocks of human DNA in the various hybrids. + indicates the presence of the marker in the hybrid cell line, - indicates its absence. ND indicates not determined.

The results obtained with the microdissection clones are shown in bold. In total, forty-nine microdissection clones were found to map to chromosome 11q. A further thirty-seven probes hybridised to MIS 7.4, but to no other chromosome 11 hybrid. These were, therefore, provisionally assigned to chromosome 1.

Table 4.2 Results of Analysis of Complete Set of Microdissection Clones (continued)

LOCUS	MIS 7.4	CF52	PG48	WJX 7.4	WJX 5.4	E67.4
MD 174	+	+	+	-	-	-
MD 139	+	+	+	-	-	-
D11S388	+	+	+	-	-	-
MD 558	+	+	+	-	+	+
MD 330	+	+	+	-	+	+
MD 173	+	+	+	-	+	+
MD 38	+	+	+	-	+	+
MD 286	+	+	+	-	+	+
MD 21	+	+	+	-	+	+
CLG	+	+	-	-	+	+
STMY1	+	+	-	-	+	+
D11S385	+	+	-	-	+	+
MD 63	+	+	-	-	+	+
MD 136	+	+	-	-	+	+
MD 27	+	+	-	-	+	+
MD 151	+	+	-	-	+	+
MD 509	+	+	-	-	+	+
MD 211	+	+	-	-	+	+
MD 116	+	+	-	-	+	+
MD 388	+	+	-	-	+	+
MD 596	+	+	-	-	+	+
MD 75	+	+	-	-	+	+
MD 399	+	+	-	-	+	+
MD 110	+	+	-	-	-	+
MD 453	+	+	-	-	-	+
MD 214	+	+	-	-	-	+
NCAM	+	+	-	+	-	+
DRD2	+	+	-	+	-	-
MD 96	+	+	-	-	+	-
MD 54	+	+	-	-	+	-
MD 162	+	+	-	-	+	-
MD44	+	+	-	-	+	-
MD 215	+	+	-	-	+	-
MD277	+	+	-	-	+	-
D11S351	+	+	ND	-	+	-
EST116	+	+	-	-	+	-
EST652	+	+	-	-	+	-
THY1	+	+	ND	-	+	ND
EST 111	+	+	-	-	-	-

For legend, see previous page.

size, the number of clones in each interval tends to decrease with increasing distance from the breakpoint. This is as expected, as it is likely that the accuracy of the cut varied over the 100 chromosomes dissected, but the region at the centre of the target area, i.e. the breakpoint, is most likely to be included in the region removed on each occasion. In accordance with the FISH painting analysis, approximately equal numbers of clones map to the two chromosomes.

There are now twelve markers, ten of which are microdissection clones, in the interval closest to the breakpoint. The genetic distance between D11S388 (which is distal to this interval) and TYR is estimated at 7.8 cM (NIH/CEPH Collaborative Mapping Group, 1992). The corresponding physical distance has been estimated at 4 Mbp, by FISH analysis of clones representing these loci (Fletcher et al., 1993). This FISH analysis indicates that the translocation breakpoint lies more or less mid-way between these markers. Thus, the distance between D11S388 and the breakpoint can be estimated at approximately 2 Mb. The proportion of this region which corresponds to the interval closest to the breakpoint is not known, but it is likely that there is now a sufficient density of markers in this region to generate a YAC contig across the breakpoint.

CHAPTER 5

5. Screening of the ICI YAC Library

5.1 Introduction

Microdissection clones from the interval immediately distal to the translocation breakpoint on chromosome 11 were used to screen the ICI YAC library. The microdissection clones chosen were those which failed to hybridise to Cot I DNA and which showed no evidence of repetitive content, as judged by the results of hybridisation to genomic digests. These were MD 122, MD 176, MD 220, MD 283, MD 471 and MD 543. A subclone (made by John Brown) of a repeat containing microdissection clone (104) was also used to screen the library.

5.2 Results of ICI YAC Library Screening

Examples of the results of screening the ICI YAC library are shown in Figures 5.1-5.3. Figure 5.1 shows representative examples of the results of hybridisation of the subclone of MD 104 to the 23 filters of gridded YACs. The colonies indicated with arrows are those which were selected as positives. However, hybridisation of MD 104 to DNA from these YACs indicated that these signals were all false positives. The same was true for all of the YACs identified using MD 543. Figure 5.2 shows representative examples of the 23 filters after hybridisation to MD 176, the colonies indicated with arrows are those which were selected as positives. The large arrows indicate those which were subsequently found to be true positives and the small arrows indicate false positives. Approximately half of the YACs selected with MD 122, MD 176 and MD 471 and 75 % of those selected with MD 283 were found to be true positives. Figure 5.3 shows examples of the 23 filters after hybridisation with the MD 220, in this case all the YACs selected as positives were subsequently found to be true positives.

5.3 Discussion

Of the markers which were successfully used in screening (i.e. those which resulted in the isolation of at least one YAC) an average of four YACs were isolated per marker. This is comparable to the estimate of the complexity of the ICI YAC library (3.5 - 4 genome equivalents). Hybridisation with MD 543

and with the subclone of MD 104 did not identify any positive YACs. It is possible that one or both of these clones contained a repetitive sequence. In retrospect, the Southern analysis with MD 543 shows a faint high molecular weight smear indicative of repetitive content. It is also possible that the genomic regions corresponding to these probes may not be represented in the ICI YAC library.

Figure 5.1: Screening of the ICI YAC Library with MD 104

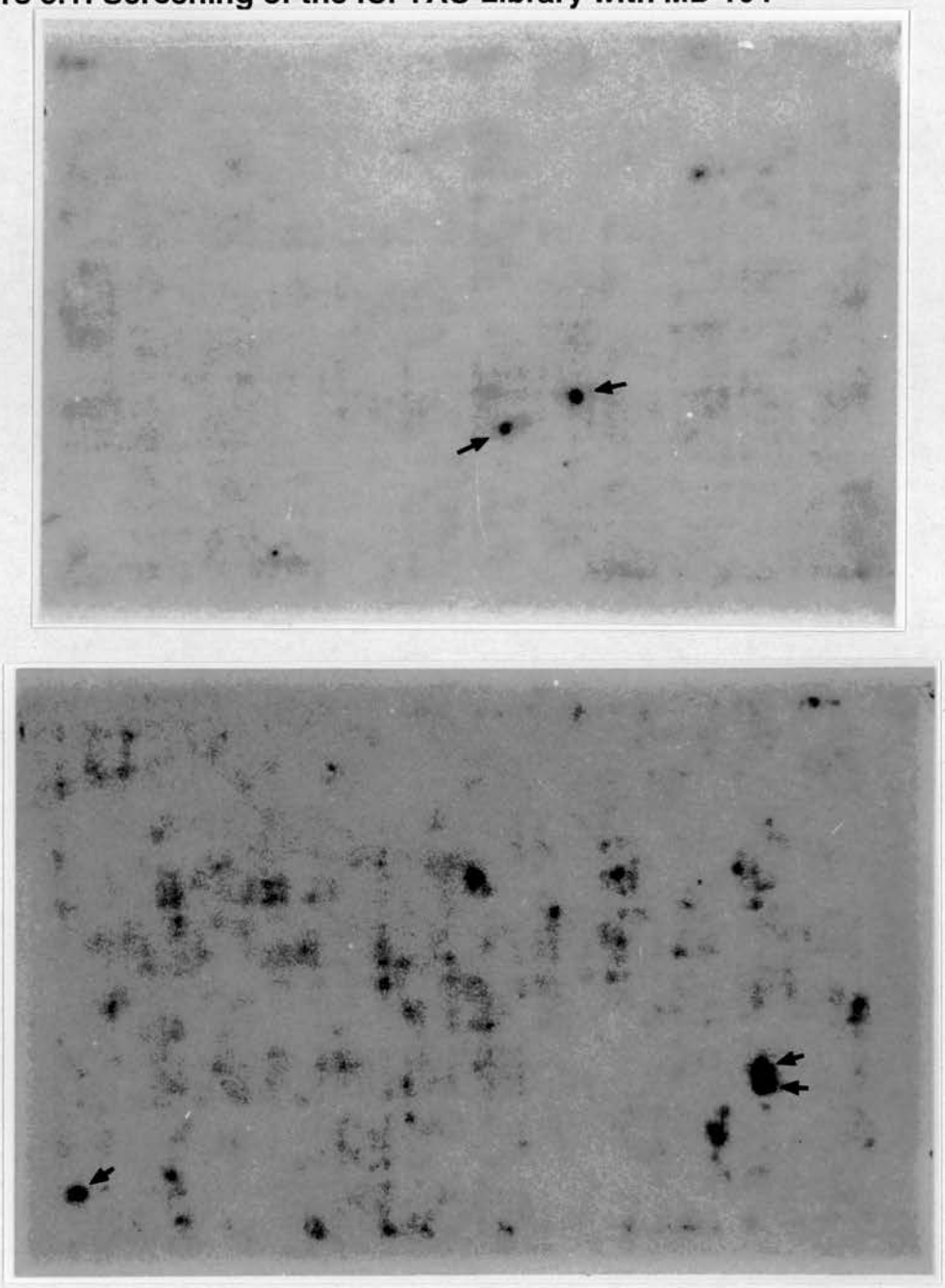


Figure 5.1 shows representative examples of the results of screening of the ICI YAC library (see section 2.3.2) with MD 104. The 23 filters were hybridised, at 68°C, with radio-labelled probe (see sections 2.11, 2.10.1 and the legend to Figure 4.1). Blots were washed to 1 X SSC and, after exposure for 1 to 7 days, hybridisation was detected by autoradiography (see sections 2.11.3 and 2.12.1). The arrows indicate YAC recombinants which were identified as potential positives. However, on further analysis (see section 5.2) these were all determined to be false positives.

Figure 5.2: Screening of the ICI YAC Library with MD 176

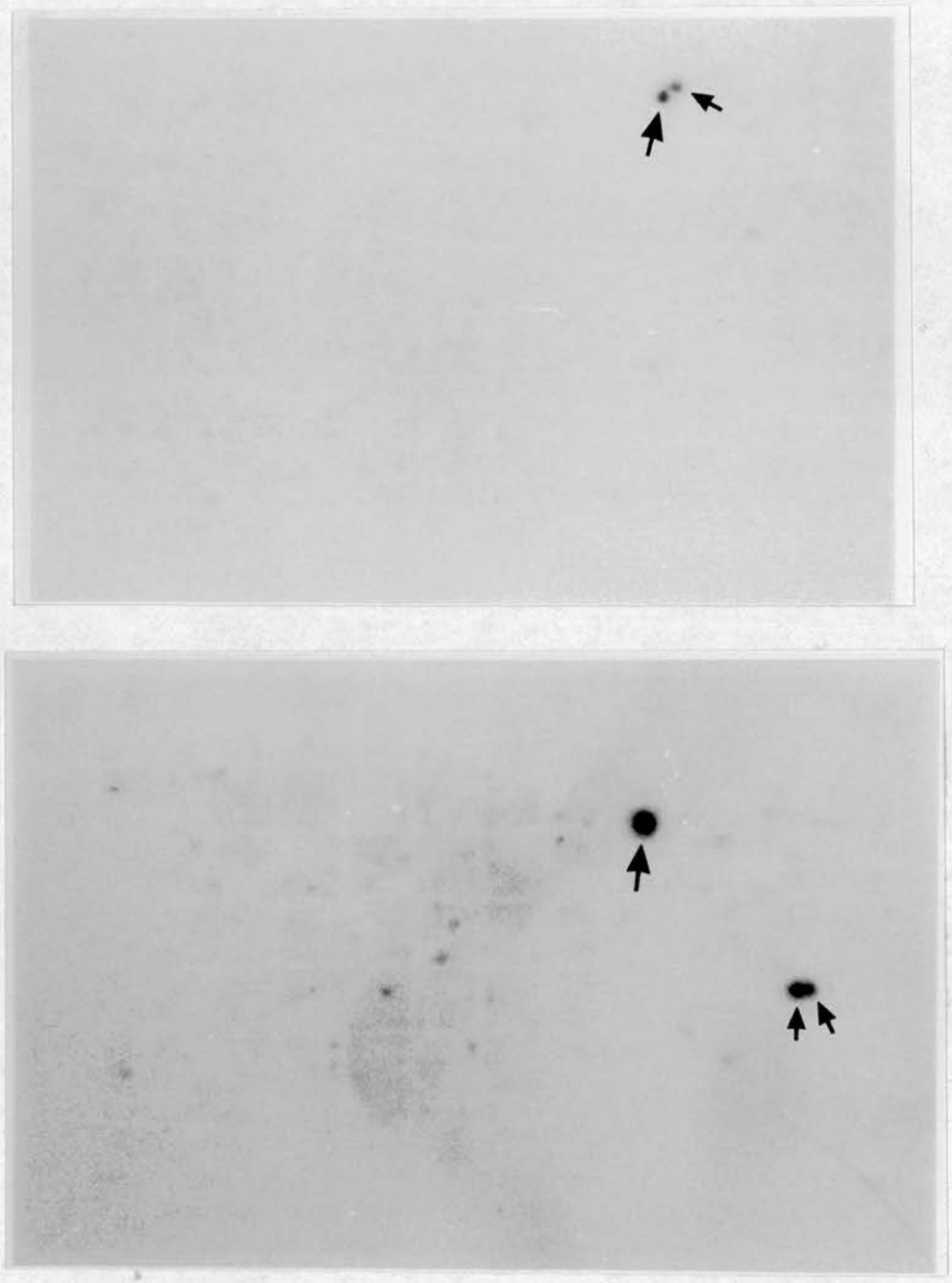


Figure 5.2 shows representative examples of the results of screening of the ICI YAC library (see section 2.3.2) with MD 176. The screening procedure is described in the legend to Figure 5.1. The arrows indicate YAC recombinants which were identified as potential positives. On further analysis (see section 5.2) it was determined that those which are marked with large arrows were true positives while those marked with smaller arrows were false positives.

Figure 5.3: Screening of the ICI YAC Library with MD 220

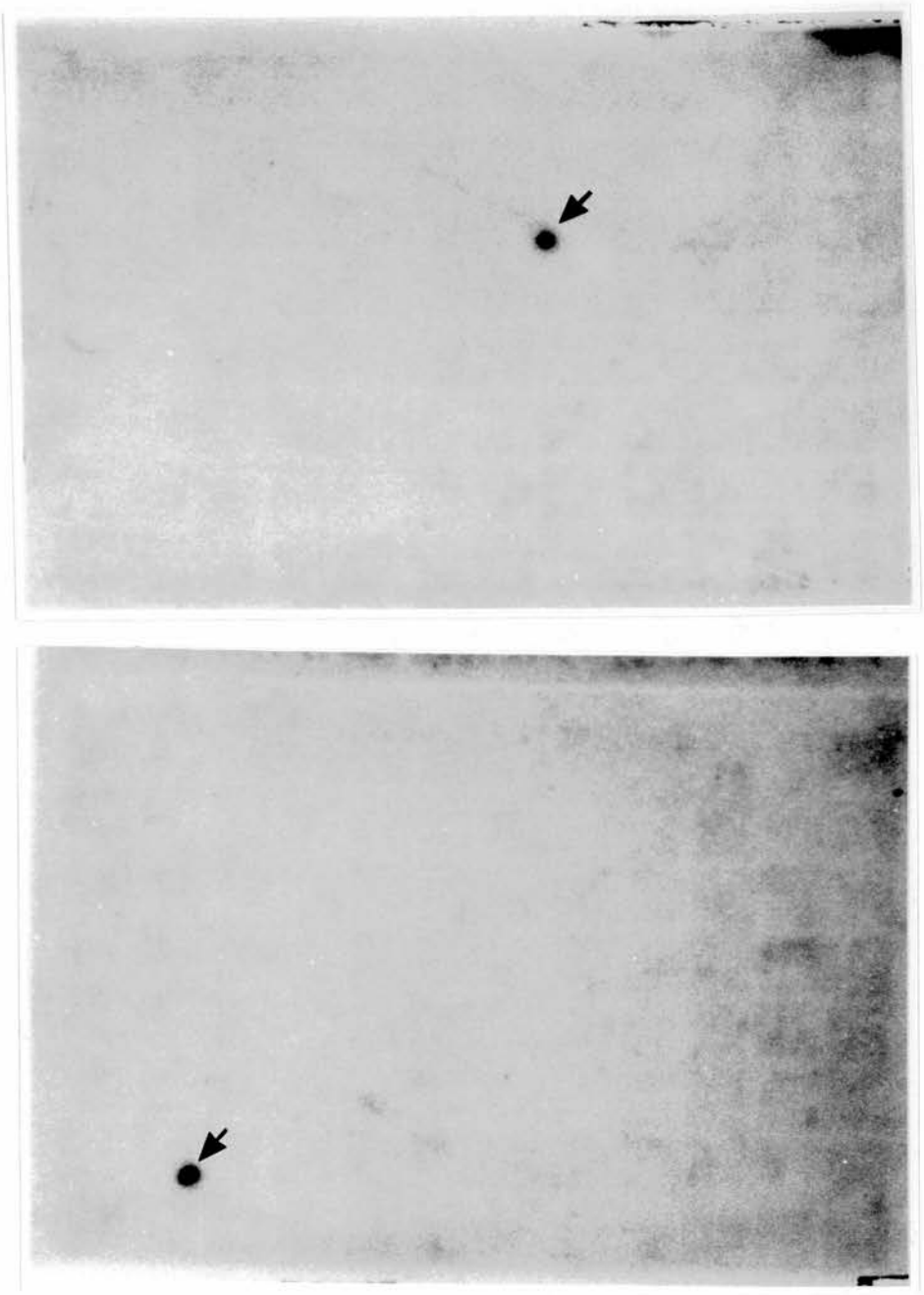


Figure 5.3 shows representative examples of the results of screening of the ICI YAC library (see section 2.3.2) with MD 220. The screening procedure is described in the legend to Figure 5.1. The arrows indicate YAC recombinants which were identified as potential positives. On further analysis (see section 5.2) it was determined that all of the recombinants identified with this probe were true positives.

