The Genetic Basis of Human Craniosynostosis syndromes.

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SUMMARY

Craniosynostosis is the premature fusion of one or more sutures of the skull. This can result in the distortion of the shape of the head and face, and affects an estimated 0.25 to 1.6 children per 1,000 live births. The aims of this project were to identify genes involved in craniosynostosis and to characterise mutations of these genes.

To this end, linkage mapping was undertaken in a craniosynostosis pedigree and a gene was localised to chromosomal region 4p16, distal to the marker D4S394. This interval contained two plausible candidate genes, *MSX1* and *FGFR3*. The localisation to 4p16 was the first evidence for the presence of a craniosynostosis syndrome in this region of the genome.

These candidate genes were screened for mutations using single strand confirmation analysis, heteroduplex analysis and sequencing. Three polymorphisms were identified in *MSX1*; however, no mutations were detected. Two polymorphisms were identified in *FGFR3* before a candidate mutation in *FGFR3* was found in a number of craniosynostosis patients, by researchers in North America. This mutation, C749G in *FGFR3* (Pro250Arg) was then demonstrated to segregate with the affected members of the craniosynostosis pedigree localised to chromosomal region 4p16. The mutation was found in a further large pedigree in which autosomal deafness was the major feature, suggesting that the Pro250Arg mutation may account for a proportion of autosomal dominant deafness in the population. The Pro250Arg mutation of FGFR3 was found in five additional unrelated patients and is now recognised as a relatively common recurrent mutation among patients presenting to craniofacial clinics.

Determination of the molecular defect responsible for this craniosynostosis syndrome, led to an investigation of the effect of the mutation on the protein and how it results in craniosynostosis. Antisera to part of the extracellular region of the FGFR3 protein were used in flow cytometry experiments on skin fibroblast cells from an affected member of the craniosynostosis pedigree and from normal controls. The hypothesis was that the receptors

from affected and unaffected cells would be distinguishable in this way, to allow further investigation. However, it was not possible to reliably detect a difference.

In parallel to the FGFR3 craniosynostosis study, patients with a clinical diagnosis consistent with FGFR2 craniosynostosis syndromes were examined for molecular defects. *FGFR2* mutations were found in 12 unrelated Apert patients, 8 unrelated Crouzon patients, 3 unrelated Pfeiffer patients and 6 unrelated patients with uncertain diagnoses. Three of the mutations found were novel; T875A, T797C and G(-1)C (a splice site mutation). Characterisation of *FGFR2* mutations will identify which regions of the gene are functionally important and will form the basis for the study of the genotype-phenotype relations in craniosynostosis disorders.

The genes causing Apert, Crouzon, Jackson Weiss, Pfeiffer and Saethre Chotzen syndromes were identified, by others, while this project was proceeding. Work carried out by