CHAPTER 6

6. Assembly of YACs into Contigs and Pulsed Field Gel Electrophoretic Analysis

6.1 Introduction

It was hoped that assembly of the isolated YACs into a contig would result in identification of the YACs which correspond to the two ends of the cloned region. The YACs identified in this way would then be localised with respect to the translocation breakpoint. The first step in contig assembly was to screen all the YACs with all the markers in the region, allowing detection of overlaps between YACs. Fingerprinting of the YACs was also carried out to confirm the overlaps between YACs. Briefly, restriction digested total yeast DNA was hybridised with a Cot I DNA probe, which identifies human repetitive sequences, producing a banding pattern for each YAC. Overlapping YACs have related banding patterns. Pulsed field gel electrophoretic (PFGE) analysis was used to provide estimates of the size of the YACs. Resolved YACs were transferred to a nylon membrane and detected by hybridisation with human Cot I DNA. PFGE analysis was also used to compare the results of hybridisation of MD 122 to normal and translocation chromosomes. DNA, contained in agarose plugs, from controls and translocation carriers was restriction digested and resolved by PFGE (control agarose plugs were a gift from John Maule). The fragments were transferred to a nylon membrane and hybridised with MD 122. This was carried out in order to determine whether this probe detected bands which varied in size between controls and translocation carriers. Detection of a variant band (or bands) would indicate that the probe hybridised to a genomic region where restriction sites varied between translocation and non-translocation individuals. It is likely that a translocation would cause such an alteration of the location of restriction sites in the region. The sizes of the altered fragments would indicate the maximum distance of a probe to the translocation breakpoint.

6.2 Results of Screening the YACs with the Markers

Figures 6.1 and 6.2 are examples of screening the isolated YACs with markers from the region closest to the translocation breakpoint. Figure 6.1 shows all the YACs from the region hybridised with MD 283, three positives

Figure 6.1: Confirmation of MD 283 Positive ICI YAC Recombinants

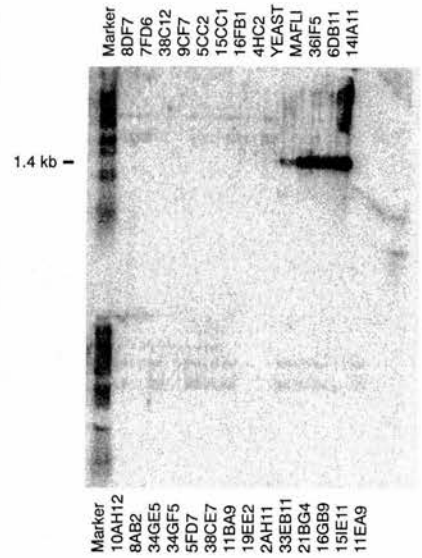


Figure 6.1 shows Southern blot analysis, with MD 283, of ICI YAC recombinants which were identified by colony hybridisation (with MD 283 and with other markers mapped to the region closest to the translocation breakpoint).

Yeast DNA (~200 ng/track) was digested with EcoRI and separated in a 1 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a radio-labelled probe (see sections 2.9.1, 2.11, 2.10.1 and the legend to Figure 4.1). Blots were washed to 1 X SSC and, after exposure for 6-8 hours, hybridisation was detected by a Molecular Dynamics PhosphorImager (see sections 2.11.3 and 2.12.2).

The marker is the 1 kb ladder (Gibco BRL). The size of the signal obtained corresponds to that of the particular insert, as the same enzyme was used to both release the insert of the microdissection clone and to digest the target DNA.

Three YAC recombinants were confirmed as positive for MD 283 and one was found to be negative.

Figure 6.2: Screening of the Isolated ICI YACs with MD 437

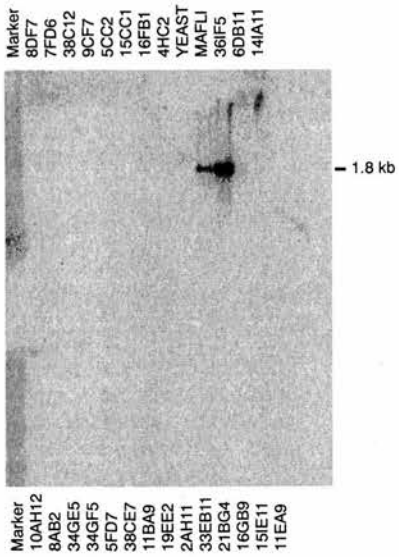


Figure 6.2 shows the results of screening the blot described in Figure 6.1, with MD 437.

Probe MD 283 was removed (see section 2.11.4) from the membrane, which was then re-hybridised, as described in the legend to Figure 6.1, with MD 437.

This analysis indicates that one of the three YACs which is positive for MD 283 is also positive for MD 437.

are identified. Figure 6.2 shows the same filter hybridised with MD 437, one of the MD 283 positive YACs is also positive for MD 437.

Table 6.1 describes the results of screening all the YACs with all the markers in the interval closest to the translocation breakpoint. It can be seen that in a number of cases YACs are positive for more than one marker. YAC contigs (described in Figure 6.7) can be assembled from this data. For example, YACs 36IF5, 6DB11 and 14IA11 clearly overlap with 36IF5 extending beyond the others at least one end (see Figure 6.7). In some cases, these results also allow determination of the relative order of the markers. For example, the three YACs mentioned above are all positive for MD 220 and MD 283. However, only YACs 36IF5 and 6DB11 are positive for MD 282 and only YAC 36IF5 is positive for MD 437. This suggests that MD 220 and MD 283 are closely linked with MD 437 at one end and MD 282 either between MD 437 and the other two, or at the other end of this contig. Thus this technique allows rapid and simple determination of relative order of the markers in the region as well as the assembly of YAC contigs.

6.3 Pulsed Field Gel Electrophoretic Analysis of YACs

PFGE analysis was carried out on a subset of the YAC clones in order to obtain estimates of the sizes of the YACs. The results of this analysis are shown in Figure 6.3. The analysis indicated that approximate sizes of the YACs are as follows: 36IF5- ~600 kb; 8AB2- ~450 kb; 16GB9- ~330 kb; 11BA9- ~250 kb; 11EA9- ~250 kb; 33EB11- ~350 kb; 19EE2- ~250 kb; 21BG4- ~250 kb and 2AH11 ~200 kb (data not shown for this YAC).

6.4 Fingerprinting of YACs

Low resolution fingerprinting of the YACs (such as that shown in Figure 6.4) confirmed the overlaps between YACs. YACs which overlap should have some bands in common, but independent recombinants should also have unique bands. This can be seen in Figure 6.4, which shows low resolution fingerprint analysis of the MD 122 and MD 176 positive YACs. In Figure 6.4, the open circle indicates an example of a band which is present in all of the YACs which are positive for MD 122, while the closed circle indicates an example of a band which is present in all of the YACs which are positive for

Table 6.1 Marker Analysis of the ICI YAC Recombinants

	MD 122	MD 176	MD 412	D11S 873	MD 471	MD 543	D11S 931	MD 104	MD 437	MD 220	MD 283	MD 282
36IF5	-	-	-	-	-	-	-	-	+	+	+	+
6DB11	-	-	-	-	-	-	-	-	-	+	+	+
14IA11	-	-	-	-	-	-	-	-	-	+	+	-
8AB2	-	-	+	+	+	-	-	-	-	-	-	-
34GE5	-	-	-	+	+	-	-	-	-	-	-	-
34GF5	-	-	-	+	+	-	-	-	-	-	-	-
5FD7	-	-	-	+	+	-	-	-	-	-	-	-
38CE7	-	-	-	+	+	-	-	-	-	-	-	-
15IE11	-	+	-	-	-	-	-	-	-	-	-	-
11EA9	-	+	-	-	-	-	-	-	-	-	-	-
16GB9	+	+	-	-	-	-	-	-	-	-	-	-
11BA9	+	-	-	-	-	-	-	-	-	-	-	-
2AH11	+	-	-	-	-	-	-	-	-	-	-	-
33EB11	+	-	-	-	-	-	-	-	-	-	-	-
19EE2	+	-	-	-	-	-	-	-	-	-	-	-
21BG4	+	-	-	-	-	-	-	-	-	-	-	-

Figure 6.3: PFGE Analysis of YAC Recombinants

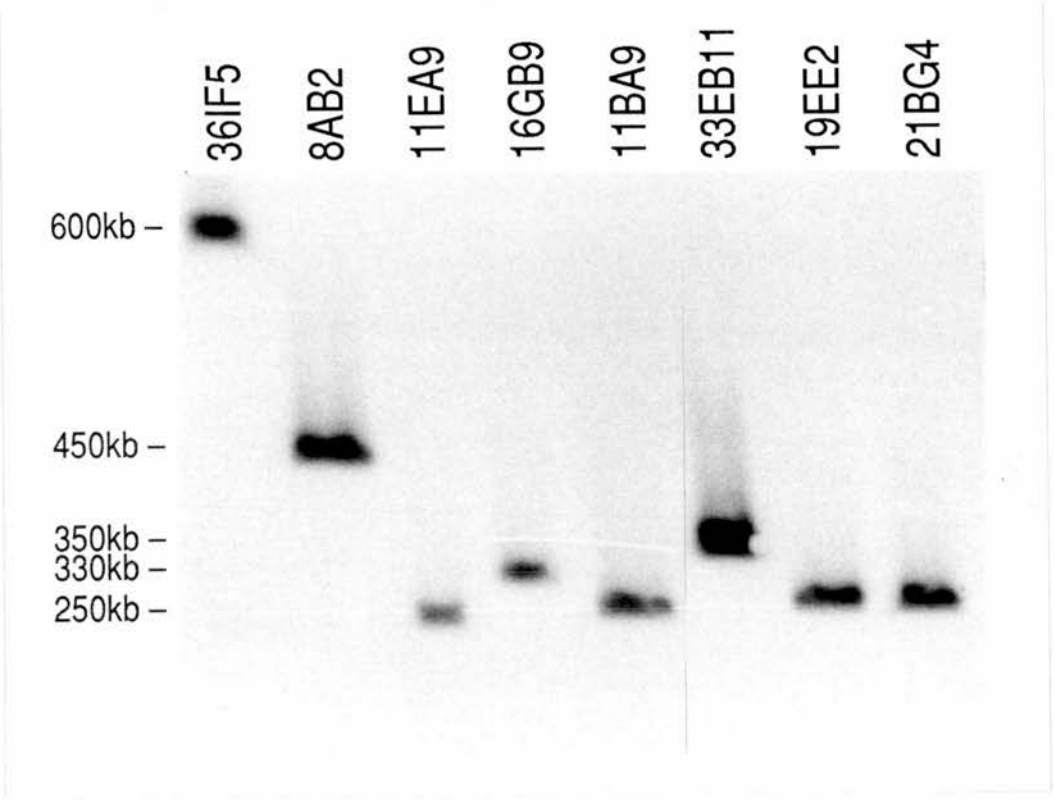
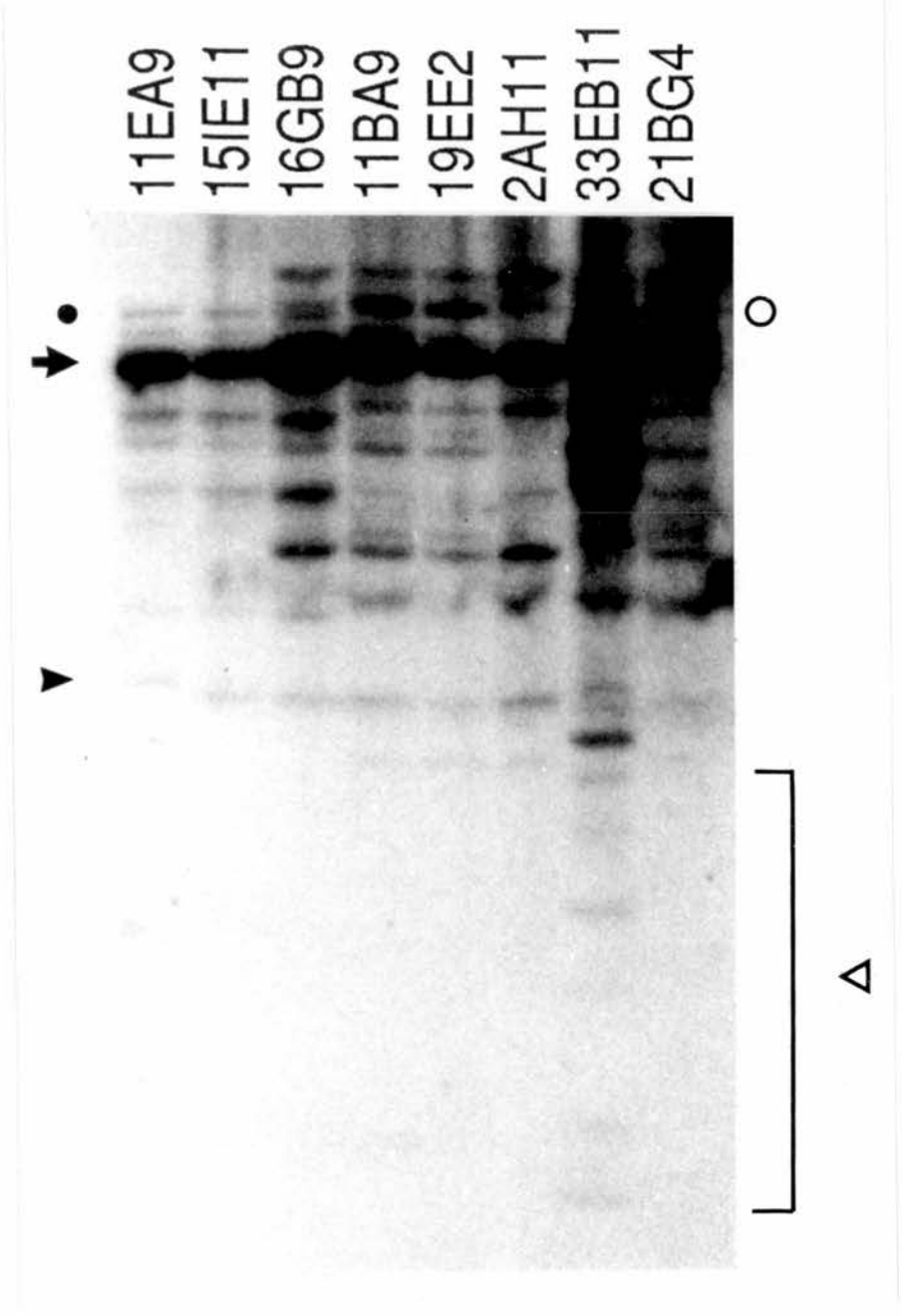


Figure 6.3 shows the results of PFGE analysis of a subset of the confirmed positive YAC recombinants. YAC DNA was resolved on a pulsed field gel and transferred to a nylon membrane (see sections 2.8.5 and 2.9.1). The membrane was then hybridised, at 68°C, (see section 2.11) with radio-labelled (see section 2.10.1) Cot I human DNA probe (which hybridises to human repetitive sequences and thus identifies the recombinant). The filter was washed to 1 X SSC and, after exposure for 3 hours, hybridisation was detected by autoradiography (see sections 2.11.3 and 2.12.1)

The approximate sizes of the YAC recombinants were determined by comparison to the position of the known size of the yeast marker chromosomes (see section 2.3.3). YAC 36IF5 is approximately 600 kb; 8AB2 is approximately 450 kb; 33EB11 is approximately 350 kb; 16GB9 is approximately 330 kb and 11BA9, 11EA9, 19EE2 and 21BG4 are approximately 250 kb.

Figure 6.4: Low Resolution Fingerprint Analysis of YAC Recombinants



For legend, see overleaf

Figure 6.4: Low Resolution Fingerprint Analysis of YAC Recombinants (continued)

Legend:

Figure 6.4 shows representative examples of the results of low resolution fingerprint analysis of the MD 122 and MD 176 positive YAC recombinants. Yeast DNA (~200 ng/track) was digested with PstI and separated in a 0.8 % agarose gel (see sections 2.7.1 and 2.8.2). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a radio-labelled Cot I human DNA probe (see sections 2.9.1, 2.11 and 2.10.1). Blots were washed to 1 X SSC and, after overnight exposure, hybridisation was detected by autoradiography (see sections 2.11 and 2.12.1).

The open circle indicates an example of a band which is present in all of the YACs which are positive for MD 122, while the closed circle indicates an example of a band which is present in all of the YACs which are positive for MD 176. The arrow indicates an example of a band which is present in all of the YACs in the contig and the arrowheads indicate examples of bands which are unique to particular YACs. Note that YAC 33EB11 shares bands with the other YACs which are also positive for MD 122, but it also has many more unique bands than any other YAC (see text for discussion).

MD 176. The arrow indicates an example of a band which is present in all of the YACs in the contig and the arrowheads indicate examples of bands which are unique to particular YACs. This technique gives confirmation of the overlaps between YACs in this contig. Independent recombinants should also have unique bands. This can be seen in many cases in Figure 6.4. This technique also indicates YACs which are very different in content. For example YAC 33EB11 shares bands with the other YACs which are also positive for MD 122, but it also has many more unique bands than any other YAC. This YAC is 350 kb in size whereas one other MD 122 positive YAC is 200 kb in size and the other four are 250 kb in size. The unique bands in this YAC correspond to a region of the genome which is not represented in the other YACs which are positive for MD 122. This means that this YAC either extends beyond the ends of the others or, alternatively, this YAC may be chimaeric and the unique bands, or least a proportion of them, may originate from a separate chromosomal region.

Higher resolution fingerprinting such as that shown in Figure 6.5 can be used to give a better indication of the overlap between YAC clones. For example, two YACs which overlap by fingerprint analysis (although not by microdissection analysis) are 11EA9 and 11BA9. These YACs have approximately the same number of bands approximately half of which are shared. The two YACs are approximately the same size, indicating that for both of these YACs approximately one half overlaps with the other while the other half is different.

6.5 Pulsed Field Gel Electrophoretic Analysis of Translocation and Normal DNA

PFGE analysis, comparing a normal and a translocation carrying individual was carried out. The results obtained after hybridisation with MD 122 are shown in Figure 6.6. While this probe detects bands of the same size in the translocation and non-translocation individuals in some restriction digests, in others (MluI, SstII and BssHII), it detects bands which differ in size. The smallest band which varies between the individuals, obtained in the MluI digest, is 300 kb. This may indicate that MD 122 hybridises to a 300 kb band in normal individuals, which is interrupted by the translocation breakpoint in carriers. However, the 300 kb band is not present in the translocation cell

Figure 6.5: High Resolution Fingerprint Analysis of YAC Recombinants

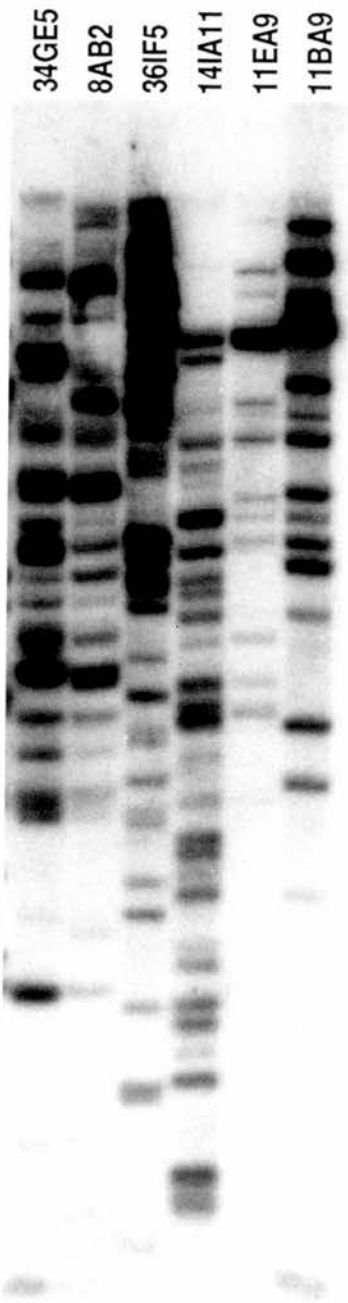


Figure 6.5 shows the results of high resolution fingerprint analysis of a subset of the YAC recombinants. The method used is the same as described in the legend to Figure 6.4, with the exception that approximately 500 ng yeast DNA was digested and this was run in a longer agarose gel (20 cm as opposed to 12 cm).

Higher resolution fingerprinting can be used to give a better indication of the overlap between YAC recombinants, than that provided by low resolution fingerprint analysis. Note that 11EA9 and 11BA9 are not linked by microdissection clone analysis, but that they share approximately half of their bands. The two YACs are of indistinguishable size, consistent with ~50 % overlap.

Figure 6.6: PFGE Analysis of Translocation and Normal DNA

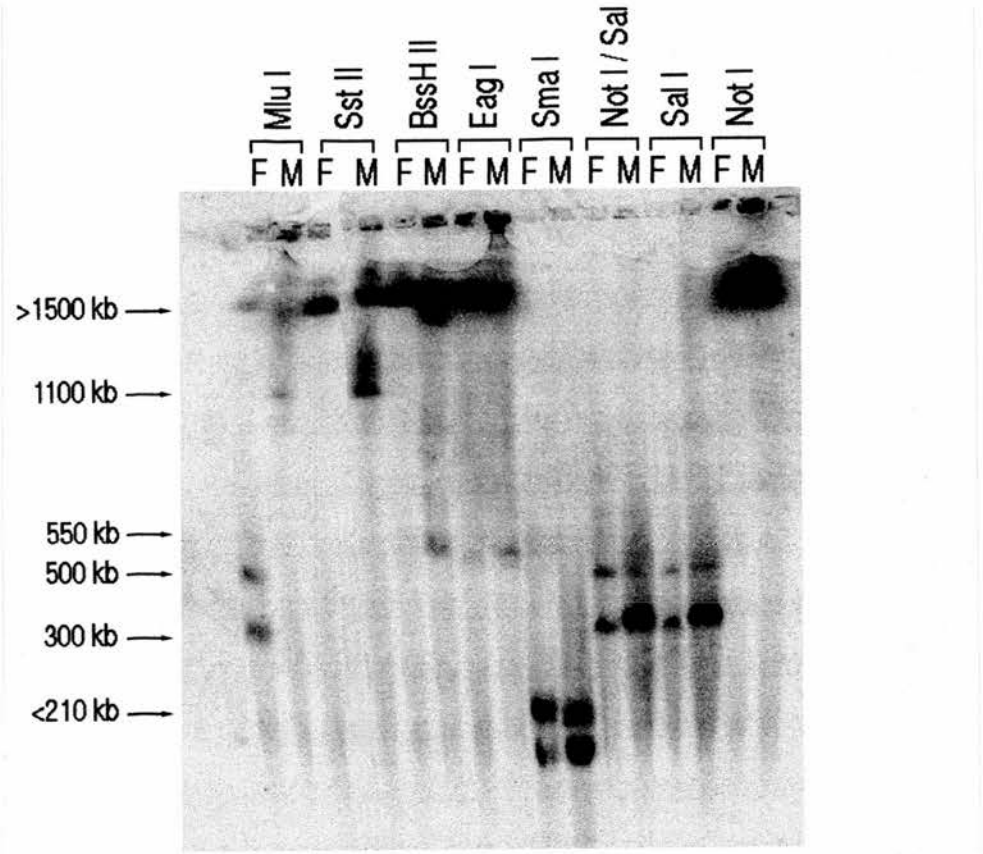


Figure 6.6 shows the results of PFGE analysis of restriction digested DNA from a translocation carrier (M=MAFLI) and a control individual (F=FATO), hybridised with MD 122. DNA contained in agarose plugs was digested with a series of restriction enzymes and resolved by PFGE (see sections 2.2.2, 2.7.2 and 2.8.5). The fragments were transferred to a nylon membrane and hybridised, at 68°C, with a radio-labelled probe (see sections 2.9.1, 2.11, 2.10.1 and the legend to Figure 4.1). The blot was washed to 1 X SSC and, after exposure for 24 hours, hybridisation was detected by a Molecular Dynamics PhosphorImager (see sections 2.11.3 and 2.12.2).

Note that the probe detects bands of the same size in the translocation and non-translocation individuals in some restriction digests, while in others (MluI, SstII and BssHII) it detects bands which differ in size (see text for discussion).

line, despite the fact that this cell line has a normal chromosome 11, as well as the translocated chromosome. This suggests that the differences observed may be caused either by differences in methylation status of the two cell lines, or by polymorphisms which result in a difference in restriction sites between the two cell lines. The results obtained in the BssHII and the SstII digest are consistent with the karyotype of the cell lines. In the BssHII digest, a large, unresolved, band is present in both cell lines, presumably corresponding to the normal chromosome 11. A band of 550 kb is present only in the translocation cell line. This suggests that MD 122 detects a band of >1500 kb in normal chromosomes which is interrupted by the translocation breakpoint in translocation chromosomes. This would indicate that MD 122 lies within 550 kb of the translocation breakpoint.

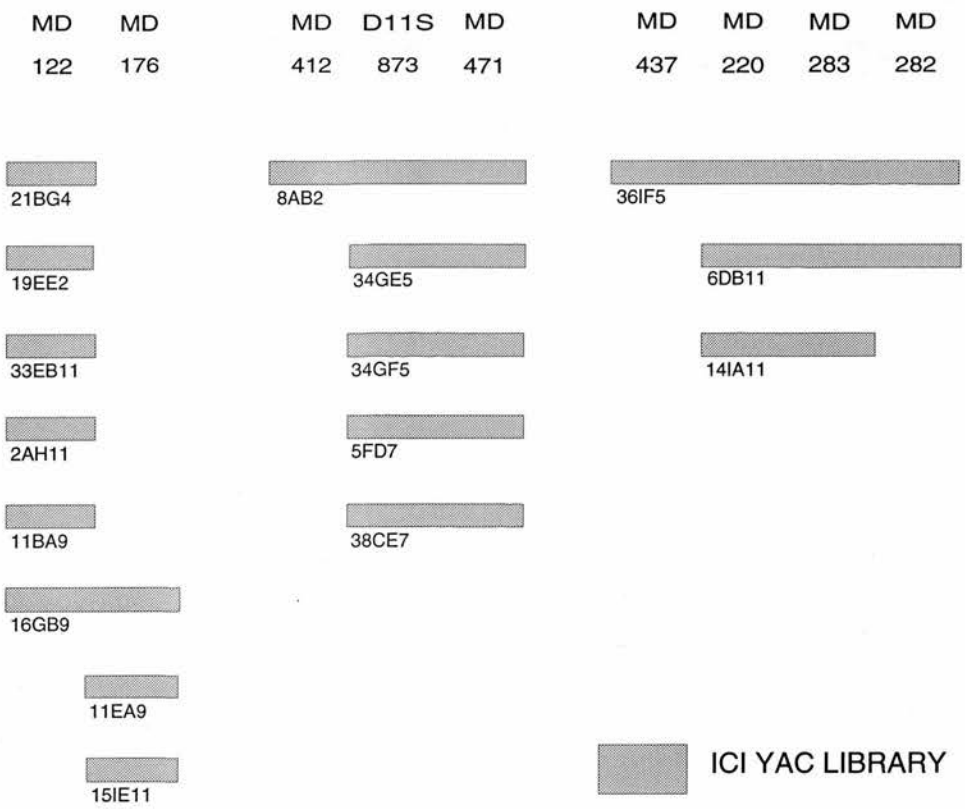
6.6 Discussion

Successful screening of the ICI YAC library with five of the twelve clones resulted in three contigs which link all but three of the markers in the region (see Figure 6.7). This suggests that there is a high density of markers in a small region and that the YACs now cover a large proportion of the region.

The sizes of the YACs can be used to estimate the density of the microdissection clones in the region. Three YACs (36IF5, 8AB2 and 16GB9) total 1380 kb and contain nine independent markers between them. This gives an estimate of one marker every approximately 150 kb and estimates the size of the region, which contains twelve markers, at 1.8 Mb. This is possibly an overestimate as it does not take into account overlap between YACs, other than that which can be detected by marker hybridisation. The figure of 1.8 Mb is compatible with our previous estimate of ~2 Mb for the interval between D11S388 and the translocation breakpoint.

Fingerprint analysis of these contigs confirmed the overlaps between the YACs. MD 122 detected an altered size restriction fragment in translocation versus normal chromosomes. Fingerprint analysis of the MD 122 positive YACs, along with the PFGE analysis which allowed determination of the size of the YACs, indicated a YAC which may extend furthest towards the translocation breakpoint. It is likely that higher resolution fingerprint analysis

Figure 6.7: Contig of the ICI YAC Recombinants



The Figure (which is not drawn to scale) shows a set of possible contigs which can be assembled from the data described in Table 6.1. Boxed areas indicate the extent of the cross hybridisation of YACs to microdissection clones. The relative order of two groups of markers: 1) MD 471, D11S873 and MD 412 and 2) MD 220, MD 283, MD 282 and MD 437 remains to be determined.

of all of the MD 122 positive YAC recombinants would provide further information on this important set of YACs.

Another YAC library (the ICRF YAC library obtained courtesy of Hans Lehrach) was screened with MD 122, MD 176, MD 220 and MD 471 (this was carried out by John Brown). The results of this screening are described in Table 6.2 and incorporated into the contig of the region (see Figure 6.8). YAC D485 is important, as it is positive for MD 104, MD 543 and D11S931, for which there were previously no YACs. It also extends the contig which previously consisted of the YACs which are positive for MD 220, MD 282, MD 283 and MD 437. Similarly, D11155 links the contig of those YACs which are positive for MD 122 and MD 176, to the contig which is positive for MD 412, D11S873 and MD 471. This means that all twelve markers are now localised to one or other of these contigs which between them are likely to cover the entire region. When this information is used to re-estimate the size of the region, a figure in line with the previous estimate, of ~1.8 Mb, is obtained.

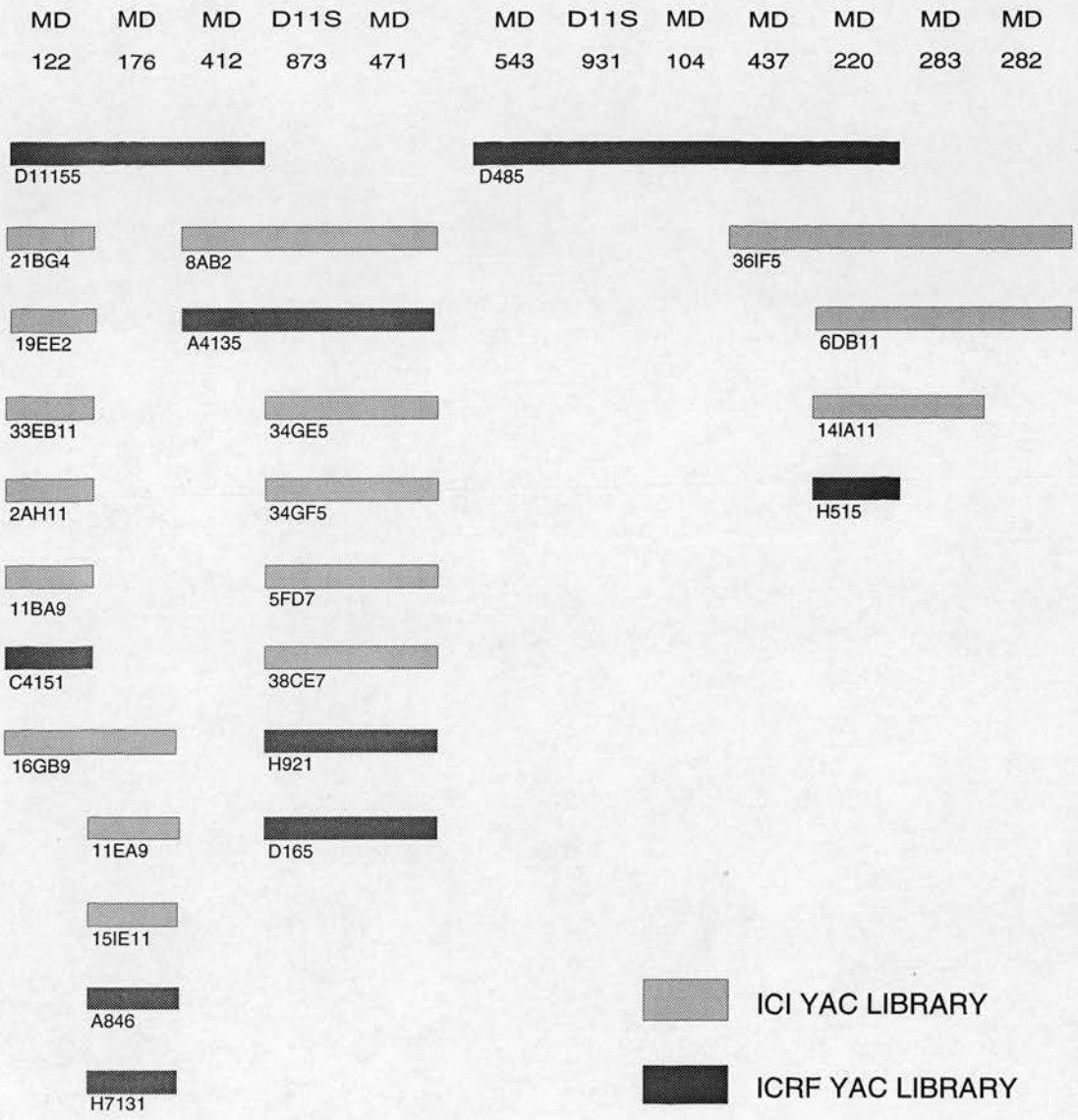
The results of PFGE analysis of digests of total human DNA, hybridised with microdissection clones provided more information which can be used to assemble a contig of the region. When SalI digests of human DNA were sequentially hybridised with MD 176, MD 471 and MD 543 the same band of approximately 240 kb was identified (this work was carried out by John Maule). This result potentially links the two contigs defined previously. MD 543 has been linked to MD 176 and MD 471 and if this 240 kb pulsed field band is also positive for D11S873 and MD 412 then the contig described in Figure 6.9 can be assembled. It is possible that these five markers do not all hybridise to the same 240 kb fragment, but that they hybridise to two, or more, fragments of indistinguishable size. MD 176, MD 412, D11S873 and MD 412 have already been linked by YAC recombinants, suggesting that these markers are indeed linked and either hybridise to the same pulsed field fragment, or if they hybridise to different fragments, that there are two adjacent 240 kb Sal I fragments. It may be possible to confirm or refute linkage of MD 543 to the other markers by the study of hybridisation to restriction digests of other enzymes and/or by analysis with end clones from potentially overlapping YACs.

Table 6.2 Marker Analysis of the ICRF YAC Recombinants

	MD 122	MD 176	MD 412	D11S 873	MD 471	MD 543	D11S 931	MD 104	MD 437	MD 220	MD 283	MD 282
D485	-	-	-	-	-	+	+	+	+	+	-	-
H921	-	-	-	+	+	-	-	-	-	-	-	-
A4135	-	-	+	+	+	-	-	-	-	-	-	-
D165	-	-	-	+	+	-	-	-	-	-	-	-
A846	-	+	-	-	-	-	-	-	-	-	-	-
H7131	-	+	-	-	-	-	-	-	-	-	-	-
D11155	+	+	+	-	-	-	-	-	-	-	-	-
C4151	+	-	-	-	-	-	-	-	-	-	-	-
H515	-	-	-	-	-	-	-	-	-	+	-	-

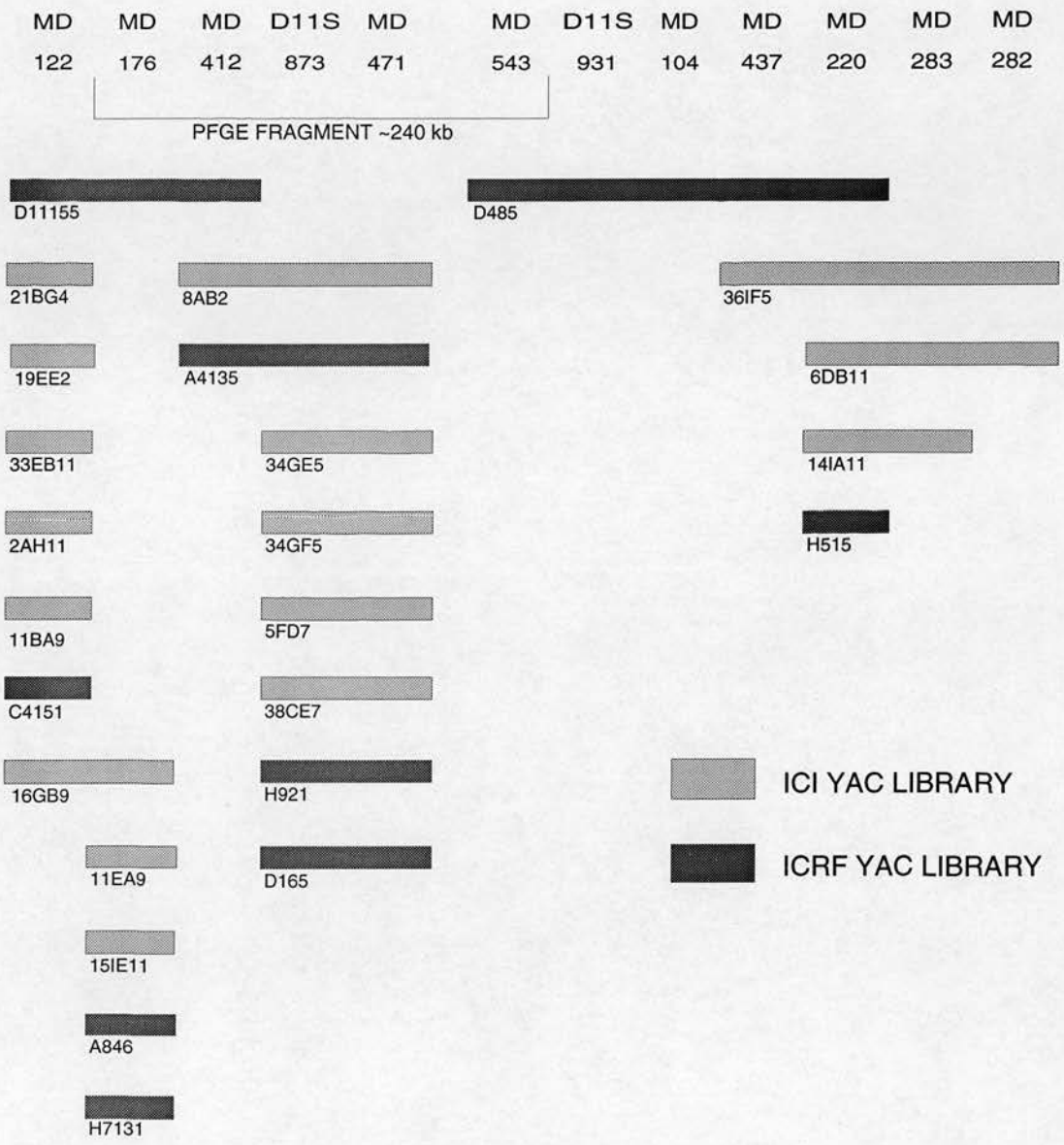
Table 6.2: This work was carried out by John Brown and Susan Maguire.

Figure 6.8: Contig of the ICI and ICRF YAC Recombinants



The Figure (which is not drawn to scale) shows two possible contigs which can be assembled from the data described in Tables 6.1 and 6.2. Boxed areas indicate the extent of the cross hybridisation of YACs to microdissection clones. The relative order of two groups of markers: 1) MD 471 and D11S873 and 2) MD 543, D11S931 and MD 104 remains to be determined.

Figure 6.9: Contig and PFGE Map of the ICI and ICRF YACs



The Figure (which is not drawn to scale) shows a possible contig which can be assembled from the data described in Tables 6.1 and 6.2 and from PFGE analysis. The boxed areas indicate the extent of the cross hybridisation of YACs to microdissection clones. A PFGE fragment of ~240 kb hybridised to MD 176, MD 471 and MD 543; MD 412 and D11S873 have yet to be tested as PFGE probes. The relative order of two groups of markers: 1) MD 471 and D11S873; and 2) D11S931 and MD 104 remains to be determined.

MD 122, while linked to MD 176, identifies a different size SalI fragment. The results of PFGE analysis, which suggest that MD 122 lies within 550 kb of the chromosome 11 translocation breakpoint, are consistent with the assembled contig (see Figure 6.9) which suggests that MD 122 lies towards one end.

The relative orientation of the contig with respect to the translocation breakpoint has also been determined by duel colour FISH analysis (carried out by Yoshiro Shibasaki). FISH analysis has been carried out with the following reagents.

1. Cosmids corresponding to microdissection clones MD 104, MD 220 and MD 283 (isolated by David Baillie and John Brown from the ICRF cosmid library which was obtained courtesy of Hans Lehrach).

2. A probe produced by catch-linker PCR from YAC 16GB9 (MD 122 and MD 176 positive) (produced by Tony Brookes).

3. Cosmids produced from YAC 16GB9 (produced by Yoshiro Shibasaki).

FISH analysis on metaphase chromosomes suggests that the cosmid corresponding to MD 104 is proximal to those corresponding to MD 220 and MD 283. Similarly, the analysis shows that MD 122 is proximal to MD 104. Thus the results of FISH analysis are consistent with the contig described in Figure 6.9.

The marker order described in Figure 6.9 is inconsistent with measurements of genetic distances between D11S901 (which maps proximal to the translocation breakpoint and TYR) and D11S873 and D11S931 respectively, which suggest that D11S931 is proximal to D11S873 (see section 3.4). The genetic distances from firstly, D11S931 and secondly, D11S873 to D11S901 are ~ 8 cM and ~8.5 cM. However, as mentioned previously, these markers have not yet been mapped on the same set of individuals and a difference of 0.5 cM over a distance of ~8 cM may not be significant.

CHAPTER 7

7. Related Studies

7.1 Production of a YAC Contig Across the Chromosome 1 Translocation Breakpoint

This work is being carried out, in parallel with the chromosome 11 work, by Benoit Arveiler. Pools of microdissection clones (from the library described in Chapter 4) were used to screen the ICI YAC library. The YAC recombinants isolated in this way were assembled into a sub-library, which was then screened with the microdissection clones which had been assigned to chromosome 1 (see section 4.4). Currently, the YACs isolated in this way are being assembled into a contig. Contig assembly is being carried out by fingerprint analysis of the YACs and by testing the YACs for co-hybridisation of microclones. The sub-library and the ICI YAC library are also being screened by PCR. This analysis is being carried out with the PCR markers D1S179, D1S251 and D1S103 which flank the chromosome 1 translocation breakpoint.

CHAPTER 8

8. Summary and Concluding Remarks

8.1 Evolution of Experimental Strategy

This thesis describes progress towards the mapping and cloning of the breakpoints of a balanced translocation t(1;11)(q42.2;q21) which segregates with schizophrenia and other major mental illness in an extensive Scottish pedigree.

No gene which predisposes to schizophrenia has yet been isolated. In fact, attempts to establish genetic linkage to schizophrenia have so far been unsuccessful. The same is true for attempts to establish linkage to the affective disorders. It is likely that this reflects the difficulties of carrying out linkage analysis on complex, polygenic disorders. A possible alternative solution is to utilise a chromosomal rearrangement which segregates with the disease to isolate the causative gene. The availability of a chromosomal rearrangement greatly simplifies the search for a gene. Firstly, the chromosomal region(s) to be cloned and examined for the presence of a gene is immediately obvious. Secondly, it is very likely that the size of the region(s) to be examined will be less than that which would be implicated by linkage analysis. Therefore, a pedigree where a balanced translocation segregated with schizophrenia represented an opportunity for identifying a gene (or genes), at or near one of the translocation breakpoints, which is responsible, at least within this family, for predisposition to schizophrenia and other major mental illnesses.

Cytogenetic banding analysis of translocation chromosomes suggested that the translocation breakpoints occurred at 11q21 and 1q42.2 (St.Clair et al., 1990). In order to refine the position of the translocation breakpoints genetic linkage analysis was carried out (by various members of the group) with polymorphic markers in these regions. Clones representing markers which showed little or no recombination with the phenotype and the translocation were used in FISH analysis (carried out by Diane Hanratty and John Gosden), resulting in the definition of initial flanking markers (Fletcher et al., 1993).

We decided to focus primarily on the cloning of the chromosome 11 translocation breakpoint. The chromosome 11 translocation breakpoint was chosen because we had already established a number of chromosome 11 resources in the laboratory. These included somatic cell hybrids and assays for markers of known location on chromosome 11. The existence of two other balanced translocations which appeared to segregate with schizophrenia and which involved chromosome 11q (Smith et al., 1989; Holland and Gosden, 1990) (the breakpoints were not accurately defined) also prompted this focus on the chromosome 11 translocation breakpoint.

We wished to define the location of the chromosome 11 breakpoint at a higher resolution, as part of a positional cloning strategy towards the isolation of a clone (or clones) which spanned the translocation breakpoint. Positional cloning of the chromosome 11 translocation breakpoint was a viable proposition because, as well as the access to chromosome 11 markers, there was considerable expertise in somatic cell genetics and molecular mapping within the group.

High resolution characterisation of the translocation breakpoints required firstly the segregation of the two translocation derived chromosomes into separate somatic cell hybrids. Segregation of the translocation chromosomes was achieved by cell-surface marker selection strategies (this was carried out by Judy Fletcher and Veronica van Heyningen) (Fletcher et al., 1993). The assembly of a panel of somatic cell hybrids, each of which contained complementary segments of chromosome 11 was also required. Four hybrids, containing fragments of chromosome 11 in the background of a Chinese hamster ovary cell line (WG3H) were produced by Judy Fletcher and Veronica van Heyningen. These fragmentation hybrids were produced by X irradiation of a chromosome 11-only hybrid J1Cl4 (Jones et al., 1984), followed by rescue of the fragments produced, by fusion with WG3H cells and finally, cell surface marker selection to isolate those cell lines which retained fragments of chromosome 11. Two independent HRAS 1-selected chromosome mediated gene transformants (CMGT), which carry single fragments of chromosome 11 material in a murine background (Porteous et al., 1986), and two other, unrelated translocation cell lines, PG48, which has a translocated chromosome 11 which lacks 11q22-11qter, in a hamster background (Gillett, et al., 1993) and CF52 which has a well characterised

t(11;16) (q13;p11) translocation chromosome as its sole human component, in the background of a murine cell line (Koeffler et al., 1981) were also obtained. This was carried out with a view to using the cell lines to map markers relative to the translocation breakpoints and to a series of intervals on chromosome 11, as defined by the segments of chromosome 11 present in the set of hybrids. This was the starting point of my PhD project. I initially analysed the cell lines for the presence of markers whose chromosome 11 regionalisation and relative order was known. Subsequently, I analysed the cell lines for chromosome 11 markers whose chromosome 11 regionalisation was unknown. These markers were localised by comparison with those of known chromosomal location.

Once the hybrid mapping panel had been established and characterised and markers of unknown location had been mapped, it was obvious that new markers would have to be generated from a region close to the chromosome 11 translocation breakpoint. This was achieved by microdissection of a 30 Mb region surrounding the translocation breakpoint in the derived chromosome 1. The dissected material was digested with EcoRI and cloned (Muir et al., manuscript in preparation). The microdissection clones generated in this way were assigned to intervals on chromosome 11, using a subset of the hybrid cell lines. This was done with the aim of identifying those markers from the microdissection library which mapped to the interval closest to the chromosome 11 breakpoint. The markers which mapped to this critical region were used to isolate YACs. The analysis of these YACs, along with PFGE analysis of the normal and translocated chromosomes (using probes from the closest interval) and FISH analysis (with probes derived from the isolated clones) were used to build up a YAC contig of the region.

8.2 Summary of results

This project has resulted in the determination of flanking markers of the chromosome 1 and chromosome 11 translocation breakpoints and the characterisation of a set of somatic cell hybrid lines. These somatic cell hybrids have been used to regionally localise genes, ESTs, microdissection clones and anonymous DNA markers on chromosome 11q. My efforts have resulted in the localisation of 13 genes, two ESTs, eight anonymous DNA markers and 18 microdissection clones and, as a group, we have mapped an

additional nine ESTs and 31 microdissection clones to a series of intervals on chromosome 11q. This hybrid mapping panel now defines 22 intervals on chromosome 11q. Thirty-seven microdissection clones have been localised to chromosome 1 and six anonymous DNA markers have been mapped relative to the translocation breakpoint on chromosome 1. The genetic distance between the distal flanking marker and one of the potential proximal flanking markers is estimated at 6 cM (Engelstein et al., 1993).

The microdissection clones were regionally localised on chromosome 11, with the aim of identifying a number of new markers in the interval closest to the chromosome 11 breakpoint. Ten microdissection clones were identified as mapping to this interval. This, together with the fact that two anonymous markers were subsequently localised to the same interval, means that there are 12 markers in a region estimated at ~ 2 Mb. Five of these markers were successfully used to screen the gridded filters of the ICI YAC library. This resulted in the isolation of 16 YACs. Overlaps between the individual YAC recombinants were detected by co-hybridisation of markers to the YACs, allowing the assembly of three YAC contigs. These three contigs correspond to nine of the twelve markers in the region under study. The validity of these contigs was confirmed by fingerprint analysis, which demonstrated the overlaps between YACs where they had been predicted. The contigs were confirmed and extended by the analysis of a set of nine YACs isolated from the ICRF YAC library (this work was carried out by John Brown and Susan Maguire). This analysis allowed assembly of the markers into two YAC contigs which covered all twelve markers in the region. The two contigs were provisionally linked by co-hybridisation of markers from the two contigs to the same pulsed field gel fragment (this work was carried out by John Maule).

The resultant contig of YACs was oriented relative to the translocation breakpoint in two ways. Firstly, using MD 122 as a probe, I detected differences in the length of PFGE restriction fragments in DNA from a translocation carrier versus that obtained in normal DNA. This analysis indicated that MD 122 is within 550 kb of the translocation breakpoint. Secondly, duel colour FISH analysis (carried out by Yoshiro Shibisaki) was carried out with a probe produced by catch-linker PCR (produced by Tony Brookes) from YAC 16GB9 (MD 122 and MD 176 positive) compared to cosmids corresponding to MD 104, MD 220 and MD 283 (isolated by David

Baillie and John Brown). This analysis indicated that 16GB9 is proximal to MD 104 which is in turn proximal to MD 220 and MD 283. It should be said that this preferred order implies that D11S873 is more centromeric than D11S931, a result which is at odds with published linkage analysis. There appears to be a need to re-evaluate the genetic map of this region.

Thus the objectives of this thesis have been achieved in part. The position of both translocation breakpoints have been refined, relative to the markers on the two chromosomes. New markers have been identified in the region immediately distal to the chromosome 11 translocation breakpoint. These markers have been used to isolate YACs, which have contributed to the assembly of a YAC contig of the region. It is necessary to confirm this contig, and it may be necessary to extend it, before the chromosome 11 translocation breakpoint can been cloned.

8.3 Future Directions

In order to confirm and complete the chromosome 11 contig a number of experiments are being carried out, by various members of the group, including myself.

1. End clones are being isolated from YACs. These clones are being used in a number of ways. They are being hybridised to Southern blots of all the YACs in the region, in order to detect all overlaps between the YACs and to confirm the order of the clones and markers in the contig. End clones from YACs at the end of the contig closest to the translocation breakpoint are being hybridised to somatic cell hybrids, in order to determine which side of the chromosome 11 breakpoint they map to. These clones are also being used in PFGE analysis in an attempt to detect alterations in restriction fragments sizes caused by the interruption of the chromosome by the translocation breakpoint.

2. Probes are being isolated from YACs located at the end of the contig closest to the translocation breakpoint, by catch-linker PCR. The probes generated are being used in a number of different ways. They are being used for FISH analysis, in order to determine whether any of the YAC recombinants at the end of the contig which is closest to the chromosome 11

translocation breakpoint, cross the breakpoint. In common with the end clones, the individually cloned catch linkered probes are being hybridised to Southern blots of all the YACs in the region, in order to detect all overlaps between the YACs and to confirm the order of the clones and markers in the contig. These probes are also being hybridised to somatic cell hybrids in order to determine which side of the chromosome 11 breakpoint they map to. Finally, individual clones are again being used in PFGE analysis, in an attempt to detect alterations in restriction fragments sizes due to the interruption of the chromosome by the translocation breakpoint.

3. YACs at the end of the contig which is closest to the translocation breakpoint are being subcloned into cosmid vectors. These cosmids will also be used in FISH analysis and as probes for hybridisation to Southern blots, both of the YACs in the region and of the somatic cell hybrids.

If the experiments described above confirm the order of the clones in the contig, but find that it does not extend to the translocation breakpoint, then it will be necessary to walk from the nearest YAC recombinant, until a clone or clones which cross the chromosome 11 translocation breakpoint have been isolated. End clones and catch linkered probes generated from the closest YAC will be used for this purpose.

A clone (or clones) which crosses the chromosome 1 translocation breakpoint is also required. As discussed in section 7.1, experiments towards this are currently underway. It may also be possible to isolate clones from the region of the translocation breakpoint on chromosome 1 using a probe adjacent to the chromosome 11 breakpoint. For example, where a probe detects restriction fragments of altered size in translocation chromosomes these fragments correspond to the piece of DNA which spans the breakpoint of the translocated chromosome. This fragment consists of DNA from chromosome 1 as well as chromosome 11. It should be possible to recover this material by coincidence sequence cloning (Brookes and Porteous, 1991) between material extracted from two bands of altered size, produced by digestion of translocation carrier DNA with two different restriction enzymes. Coincidence sequence cloning should result in the selective recovery of the DNA at the translocation breakpoint as, although there will be DNA from other chromosomal regions present in both of the bands, the only DNA in common

to the two bands should be the fragment which spans the translocation breakpoint.
An alternative method for recovery of the breakpoint region from one or both of the translocation chromosomes would be to generate sequence from the chromosome 11 side of the breakpoint and to use this sequence to design inverse PCR primers (i.e. primers which are designed to amplify out from the cloned material in opposite directions). Digestion of DNA from a translocation carrier with an enzyme which doesn't cut between the distal primer and the translocation breakpoint would then be followed by ligation, under conditions where circularisation of individual molecules is the most likely outcome. The piece of DNA which spans the breakpoint will now exist as a circle and thus sequence flanking the chromosome 1 translocation breakpoint can be obtained by inverse PCR.

Once DNA fragments from the regions flanking the translocation breakpoints have been isolated, by either of the above methods, those which are found to originate from chromosome 1 can be used to screen cosmid and YAC libraries, resulting in the isolation of clones from the breakpoint region on chromosome 1.

Once clones which span the translocation breakpoints have been isolated then the next step is the analysis of the translocated chromosomes and the isolation of candidate genes. Again, a number of complementary methods will be used.

1. Molecular analysis will be carried out on the translocation breakpoint regions in various affected individuals and compared with the appropriate normal chromosome region. Probes from the region will be hybridised to restriction digests of DNA from normal individuals and translocation carriers. This will determine whether the translocation involved clean breakage of the chromosomes or whether events such as insertions, inversions or deletions have occurred. This information will indicate the likely locations of candidate genes.

2. Identification of the CpG islands closest to the translocation breakpoints, by PFGE analysis of restriction digests of YACs and cosmids which span the translocation breakpoints, will point to the locations of candidate genes.

3. Probes which map close to a translocation breakpoint and which, by Zoo blot analysis, show cross-species conservation will be used to screen cDNA libraries to obtain candidate expressed sequences.

4. Coincidence sequence cloning between foetal brain cDNA and an appropriate genomic template will be used to isolate candidate genes (Brookes and Porteous, 1991). The appropriate genomic templates include catch linkered PCR products from a clone which spans a translocation breakpoint, a cosmid which spans the translocation breakpoint or the microdissection clones which map to an appropriate region. A number of candidate genes have already been isolated by coincidence sequence cloning between foetal brain cDNA and a proportion of the repeat free microdissection clones (this was carried out by Euan Slorach and Tony Brookes).

Thus it should be possible, by using a combination of strategies, to clone both translocation breakpoints and to isolate genes which are likely to be affected by this rearrangement.

Once candidate genes have been identified, their location, with respect to the translocation breakpoints, will be determined. If one of the breakpoints interrupts the gene, then this would indicate that it is involved in the phenotype in this family. The expression of the candidate genes will be studied, in order to detect any differences between translocation carriers and control individuals. Expression of candidate genes will also be examined in archival and autopsy material, from schizophrenics (without the t(1;11)(q42.2;q21) translocation) and from control individuals. Other individuals with schizophrenia will be tested for mutations in the candidate gene. Having a candidate gene (or genes) would allow a variety of experiments into the likely role of the gene to be designed. Any genes which are isolated in this way will also be used to identify biochemically or evolutionarily related genes or proteins which may be mutated in other cases of schizophrenia, or related mental illnesses.

Thus the molecular analysis of the translocation promises not only to provide insight into the cause of the disorder in this family, but also to provide a route

towards a broader understanding of the molecular basis of this important, but complex disorder.

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Human olfactory marker protein maps close to tyrosinase and is a candidate gene for Usher syndrome type I

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ABSTRACT

Olfactory marker protein (OMP) shows olfactory neuron-specific expression in rodents [1,2,3]. We recently reported tight linkage on mouse chromosome 7 of OMP to the *shaker-1* deafness mutant, between the tyrosinase and globin loci [4]. Here we isolate and map the human homologue. Our results show that OMP maps immediately centromeric to tyrosinase on the long arm of human chromosome 11. Genetic linkage to this region has recently been established for Usher Syndrome Type I [5], an autosomal recessive blindness and deafness disorder and a putative homologue of the *shaker-1* mutant. OMP is thus a candidate gene for both congenital deafness defects.

INTRODUCTION

Olfactory marker protein (OMP) is of unknown biological function. It is found in a wide variety of vertebrates and is expressed predominantly in mature, functioning olfactory receptor neurons where it is present as a highly abundant cytoplasmic protein [1,2,3]. Molecular genetic mapping of the mouse *Omp* gene to a series of radiation induced albino deletion chromosomes established that it mapped distal to both tyrosinase and mod-2 and proximal to the globin locus, in an interval of approximately 2cM [6]. This analysis placed *Omp* in the same minimal deletion interval as the mouse mutant *shaker-1*. *Shaker-1* is an autosomal recessive deafness mutant which shows associated circling and head-tossing behaviour and hyperactivity [7]. Homozygotes have abnormalities in eighth-nerve and cochlear potentials, in the absence of detectable structural changes in the cochlea. Electrophysiological observations suggest that the inner hair cells or afferent innervations are the primary site of the defect and likely site of *sh-1* gene expression [8]. As a prelude to positional cloning of the defect, we previously undertook to refine the genetic location by RFLV analysis of an extensive intraspecific backcross segregating for the *shaker-1* mutation. Typing for *Omp*, we observed a single recombinant in one thousand progeny [4]. This result suggested that *Omp* maps within 200kbp of *sh-1* and might in fact constitute the locus.

Similarities between a number of hereditary deafness syndromes in mice and humans have been noted [9]. New insights may come from further comparative studies of orthologous chromosomal regions [10]. We therefore undertook to isolate the human OMP homologue and establish its map location. Previous comparative studies of genes on mouse chromosome 7 have established extensive homology with human chromosome 19q, 11p and 11q and to a lesser extent 15q [11]. *Mod-2* is exceptional in that the human homologue, ME2, has been assigned to 6p [12]. The mouse map for the sub-region of current interest, with the corresponding human chromosome locations in parenthesis, can be summarised as follows: *Fes* (15q)–*Tyr* (11q21)–*Mod-2* (6p)–*Calc I/ Hbb* (11p15). Our genetic linkage studies in the mouse place *Omp* and *sh-1* 5.36cM distal to *Tyr* and 1.2cM proximal to *Hbb* [4]. Taken together with the complementary deletion mapping studies [6], our *a priori* expectation was that OMP in the human would most likely map to 11p15, or possibly 6p. Interestingly, recent studies have indicated linkage of Usher Syndrome I (an autosomal recessive disorder characterised by profound congenital deafness, vestibular areflexia and progressive retinitis pigmentosa) close to TYR on 11q [5], the region to which we now localise OMP.

RESULTS

Isolation of the human OMP homologue

In the mouse, like the rat [2], OMP is a single-copy gene encoded by one exon of 490bp [4]. Primers derived from the rat sequence were used successfully in cross-species PCR to amplify an intraexonic fragment of identical size from human genomic DNA (data not shown). This was used as a hybridization probe to screen a human cosmid library. A single recombinant was colony purified and confirmed to correspond to the probe by sequence analysis of an appropriately amplified fragment (data not shown).

Mapping of the human OMP homologue

a) Segregation in somatic cell hybrids carrying derived human chromosomes 11. A single-copy sub-fragment of the human cosmid which encompassed the OMP gene was used to probe a panel of somatic cell hybrids carrying various portions of human chromosome 11. Figure 1 shows the results which localise the gene on the long arm of chromosome 11 close to TYR. MIS 7.4 and 39.8 carry the t(1;11)(q43;q21) reciprocal translocation chromosomes we have described previously segregating with

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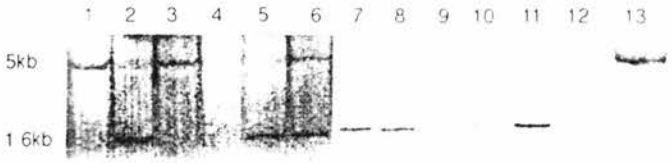

Figure 1. Mapping of human OMP to translocation, X-irradiation, and CMGT human-rodent somatic cell hybrids carrying portions of human chromosome 11. The BglII digests (5μg per track) loaded from left to right in Tracks 1 to 13 were, MIS 7.4 (−ve), MIS 39.8 (+ve), X63 (−ve), E67-1 (−ve), E67-4 (+ve), CF52 (+ve), MAFLI (+ve), J1 (+ve), WG3H (−ve), WJX 3.4 (+ve), WJX 7.4 (+ve), WJX 5.4 (−ve), WJX 11.2 (−ve). A Molecular Dynamics PhosphoImager was used to detect the hybridization. Human specific hybridization is at ~1.6Kbp (scored above as +ve or −ve for each sample tested) while the rodent specific hybridization is at ~5Kbp. MAFLI is a lymphoblastoid cell line established from a patient with schizophrenia [13] and a reciprocal t(1;11)(q43;q21) translocation which has been segregated in the MIS 7.4 and 39.8 hybrids [15]. J1 is a human chromosome 11 only hamster hybrid [26]. X63 and WG3H are mouse and hamster cell lines, respectively. CF52 is an t(11;16)(q13;p11) translocation hybrid [14]. The WJX series are radiation reduced hybrids derived from J1 [15], while E67-1 and E67-4 are HRAS1-CMGT reduced hybrids [16].

major mental illness, comprising schizophrenia and schizoaffective disorder, in a large Scottish pedigree [13]. CF52 carries a well characterised t(11;16)(q13;p11) translocation breakpoint [14]. The four radiation fusion hybrids (WJX series) [15] and two HRAS1-CMGT hybrids (E67-1 and E67-4) [16] each retain one or more sub-fragment of human chromosome 11 and help to accurately place each marker tested. The mapping results with OMP place the human gene on the long arm of chromosome 11 telomeric to FGF4 (and therefore also FGF3, see later), close to TYR and centromeric to the schizophrenia translocation breakpoint. Table 1 shows a full comparison of the OMP mapping result with other markers tested previously [15].

b) DNA in situ hybridization. To confirm and refine the OMP map location, we carried out DNA *in situ* hybridization with fluorescently labelled cosmids to banded human metaphase chromosomes, as previously described [15,17]. Figures 2a and b show the results of hybridization with a cosmid clone for tyrosinase [18] and the OMP cosmid respectively, analysed by scanning fluorescent confocal microscopy. Seventeen R banded chromosomes showing hybridization were examined for each cosmid; we conclude that tyrosinase maps to 11q14.3 and that OMP maps to 11q13.5. Fractional length measurements from the tip of the short arm to the site of hybridization, determined on the same set of images, gave FLPTER measurements of 0.6 (range 0.58−0.64) for OMP and 0.68 (range 0.64−0.71) for tyrosinase. To confirm the more centromeric location of OMP, we performed a double hybridization experiment. OMP and the chromosome 11 specific alphoid repeat probe PLC 11A [19] were both labelled with biotin and visualised with avidin-texas red. Tyrosinase was labelled with digoxygenin and visualised with FITC-labelled anti-digoxygenin. The results are shown in Figure 2c. High resolution mapping to interphase chromosomes under otherwise identical hybridization conditions is shown in Figure 2d. The relative positions of hybridization is unchanged, but the absolute distance separating the hybridization signals is altered, reflecting differential chromosome (de)condensation. Figure 3 summarises the results obtained with TYR and OMP on R-banded chromosomes.

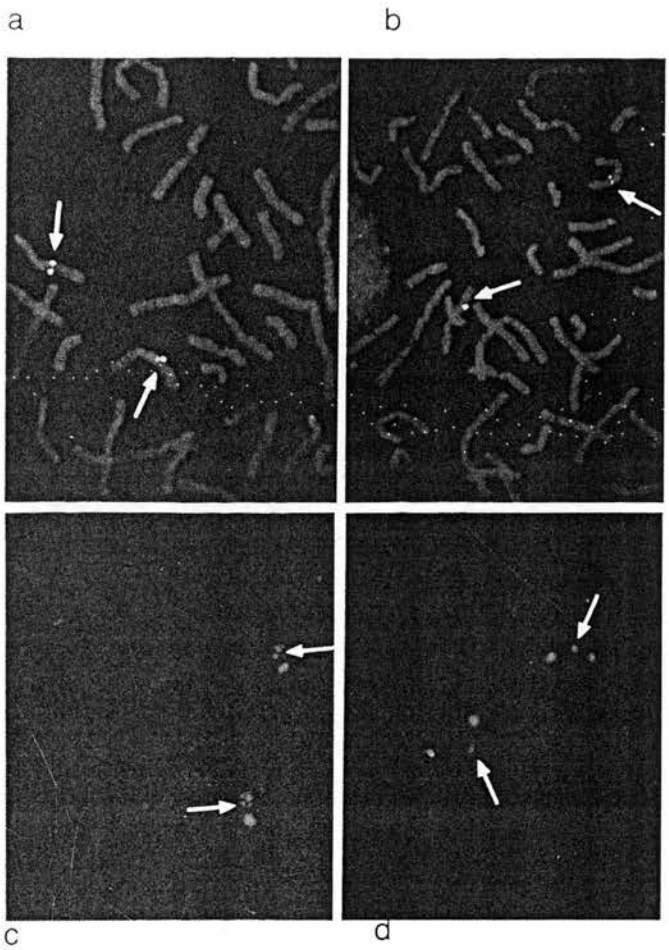

Figure 2. *In situ* localisation of TYR and OMP. **a**: Representative fluorescence in situ hybridization with OMP. Hybridization and detection was as described in the Materials and Methods. The site of OMP hybridization on each of the two human chromosomes 11 is arrowed. **b**: Representative fluorescence in situ hybridization with TYR. Hybridization and detection was as described in the Materials and Methods. The site of TYR hybridization on each of the two human chromosomes 11 is arrowed. **c**: Dual hybridization of metaphase chromosomes with TYR and with OMP plus PLC 11A. Hybridization and detection was as described in the Materials and Methods. The site of OMP hybridization on each of the two human chromosomes 11 is arrowed. The strong red signal corresponds to the hybridization of PLC 11A to the centromere and the green signal to TYR. **d**: Dual hybridization of interphase chromosomes with TYR and with OMP plus PLC 11A. Hybridization and detection was as described in the Materials and Methods. The hybridization of OMP to each of the two human chromosomes 11 is arrowed. The strong red signal corresponds to the hybridization of PLC 11A to the centromere and the green signal to TYR.

DISCUSSION

The albino locus in the mouse, which encodes tyrosinase, has been extensively studied. The availability of a number of experimental and spontaneous deletion mutants has allowed the fine mapping of flanking genes. Comparative studies show that the region shares homology with at least three human chromosome segments represented by *Tyr* (11q21), *Mod-2* (6p) and *Hbb* (11p15) [11]. As murine *Omp* maps between *Mod-2* and *Hbb* [4,6], our *a priori* expectation was that human OMP would map to 6p or 11p15. However, our present study excludes linkage to HBB and clearly shows that human OMP maps at 11q13.5 in the human, close to TYR. The present result adds

able 1. Marker analysis of translocation, X-irradiation, and CMGT human-rodent somatic cell hybrids carrying portions of human chromosome 11

OCUS	MIS 7.4	MIS 39.8	CF 52	WJX 3.4	WJX7.4	WJX5.4	WJX11.2	E67.1	E67.4
BB	−	+	ND	−	−	+	−	+	−
	−	+	ND	−	−	−	−	ND	ND
YR LI	−	+	−	+	−	+	−	−	+
GA	−	+	−	+	+	−	−	+	−
DU 1	−	+	−	+	+	−	−	−	−
STF1	−	+	−	+	−	−	−	−	−
ST 3	−	+	−	+	−	−	−	+	+
11S527	−	+	+	+	−	−	−	−	−
11S533	−	+	+	+	+	−	−	−	+
MP	−	+	+	+	+	−	−	−	+
YR	−	+	+	−	+	−	+	−	+
11S388	+	−	+	−	−	−	+	−	−
TMY	+	−	+	−	−	+	+	−	+
LG	+	−	+	−	−	+	+	−	+
11S385	+	−	+	−	−	+	+	−	+
CAM	+	−	+	−	+	−	+	−	+
RD2	+	−	+	−	+	−	+	−	−
11S351	+	−	+	−	−	+	+	−	−
HY1	+	−	+	−	−	+	+	−	−
IC 9	+	−	+	−	−	+	+	−	−

e legend to Figure 1 for details of DNA's. Marker analysis for OMP was as described in Figure 1. All other markers were mapped by Southern blot analysis d/or PCR analysis, as described and published in detail elsewhere [15], with the exception of D11S527 for which the original PCR conditions [22], with slight odification, were used.

the comparative map and suggests that the divergent evolution this region between mouse and man is even more complex an thought previously; we must now account for the presence a second region of homology to 11q on mouse chromosome in the region between *Mod-2* (6p in human) and *Hbb/Calc I* 1p15 in human). Equally, it should be possible to use this new mparative map information to further define the transcription ap of the region and therefore the *shaker-1* locus, as any ansition from one syntenic region in the human to another will ly occur between rather than within homologous genes.

Usher Syndrome I is the most common combined cause of afness and blindness in the developed world [5]. Recent genetic udies have provided evidence for linkage of Usher Syndrome o 14q in French families from the Poitou-Charentes region [20], 11p in an isolated Louisiana Acadian population [21], and to q in other families from the USA, Sweden, Ireland and South frica [5]. The 11q linkage studies place the latter Usher I locus stal to FGF3 (and therefore also FGF4) and almost certainly oximal to TYR [5]. The highest LOD score was observed with e polymorphic marker D11S527 [22] ($Z=4.15$, $@=0.1$). 11S527 was isolated from a cosmid 'contig' which has been apped by fluorescence *in situ* hybridization to 11q13.5 [23]. is matches closely our localisation of OMP. It is of note that observe discordance between OMP and D11S527 and between MP and TYR each in two (different) reduced hybrids, whereas re is complete concordance between OMP and another closely ked and highly polymorphic marker, D11S533 [24]. This servation suggests that D11S533 will be a useful marker in ther genetic linkage studies in Usher Syndrome Type I families l at the same time illustrates the power of fragmentation hybrids high resolution physical mapping.

We estimate from our previous genetic studies of mouse OMP t the gene lies within 200kbp of the *shaker-1* deafness mutant . The similarities in the mode of inheritance, penetrance and ic pathology of the hearing defect in *shaker-1* mice and Usher drome I patients are provocative. We have examined mice rying several independent *sh-1* alleles by electroretinography l although we have found no evidence for visual impairment in young adult mice (Steel and Brown, unpublished), a careful study of ageing mice for evidence of eye degeneration is justified.

The function of OMP is unknown, but expression studies suggest an involvement in olfaction and a direct or indirect role

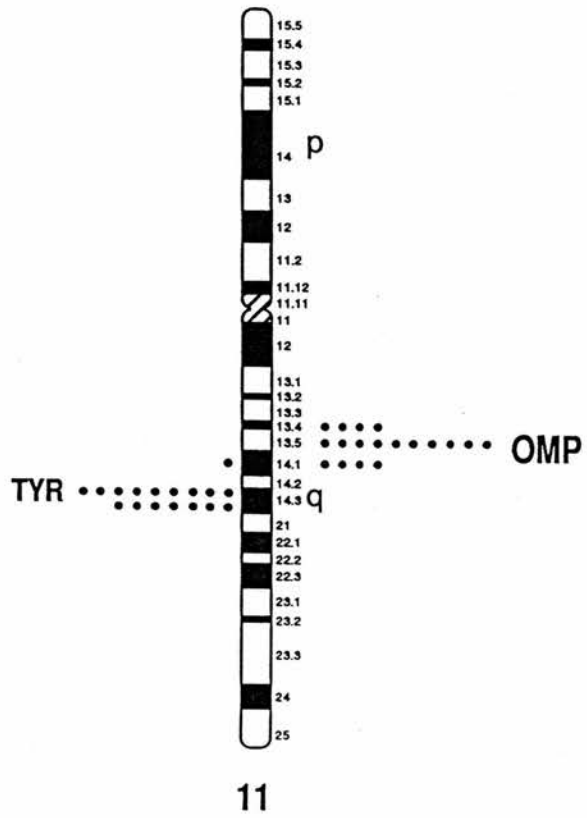

Figure 3. Summary of hybridisation results with TYR and with OMP on R-banded chromosomes. Hybridization and detection was as described in the Materials and Methods. The site of hybridization of TYR (17 metaphases) and of OMP (18 metaphases) is shown.

in neurotransmission is possible [1,2,3]. Some Usher Syndrome patients show increased numbers of compound nasal cilia with abnormal arrangements of the axonemal microtubules [25]. Since the outer limbs of the photoreceptors are modified cilia and the sensory epithelium of the inner ear is derived from ciliated epithelial cells, a unifying hypothesis would be that the spectrum of sensory abnormalities in Usher Syndrome I stem from a single basic defect in cilial development. Taken together with the genetic linkage in Usher Syndrome I and in *sh-1*, it is tempting to speculate that these defects are true homologues and that OMP is either directly implicated in or very closely linked to both disorders. We are carrying out further molecular cloning and mutation screening experiments to test both hypotheses.

MATERIALS AND METHODS

Isolation of a human OMP cosmid

A PCR reaction was performed on total human DNA using primers and conditions as described previously for the mouse [4]. The resultant 490bp product was radiolabelled by random priming and used to screen a total genomic DNA library (generous gift of Dr W Bickmore) prepared by partial digestion with Sau3A and insertion at the BamHI site of the Lawrist cloning vector. A single positive hybridising colony was identified, purified and confirmed as being human OMP by PCR amplification using the above protocol and direct sequencing of the product (data not shown).

Hybrid cell culture, DNA analysis and marker analysis

Somatic cell hybrids were maintained and characterised, and DNA prepared for Southern blot and PCR analysis as described previously [15,16]. A ~900bp PstI fragment encompassing the OMP gene was subcloned from the OMP cosmid and used as a hybridization probe under standard conditions to 5μg aliquots of BglII digested human, rodent and human-rodent somatic cell hybrid DNA. A Molecular Dynamics PhosphoImager was used for hybridization detection.

In situ hybridization

Metaphase spreads were prepared from human peripheral lymphocyte cultures and hybridised to biotin labelled cosmids; the signals were detected using alternate layers of avidin-FITC, biotinylated anti-avidin, and avidin-FITC as described previously [15,17]. The slides were mounted in antifadent containing propidium iodide and DAPI to produce an R banded propidium iodide pattern. Images were obtained and analysed with a Biorad MRC 600 scanning confocal microscope. In double hybridization experiments, the tyrosinase cosmid was labelled with digoxygenin and detected with FITC-conjugated sheep anti-digoxigenin Fab fragments (Boehringer-Mannheim) while the OMP cosmid and the chromosome 11 specific centromere probe PLC 11A were labelled with biotin and detected with alternate layers of avidin-Texas red, biotinylated anti-avidin and avidin-Texas red.

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Schizophrenia-associated Chromosome 11q21 Translocation: Identification of Flanking Markers and Development of Chromosome 11q Fragment Hybrids as Cloning and Mapping Resources

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Summary

Genetic linkage, molecular analysis, and in situ hybridization have identified TYR and D11S388 as markers flanking the chromosome 11 breakpoint in a large pedigree where a balanced translocation, t(1;11)(q43;q21), segregates with schizophrenia and related affective disorders. Somatic cell hybrids, separating the two translocation chromosomes from each other and from the normal homologues, have been produced with the aid of immunomagnetic sorting for chromosome 1– and chromosome 11–encoded cell-surface antigens. The genes for two of these antigens map on either side of the 11q breakpoint. Immunomagnetic bead sorting was also used to isolate two stable X-irradiation hybrids for each cell-surface antigen. Each hybrid carries only chromosome 11 fragments. Translocation and X-irradiation hybrids were analyzed, mainly by PCR, for the presence of 19 chromosome 11 and 4 chromosome 1 markers. Ten newly designed primers are reported. The X-irradiation hybrids were also studied cytogenetically, for human DNA content, by in situ Cot1 DNA hybridization and by painting the Alu-PCR products from these four lines back onto normal human metaphases. The generation of the translocation hybrids and of the chromosome 11q fragment hybrids is a necessary preliminary to determining whether a schizophrenia-predisposition gene SCZD2 is encoded at this site.

Introduction

A large Scottish pedigree (K26) has been described (St. Clair et al. 1990) in which major mental illness, classifiable as schizoaffective disorder, was seen to segregate with a balanced translocation, t(1;11)(q43;q21). The family had been initially ascertained through a cytogenetic survey of mental hospitals and borstals (Jacobs et al. 1970). Subsequently (St. Clair et al. 1990) it was found that 23 members of this family had psychiatric diagnoses conforming to the details set out in Research Diagnostic Criteria for mental and/or behavioral disorders (Spitzer et al. 1987). Thirty-four of the 77 individuals karyotyped carried the translocation. Sixteen of the 23 family members who had psychiatric diagnoses had major mental illness; two others, not analyzed cytogenetically, had committed suicide. The five remaining cases with psychiatric diagnoses but without the translocation did not have major mental illness (one had generalized anxiety, one had minor depressive disorder, and three were alcoholics). No physical dysmorphism or mental handicap had been observed in this carefully studied family.

Pedigrees with familial aggregations of common diseases, with both genetic and environmental components, are now used to unravel the specific genetic contributions to such disease, in the hope of gaining insight

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into the biochemical pathways affected, thus allowing us to think about therapy and prevention. A large pedigree where the disease segregates tightly with a cytogenetic abnormality is an ideal starting point for identifying the genetic component implicated at least in that family. Obviously, a cosegregating translocation may result in functional inactivation of a particular gene at or near the breakpoint site, but we are aware that the breakpoint may only be a closely linked marker for a dominantly acting disease-predisposing allele that happens to be present in this family, at a nearby locus. Two other, smaller families have been described with less clear-cut cosegregation of mental illness with two independent chromosome 11q translocations, one with chromosome 9 and one with chromosome 6 (Smith et al. 1989; Holland and Gosden 1990). The breakpoints were not accurately defined. However, their existence strengthened our interest in defining the exact site of the translocation breakpoint in pedigree K26.

Cytogenetic banding analysis was carried out on peripheral blood chromosomes from selected individuals, to confirm and refine the position of the breakpoint. Lymphoblastoid cell lines were set up from key family members, both translocation carriers and noncarriers. Genetic linkage analysis was carried out with available polymorphic markers in the region of the translocation, by using markers both from chromosome 1 and from chromosome 11. We expected to see suppression of recombination in the vicinity of the translocation but hoped to define more accurately the site of the breakpoint. Using markers which showed little or no recombination with the disease and the translocation in this family, we used chromosomal in situ hybridization to determine the position of the breakpoint, with the aid of suitable large insert markers. However, with a view to defining the breakpoint much more closely by positional cloning strategies, we also embarked on segregating the two translocation-derived chromosomes into separate somatic cell hybrids, using cell-surface marker selection strategies which we had developed for our work on the short arm of chromosome 11 (Seawright et al. 1988). The two independent pairs of mouse-human hybrids so produced showed TYR to be above the breakpoint and showed STMY1 to be below it. In situ hybridization confirmed the tyrosinase assignment to the derived chromosome 11 and showed the next most closely linked distal 11q marker, D11S388 (cosmid CJ52.4) (Julier et al. 1990), to be below the breakpoint. The chromosome 1 breakpoint was also defined with available markers and was found to be between D1S103 and D1S8. We then used DNA from the reciprocal translocation hybrids MIS7 and MIS39 to set up and refine PCR-based analyses for a number of chromosome 11q loci.

Cell-surface marker analysis revealed early on that the two chromosome 11q markers MDU1 and MIC9 were, respectively, above and below the translocation breakpoint. Therefore we set out to produce stable chromosome-fragment hybrids, by X irradiation of the chromosome 11–only hybrid J1CI4 (Jones et al. 1984), selecting for each marker separately. These were destined to be both a mapping and a cloning resource, for the production of new markers in regions defined by earlier markers.

Material and Methods

Genetic Analysis of Selected Family Members

DNA was available for linkage analysis, from 10 translocation carriers and 5 noncarriers in the pedigree. Individuals came from five branches of the family, each separated by several meiotic events. Analysis was carried out either by DNA blotting using informative restriction digests or by PCR followed by sizing of dinucleotide repeats on sequencing gels. Suitable markers were chosen above and below the breakpoint positions suggested by cytogenetic banding analysis on both translocation chromosomes: on chromosome 11—D11S97 (Jeffreys et al. 1988), TYR (Spritz et al. 1990; Giebel et al. 1991), D11S388 (Julier et al. 1990), and D11S35 (Litt et al. 1990); on chromosome 1—D1S81 (Nakamura et al. 1988), D1S103 (Weber et al. 1990), and D1S8 (Royle et al. 1988). Of these, D11S35, D1S103, and, latterly, TYR (Morris et al. 1991) were analyzed as dinucleotide repeats.

Cell Culture and Fusion

Peripheral blood lymphocytes were isolated from a number of cytogenetically normal and translocation-carrying members of the pedigree. In most cases, permanent B lymphoblastoid cell lines were produced by Epstein-Barr virus (EBV) transformation (van Heyningen, in press). Two cell lines, MAFLI and ROMAR, from translocation individuals with major mental illness have been used for most of the marker analyses. Culture of peripheral lymphocytes and EBV cell lines was in RPMI 1640 medium with 10% FCS. Preexisting cell hybrids were grown in appropriate medium: CF37, carrying an X-11 translocation chromosome (Mohandas et al. 1980) was grown in HAT medium; CF52 has an 11-16 translocation chromosome as its sole human chromosome complement and was grown in medium selective

for APRT (Koeffler et al. 1981). CF37 and CF52 were the gift of Dr. T. Mohandas. The CMGT hybrids E67.1 and E67.4 have been described elsewhere (Porteous et al. 1986) and have been analyzed in detail for short-arm markers (Porteous et al. 1987). They were grown in RPMI 1640 with 10% FCS.

The thioguanine-resistant, HPRT$^-$ mouse cell lines used for fusion were Sp2/0-Ag4 (Shulman et al. 1978) and RAG (Klebe et al. 1970). Polyethylene glycol (PEG)–induced fusion, with MAFLI as the human parent, was carried out according to a method described elsewhere (van Heyningen, in press). The Sp2/0 hybrids, MIS series, grew in suspension and were subcloned by limiting dilution. The attached cell hybrids, MAR series, derived from RAG were subcloned by seeding about 100 cells into small Falcon flasks and transferring cell-surface marker–positive colonies into 2-ml wells 10–14 d after plating.

J1CI4 (a gift from Dr. Carol Jones; Jones et al. 1984) is a hamster cell hybrid carrying an intact chromosome 11 as its only human component. WG3H is an HPRT$^-$ hamster cell line used as the recipient in X-irradiation fusions (Westerveld et al. 1971). Both of these cells were routinely grown in RPMI 1640 with 10% FCS.

Cell-Surface Marker Selection and Analysis

Hybrid cell selection for the expression of cell-surface markers expected on each "half" of the translocation chromosome was carried out under sterile conditions by using magnetic beads. The monoclonal antibodies used were in the form of sterile culture supernatant produced in our laboratory from hybridoma cells which we had bought or received as gifts. The hybridomas to detect MIC10 (on chromosome 1), MIC11, MIC4 (=CD44), MDU1, and MIC9 were, respectively, TRA2.10 (Andrews et al. 1985), from Dr. Peter Andrews; 163A5 (Woodroofe et al. 1984), from Dr. Frank Walsh; F10.44.2 (Goodfellow et al. 1982), from Dr. John Fabre; 4F2, from ATCC (HB-22); and 4D12 (Jones et al. 1984), from Dr. Peter Goodfellow.

Dynabeads M450 (sheep anti-mouse immunoglobulin conjugated) were used. The stock suspension is 4×10^8 beads/ml (30 mg/ml). The most efficient separation is expected if all the available second antibody sites are saturated with specific monoclonal antibody. One-milligram (33 μl = 1.3×10^7) aliquots of beads were washed three times in sterile buffer (PBS and 1% BSA). They were then incubated with end-over-end rotation in 1 ml of each antibody in separate Eppendorf tubes for 10 min. The antibody was discarded after the beads were separated using the Dynabeads magnetic separator, and the coated beads were washed three times in buffer, to remove any unbound antibody.

A very small number (1–2 beads/cell) of coated beads were gently added to the cells suspended in PBS and incubated at room temperature with rotation of the samples, for 30 min. The beads, some with cells attached, were then separated magnetically and were washed three times in sterile serum-free medium and finally were gently resuspended in culture medium and were transferred to 2-ml wells.

For attached cells (the MAR hybrids) direct antigen-positive clone selection of attached cells is possible in a Falcon flask. The 30-min incubation of a 25-cm^2 flask with 10^4 coated beads/1 ml PBS is followed, without washing but with gentle rocking of the flask, by inspection, under the microscope, for bead-binding clones which can then be transferred into a 2-ml well by using separate sterile bacterial loops for each.

Cells with beads attached are handled as little as possible, to prevent mechanical damage. They continue to proliferate, and the beads are diluted out. Selection can be repeated and may be alternated with subcloning and quantitative fluorescence-activated cell sorter (FACS) analysis.

FACS analysis was carried out according to a method described elsewhere (Seawright et al. 1988; van Heyningen, in press), except that the cell sorter used was a Becton Dickinson FACSCAN. Anti-AFP monoclonal antibody was used as a negative control. The proportion of cell-surface marker–positive cells was estimated from the machine-derived plot of cell number against the log of fluorescence intensity.

Production of Cell-Surface Marker–selected X-Irradiation Hybrids

The chromosome 11–only hybrid J1CI4 (Jones et al. 1984) was used as the potential fragment donor, and the HPRT$^-$ Chinese hamster cell line WG3H was used as the rodent fusion partner. A total of 2×10^7 log-phase J1CI4 cells were harvested by gentle trypsinization and were washed and resuspended in 2 ml of fresh medium containing 10% FCS in a bijou bottle which fits our gamma-irradiating cobalt source. A total of 16,000 rads were delivered to these cells. The cells were then washed in serum-free medium and fused with 1.7×10^7 freshly harvested and washed WG3H cells, by using our routine PEG fusion protocol. The fusion products were plated into 8×75-cm^2 flasks. HAT selection was implemented 16 h later. Two weeks after fusion, MDU1-positive and MIC9-positive clones were sought in the flasks, initially by using Dynabeads coated

with both antibodies. The positive colonies were picked with bacterial loops, were grown up as clones in 25-cm^2 flasks, and were reselected with the two antibodies separately and sequentially. Clones were FACS analyzed for all four chromosome 11–encoded cell-surface markers, and samples were cryopreserved at every stage. After repeated subcloning we obtained four relatively stable independent hybrid clones, two carrying each cell-surface marker in the absence of the others: WJX3 and WJX7 are 100% MDU1 positive; and WJX5 and WJX11 are 100% MIC9 positive.

Mitotic Chromosome Preparation

The techniques used for both lymphoid cells and somatic cell hybrids were essentially as described elsewhere (Fletcher, in press). For chromosome analysis by in situ hybridization, cell synchronization with fluorodeoxyuridine is recommended. Mitotic cell preparations can be stored in methanol:acetic acid (3:1) for at least 6 mo at −20°C. Spreads on slides were made as required.

Chromosomal In Situ Hybridization

The markers used for this analysis were suggested by preliminary linkage and hybrid cell results on determining flanking markers. Probes were labeled with biotin by nick-translation using routine methods (described in detail in Gosden 1990).

Slides were prepared 3–4 d before the hybridization was carried out. The hybridization protocol was as described by Gosden (1990).

Detection was essentially as described by Pinkel et al. (1986), using (*a*) avidin-FITC (Vector Labs) as reporter and (*b*) linking layers of FITC with biotinylated anti-avidin. The slides were counterstained with propidium iodide and were mounted in Citifluor antifade:glycerol. They were examined on a Leitz Ortholux II microscope equipped with Ploemopak fluorescence filters, and suitable metaphase spreads were scanned and stored with the BioRad Lasersharp MRC 600 confocal laser scanning system attached to the same microscope.

The markers used to define the breakpoint were a lambda phage clone of tyrosinase and the next distal marker, cosmid CJ52.4. The latter defines the locus D11S388 (Julier et al. 1990).

Cytogenetic Analysis of X Irradiation–Fragment Hybrids

The human DNA content of these hybrids was analyzed in several different ways. Mitotic chromosome preparations from the subcloned stabilized hybrids were hybridized with biotinylated human Cot1 DNA (GIBCO BRL), and signal was developed with a single layer of avidin-FITC and was counterstained with propidium iodide in Citifluor by the method above. Hybridization conditions used were as described by Gosden and Hanratty (1991*b*).

The origin of the human fragments in these hybrids was also determined by chromosome painting. For this the hybrid cell DNA was subjected to interrepeat sequence (IRS)–PCR using several primers for the human Alu and L1 interspersed repeated sequences (table 1; Gosden and Hanratty 1991*a*; Dorin et al. 1992). The PCR products were labeled with biotin by nick-translation and were used as a probe for in situ hybridization back onto normal human mitotic spreads of relatively extended chromosomes. Considerable suppression, by adding 500 ng of unlabeled human Cot1 DNA to 50 ng labeled IRS-PCR product for each slide, was required to give chromosome-specific results. Hybridization and detection were essentially as described above for specific marker in situ analysis.

Marker Analysis

DNA was prepared, by standard procedures described elsewhere (van Heyningen, in press), from frozen cell pellets, for all of the cell lines. Most marker analysis was by PCR, and the products were analyzed on agarose (1.5% for fragments of 500 bp). DNA blot analysis was carried out for the chromosome 1 probes D1S8 and D1S81.

Designing and Setting Up PCR Assays for Chromosome 11 Markers

The aim was to set up PCR-based assays to quickly analyze somatic cell hybrids for the presence of human chromosome 11 markers on both mouse and hamster backgrounds. Except for a few short-arm markers, we concentrated on well-spaced long-arm markers. Where no published primer sequences were available, we designed our own, either from published gene sequences or, in the case of D11S388, D11S385, and D11S351, from data obtained by sequencing a fragment of the insert from cloned probes. In order to try to keep the PCR human-specific, primers were designed from untranslated regions or introns, wherever such information was available. Theoretical conditions were calculated for each primer pair and then were refined by experimentation. Final primer sequences and annealing temperatures are shown in table 1. For chromosome 1 markers—renin (REN) (Theune et al. 1991) and

Table 1

PCR Primers Used to Detect Genes in Somatic Cell Hybrids and for IRS-PCR for Painting Human DNA Content of Hybrid Cells back to Normal Karyotype

Repeat	Primer(s)	Annealing Temperature (°C)	Fragment Size (bp)	Reference (remarks)
HBB	5′ ATG GTG CAC CTG ACT CCT GAG G 3′ 5′ GCC ATC ACT AAA GGC ACC GAG C 3′	65	355	In-house design
D11S87	5′ CCC TGG AAA CAC TTT CTG CC 3′ 5′ GGC TGG GTT GGA GGC AAG G 3′	65	120	Lewis et al. 1988; in-house sequence and design
TYRL1	5′ ACA ATA TGT TTC TTA GTC TG 3′ 5′ TGG TAA CAC TAG ATT CAG C 3′	51	260	Giebel et al. 1991 (exon 4 oligos P1 and P2; *Msp*I to distinguish from TYR)
PGA	5′ GCA TCT CTG ACA CCA ATC AG 3′ 5′ TGG AGA AGA GAC AGA TGG AG 3′	54	188	W. Cookson, personal communication
MDU1	5′ TCT TCA AAG CCT CTG CAG TAC C 3′ 5′ CTC ATC TCC AAC CTG TCT AAC C 3′	57	859	Gottesdiener et al. 1988; in-house design (nt −1023 to −174)
FGF4	5′ GAT GAG TGC ACG TTC AAG GAG 3′ 5′ CAG AGA TGC TCC ACG CCA TAC 3′	62	542	Yoshida et al. 1987; in-house design (nt 4161 to 4703)
GST3	5′ GGG AGG GAT GAG AGT AGG ATG 3′ 5′ GGA GGT TCA CGT ACT CAG GGG 3′	60	450	Cowell et al. 1988; in-house design (exons 5–7)
TYR	5′ GCA AGT TTG GCT TTT GGG GA 3′ 5′ CTG CCA AGA GGA GAA GAA TG 3′	55	512	Giebel and Spritz 1990 (exon 1 oligos)
D11S388	5′ CAT CCA CGC TGT TGG TCT GC 3′ 5′ CAG TAA GAA CCA AAG AAA GGT TAC 3′	51	118 (variable)	Julier et al. 1990; in-house sequence and design
STMY1	5′ CAG TTT TCT CCT CTA CCA AGA C 3′ 5′ ACT GGC TTT ACT TAG CTC TAT G 3′	50	300	Sirum and Brinkerhoff 1989; in-house design (nt −300 to −4)
CLG	5′ AGT CAG TAC AGG AGC CGA ACA G 3′ 5′ GGA GAA AAG CTG TGC ATA CTG G 3′	59	517	Angel et al. 1987; in-house design (nt −434 to +83)
D11S385	5′ TTT TAT AGG GAC AGG ATC TTG C 3′ 5′ GGC TGT ATA ATC TTG TGT TCT C 3′	54	286	Julier et al. 1990; in-house sequence and design
NCAM	5′ TGG AAA TCT CTT CCA AAC ATC GGA G 3′ 5′ AAT TAG AAC TTT GGA GAG GGA TGG G 3′	55	179	Barton et al. 1990 (nt −848 to −663; designed by G. Gillett)
DRD2	5′ GAG GCC CTC TCA CTG ACA C 3′ 5′ AGT GCA GGG CCC TGC TGG A 3′	55	390	R. Todd, personal communication (exon 2)
D11S351	5′ CTT GGG TAG CTG GTA CTA CAG G 3′ 5′ AGG TCA CTA CAC ATC AAA ACA GC 3′	59	209	Julier et al. 1990; in-house sequence and design
THY1	5′ CAG AAG GTG ACC AGC CTA ACG 3′ 5′ CTG AGC ACT GTG ACG TTC TGG 3′	40	324	Cotter et al. 1989 (with slight modification)
D1S103	5′ ACG AAC ATT CTA CAA GTT AC 3′ 5′ TTT CAG AGA AAC TGA CAT GT 3′	55	>85 (variable)	Weber et al. 1990

(*continued*)

Table 1 (continued)

Repeat	Primer(s)	Annealing Temperature (°C)	Fragment Size (bp)	Reference (remarks)
5′ End Alu Consensus	5′ AAAGTGCTGGGATTACAGG 3′ 5′ GTGGCTCACGCCTGTAATCCC 3′	60	Various	Dorin et al. 1992
3′ End Alu Consensus	5′ GTGAGCCGAGATCGCGCCACTGCACT 3′ 5′ TGCACTCCAGCCTGGGCAACA 3′	60		
L1H	5′ CATGGCACATGTATACATATAGTAAC(A/T)AACC 3′	60		
HumanL1A	5′ CACAGGAAGGGGAACATCACA 3′	60		
HumanL1B	5′ GGGGAGGGATAGCATTAGGAG 3′	60		

D1S103 (Weber et al. 1990)—published PCR conditions were used.

A problem which had to be overcome in the tyrosinase analysis was the existence of the tyrosinase-like gene (TYRL1), which was described in detail while the present work was in progress (Giebel et al. 1991). This allowed us to resolve TYRL1 from TYR by using primers to TYR exon 4, which is common to both loci; but, after *Msp*I or *Hpa*II digestion of the PCR product, TYR gives rise to 120-bp and 140-bp fragments, while the TYRL1 products are 29 bp, 91 bp, and 149 bp (data not shown). For the detection of TYR alone, primers for exon 1 were used (table 1).

Results

Genetic Analysis

With the more distant but highly informative chromosome 11 breakpoint-flanking markers D11S97 and D11S35, two and three obligate recombinants, respectively, were found among the 10 translocation carriers. For the closer but less informative flanking markers TYR and D11S388, no recombinants were found, even when DNA was reanalyzed for the recently described TYR dinucleotide-repeat polymorphism (Morris et al. 1991). On chromosome 1 several recombinants are seen with the highly informative distal marker D1S8, but none was found with the proximal markers D1S103 or D1S81—both of which are highly polymorphic reference markers. These results were emerging as the cell hybrid analysis and the in situ hybridization were getting under way and gave us an idea of the markers to study, especially for the latter technique.

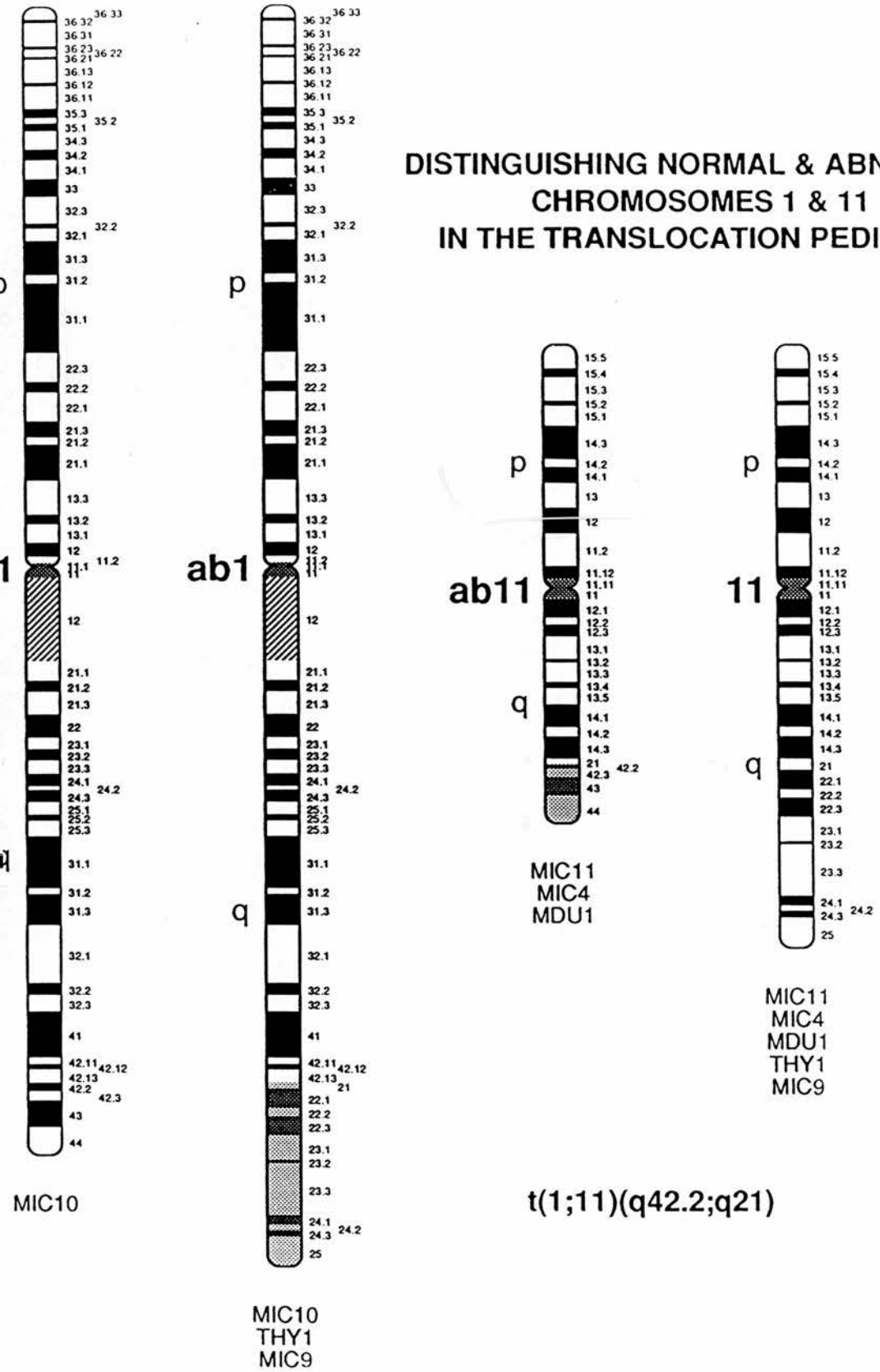
Marker Analysis of the Hybrid Cell Panel

The stabilized, repeatedly subcloned, cell-surface marker–selected translocation hybrids were analyzed mainly by PCR, once segregation of the two derived chromosomes from their normal homologues had been ascertained by cell-surface marker expression (fig. 1). The X irradiation–fragment hybrids were screened in the same way. Included in the panel for analysis, both by PCR and for cell-surface markers, were potentially useful preexisting translocation hybrids and earlier CMGT fragment hybrids. High-molecular-weight DNA was used for all analyses.

The PCR primers shown in table 1 were used to analyze the complete hybrid cell panel, including some earlier CMGT chromosome-fragment hybrids. The complete data set is shown in table 2, where the position of the translocation breakpoint is clearly defined by hybrids MIS7 (=MAR12) and MIS39 (=MAR1), and the marker analysis of the fragment hybrids gives an idea of what chromosome 11q regions are present for future marker isolation.

Molecular Cytogenetic Analysis of the X Irradiation–Fragment Hybrids

The human DNA content of the WJX hybrids was analyzed by in situ hybridization using human Cot1 DNA. Figure 2 shows the distribution of the chromosome 11 fragments in each hybrid. In three of four cases there appears to be a single block of human DNA, but in WJX7 three separate blocks are seen, although all three are located on the same recipient chromosome. The appearance of the single extended region in WJX5 is nonuniform.



=ure 1 Strategy for distinguishing hybrid cells carrying normal and derivative chromosomes 1 and 11, using cell-surface marker :tion.

Table 2

Marker Analysis of Translocation, X Irradiation, and CMGT Hybrids

	Status of Cell Line(s)[a]								
	MIS7 and MAR12	MIS39 and MAR1	CF37 and CF52	WJX3	WJX5	WJX7	WJX11	E67.1	E67.4
CHR 11 markers:									
HBB	−	+	ND	−	+	−	−	+	−
D11S87	−	+	ND	−	−	−	−	ND	ND
MIC11	−	+	−	−	−	−	−	+	+
CD44	−	+	−	−	−	−	−	−	+
TYRL1	−	+	−	+	+	−	−	−	+
PGA	−	+	−	+	−	+	−	+	−
MDU1	−	+	−	+	−	+	−	−	−
FGF4	−	+	−	+	−	−	−	−	−
GST3	−	+	−	+	−	−	−	+	+
TYR	−	+	+	−	−	+	+	−	+
D11S388	+	−	+	−	−	−	+	−	−
STMY1	+	−	+	−	+	+	+	−	+
CLG	+	−	+	−	+	+	+	−	+
D11S385	+	−	+	−	+	+	+	−	+
NCAM	+	−	ND	−	−	+	+	ND	ND
DRD2	+	−	ND	−	−	+	+	ND	ND
D11S351	+	−	+	−	+	−	+	−	−
THY1	+	−	+	−	+	−	+	ND	ND
MIC9	(+)	−	+	−	+	−	+	−	−
CHR 1 markers:									
MIC10	+	−	ND	ND	ND	ND	ND	ND	ND
REN	+	−	ND	ND	ND	ND	ND	ND	ND
D1S103	+	−	ND	ND	ND	ND	ND	ND	ND
D1S8	−	+	ND	ND	ND	ND	ND	ND	ND

[a] A plus sign (+) denotes presence of marker; and a minus sign (−) denotes absence of marker. ND = not determined.

Chromosome painting onto a normal human metaphase spread was also carried out to study directly the chromosome 11 regions from which the human fragments were derived. Figure 3 reveals an apparently single contiguous region of chromosome 11q in WJX3, WJX7, and WJX11. Three distinct regions, two on the short arm and one on the long arm, are seen only in WJX5, as expected on the basis of marker analysis (table 2). Marker analysis would suggest the presence of discontinuity in WJX7 too. Cot1 analysis of the hybrid showed three separate blocks of human DNA in WJX7, and the results in table 2 reveal two gaps—first, in the HSTF1,GST3 region and, second, around D11S388. This suggests that two small interstitial deletions have interrupted contiguity, and the three separate blocks of human DNA seen in the hybrid cell line may represent the separate insertion of each contiguous segment. However, as the gaps are small, the human DNA content of WJX7 appears as a single block when painted back onto normal human chromosomes.

In Situ Hybridization to Determine the Site of the Translocation Breakpoint

Figure 4 shows the results with the flanking markers on mitotic chromosomes prepared from peripheral leukocytes of translocation carrier and affected patient ROMAR. Figure 4*a* shows the breakpoint-distal marker D11S388 hybridizing to the large derived chromosome 1 and to the normal chromosome 11 homologue, and figure 4*b* shows the TYR signal on the normal chromosome 11 and on the visibly smaller, derived chromosome 11.

Discussion

Schizophrenia and related major mental illness are very common in the general population (Gottesman

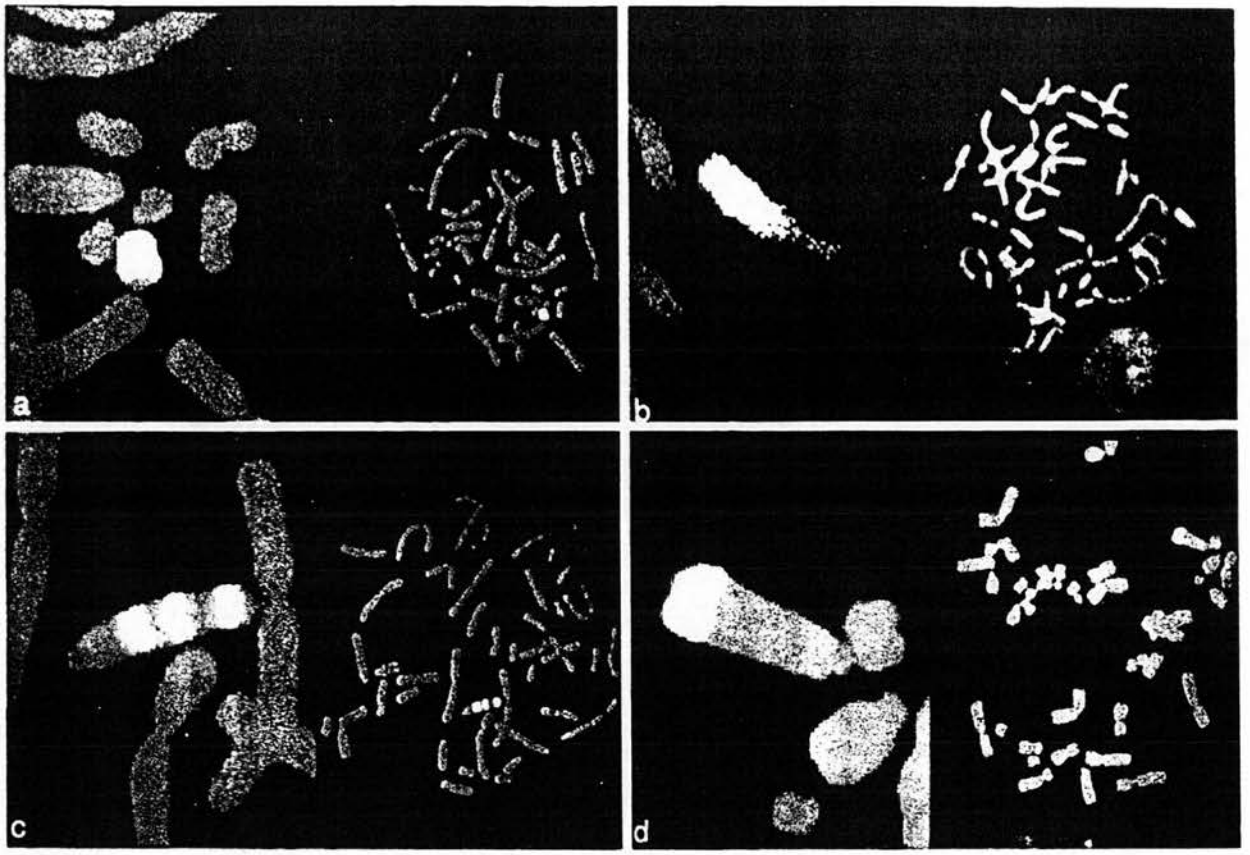


Figure 2 Human DNA content of the WJX hybrids, revealed by in situ hybridization with Cot1 fraction of human DNA. *a*, WJX3. *b*, WJX5. *c*, WJX7. *d*, WJX11. For each cell line a representative mitotic spread is shown on the right, with an enlarged view of the human fragment-bearing chromosome in the left panel.

and Shields 1982). The etiology of these diseases is not understood but is almost certainly heterogeneous. Social, environmental, and genetic components interact to cause disease. Twin studies point to a major genetic contribution (Gottesman and Shields 1982), which is, however, likely to be due to several different interacting genes. Any insight into the physiological/biochemical abnormalities which are involved in the onset of disease may give a lead in designing improved therapy and, perhaps, in devising preventive measures. Understanding the underlying pathology in one form of the disease may lead to other candidate genes or to environmentally alterable targets.

With this philosophy in mind, we set out to identify, as a pointer to one candidate genetic component, the translocation breakpoint which cosegregates closely with schizophrenic illness in this large kindred. We have succeeded in showing that the breakpoint lies between two adjacent genetically linked markers, TYR and D11S388, initially placed 4 cM apart (C. Julier, unpublished results) but most recently estimated to be about 7.8 cM apart at 11q14-q21 (NIH/CEPH Collaborative Mapping Group 1992). The DNA distance between two such markers is impossible to predict, since the relationship between genetic and physical distance is not constant, so that in part of the nearby 11q13 MEN1 region 1 cM corresponds to only 300 kb (Janson et al. 1991). The next step is to identify the exact site of the breakpoint, with the aid of further markers in the TYR-D11S388 interval. With this in mind we segregated the reciprocal translocation chromosomes into stable mouse-human hybrids, for quick assignment of any newly isolated markers. We have also produced the four WJX series of X-irradiation hybrids carrying 11q fragments selected with cell-surface markers known to map above and below the 11q21 breakpoint. Of these, WJX11 in particular reveals a contiguous block of markers which appear to cross the transloca-

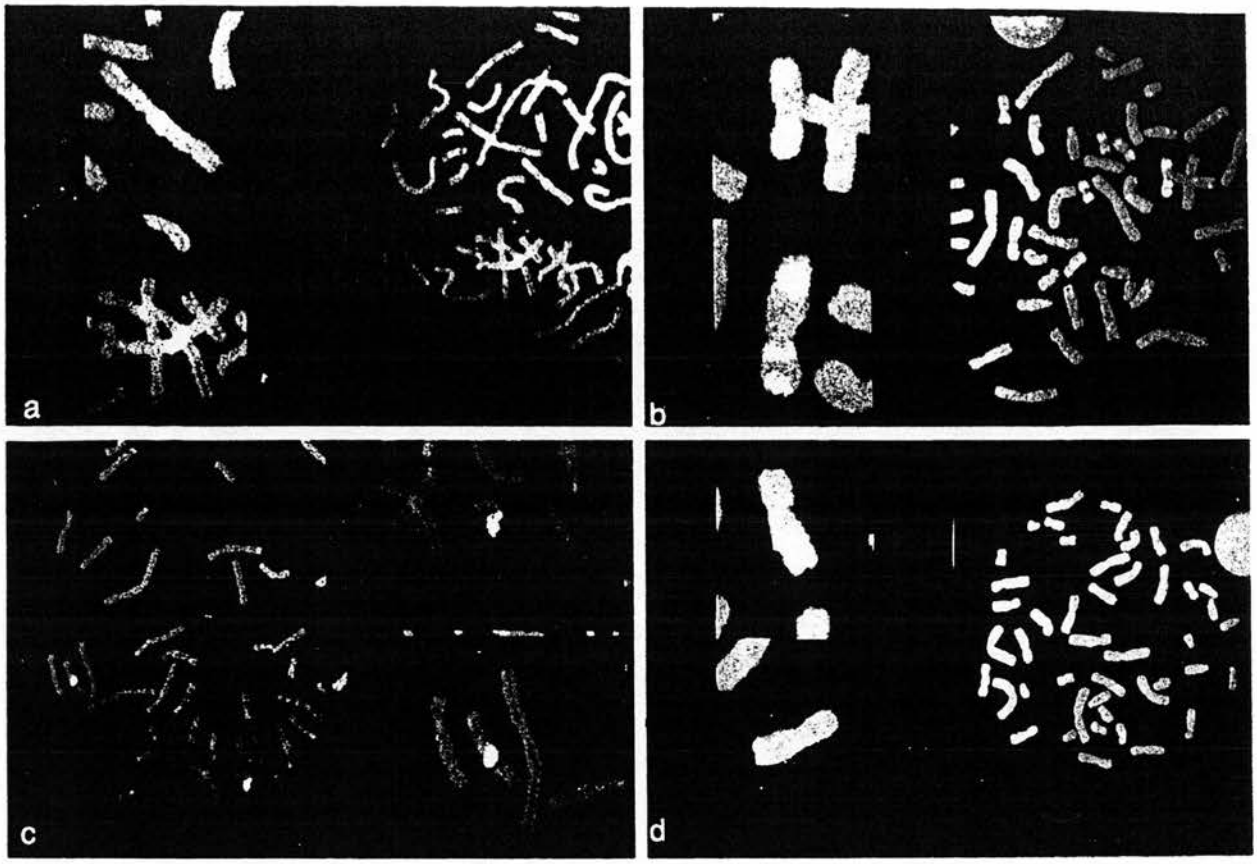


Figure 3 Human DNA from each WJX hybrid, isolated by Alu-PCR and painted back onto normal human mitotic spreads. *a*, WJX3, showing a subcentromeric block of DNA on 11q. *b*, WJX5, revealing the presence of three distinct blocks, from 11pter, 11p11-p12, and 11q23-q24 (the degree of Cot1 suppression was not quite sufficient in this case, showing hybridization to some other chromosomes). *c*, WJX7, showing a single relatively centromere-proximal block on 11q. *d*, WJX11, with a more distal 11q block of DNA. Each chromosome 11 homologue is shown enlarged separately in a small panel.

tion breakpoint. We cannot be certain of the integrity of the DNA without PFGE analysis, since, for example, in CMGT hybrids (of which E67.1 and E67.4 are representative), we almost invariably find the human DNA components to be rearranged when looked at by long-range techniques (Bickmore et al. 1989). Alu-PCR painting similarly reveals discontinuity in some chromosome-fragment hybrids (Dorin et al. 1992). Nevertheless, such hybrids provide an excellent source of new markers for the chromosome regions indicated to be present by the preexisting markers (Porteous et al. 1987; Bickmore et al. 1989). However, to narrow marker isolation more closely to the region of interest, we propose to try coincidence sequence cloning (Brookes and Porteous 1991)—for example, between MIS39 (=MAR1) and WJX11, which carry overlapping human fragments on a mouse cell background and hamster cell background, respectively. Using this technique should allow us to isolate new markers solely for the human region which the two cells have in common, which is around the translocation breakpoint. Other approaches in progress that are seeking to produce markers for the region of interest include (*a*) the production of microdissection libraries and (*b*) the isolation of TYR and D11S388 yeast artificial chromosome (YAC) clones, followed by YAC walking between these clones. New markers produced are quickly mapped onto the translocation hybrids MIS7, MIS39, and CF37. Markers present in both MIS39 and CF37 or WJX11 are automatically in the region of interest. Candidate YAC clones for crossing the breakpoint can be identified by in situ hybridization (Breen et al. 1992) onto the translocation cell line MAFLL.

In the process of producing and analyzing the so-

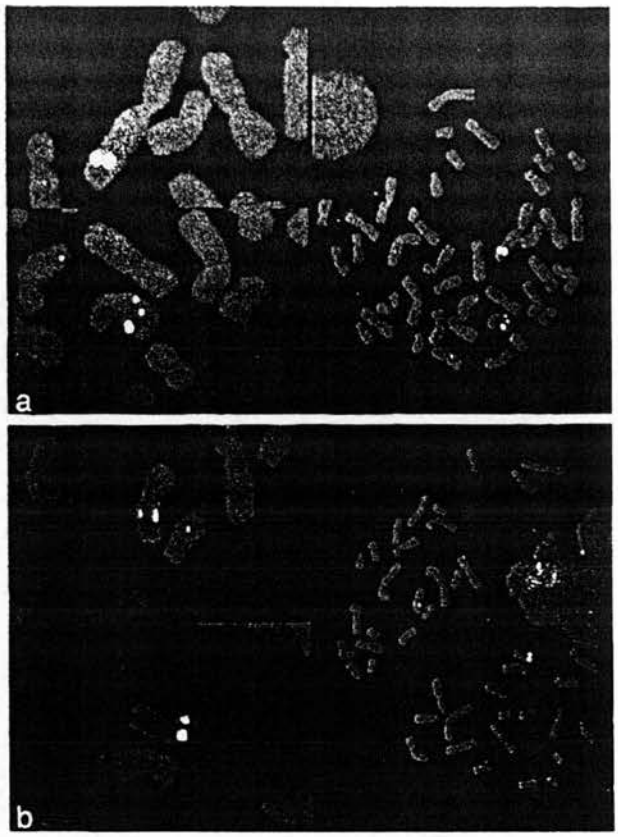


Figure 4 In situ hybridization of the two adjacent flanking markers on mitotic spreads from an affected translocation carrier. *a*, D11S388, which hybridizes to the derived 1 (upper left) and to the normal 11 homologue (lower left). *b*, TYR signals, seen on the normal 11 homologue (upper left) and on the truncated-looking derived 11 (lower left).

matic cell hybrids described, we have contributed to the mapping effort on chromosome 11q. We have devised human-specific PCR assays for a number of gene-specific markers and have developed new sequence-tagged sites for the D11S351, D11S385, and D11S388 (Julier et al. 1990) loci. The localization of the FACS-selectable cell-surface marker MDU1 (Seawright et al. 1988), which is also the cloned gene 4F2 (Gottesdiener et al. 1988), has been refined to the region proximal to TYR but deleted in J1-44 on 11q (Jones et al. 1984; Tanigami et al. 1992). It is interesting to note that, although there is considerable fragmentation of the retained chromosome fragments in WJX5 and WJX7, markers known to be closely linked, such as the CLG/STMY1/D11S385 complex and NCAM/DRD2 (McConville et al. 1990), are not separated. These results suggest that such fragment hybrids will be useful for short-range mapping.

Linkage studies of chromosome 11q markers with schizophrenia have recently been carried out (Muir et al. 1991). Interest in a possible disease association with this chromosomal region has been influenced by (1) the finding of three 11q21-q23 translocations (Smith et al. 1989; Holland and Gosden 1990; St. Clair et al. 1990), (2) the existence of several published cases of schizophrenia in albino patients, implicating the tyrosinase region (Baron 1976; Clarke and Buckley 1989), and (3) the presence of possible candidate loci, such as the dopamine D_2 receptor (Grandy et al. 1989; Seeman et al. 1989). Although the results of these studies generally have been negative, it is not possible to exclude the involvement of a major locus SCZD2 in this region, in as many as 20% of cases, making the isolation of genes from the breakpoint region an important approach for identifying specific schizophrenia-predisposition genes which may be implicated in only a proportion of cases.

Acknowledgments

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nucleotide repeat morphism (D9S202) in the reich's ataxia region on mosome 9q13-q21.1

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Description: The gene mutated in Friedreich's ataxia has been localized on chromosome 9q13-q21.1 within m the polymorphic DNA segments D9S15 and D9S5. ıtly determined gene order is D9S15-D9S5-FRDA (1,2). ined YAC clones extending to 330 kb beyond D9S5 he candidate FRDA region, and we isolated DNA s containing GT repeats from these YACs by polymerase ction (PCR). A $(TG)_{12}TA(TG)_4A(GT)_5$ repeat (MLS1) ıer localized near a CpG island 200 kb from D9S5 by on YAC-derived cosmids.

equences:
(CA strand): 5'-CTTGTCTGTGATCGTGGTCACCTGTACC-3'
(GT strand): 5'-AACATCTTGCATAATTATAGAATAA-3'

y: Estimated from 88 chromosomes of unrelated n individuals:

bp	Frequency
203	0.03
201	0.39
199	0.58

l heterozygosity = 0.50.

omal Localization: The D9S15/D9S5/FRDA region is on chromosome 9q13−q21.1.

in Inheritance: Co-dominant inheritance was ated in 30 informative families.

ditions/Comments: PCR was performed on 100−200 iomic DNA in a 20 μl volume with 10 pmoles of ^{32}P ed MLS1 F primer, 10 picomoles cold MLS1 R primer, Mg^{2+}, and consisted of 25 cycles of denaturation at 1 minute, annealing at 57°C for 1 minute, and extension or 1 minute. Amplification products were separated on crylamide-urea gels, and detected by autoradiography. ely, PCR was performed for 35 cycles with cold and detection was obtained by chemiluminescence after ıe amplification products from the polyacrylamide-urea a nylon membrane, which was then hybridized with a led amplification product (manuscript in preparation).

dgement: The support of a Telethon Italy grant to M.P. lly acknowledged.

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Dinucleotide repeat polymorphism at the human olfactory marker protein (OMP) locus on chromosome 11q13.5 near tyrosinase (TYR)

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Source/Description: The human OMP gene was isolated and subcloned into an O2/B cosmid (1,2). A 717 bp Sau3A I fragment was selected by hybridization to poly(dC-dA) poly(dG-dT) and subcloned into the BamHI site of pBS(+/−) phagemid. Sequencing revealed a $(CA)_5GA(CA)_{15}$ DNR.

Primer Sequences:
CTG AAA GGT AAA ACA ATA ATG C (22-mer) CA strand
TGT CCT CCT ACC ACT AAT GC (20-mer) GT strand

Polymorphism: Oligonucleotide primers were constructed from OMP sequences flanking the repeat region. These primers were used to amplify a 254 bp fragment. Four alleles were observed in genotypes of 54 unrelated persons. Heterozygosity=0.62. Genotypes of CEPH individuals K-1331 GMO6990 and K-1331 GMO7057 are A2,A3 and A3,A3, respectively.

Allele frequencies:
A1 (254 bp) = 0.12; A2 (250 bp) = 0.45; A3 (248 bp) = 0.41; A4 (244 bp) = 0.02

Chromosomal Localization: Human OMP has been localized to 11q13.5 (immediately centromeric to TYR) by somatic cell hybrid panel and fluorescent in situ hybridization (2). Linkage analysis places OMP near D11S527 (θ=.001, Zmax=9.414).

Mendelian Inheritance: Co-dominant segregation was observed in 17 multigenerational informative families.

PCR Conditions: PCRs were done in 25 μl with 20 ng genomic DNA, 1.5 mM $MgCl_2$, 10 mM Tris−Cl pH 8.3, 50 mM KCl, 0.01% gelatin (Promega 10× Taq polymerase buffer), 200 μM each of dCTP,dGTP, dTTP, and 2.5 μM dATP, 1 μCi α P^{32} dATP (NEN Du Pont, 800 Ci/mmol, 10 μCi/μl), 10 ng each oligonucleotide primer, 1 unit of Taq DNA polymerase (Promega). PCR conditions were set at denaturation at 94°C for 1 min., annealing at 55°C for 1 min., and extension at 72°C for 2 min. for 29 cycles. A final cycle ended with 10 min. of extension at 72°C. Fragments were separated on 6% polyacrylamide denaturing gels. Dideoxy sequencing ladders of M13mp18 DNA were used as fragment size standards.

Other Comments: The human OMP sequence has been assigned EMBL accession number Z18943.

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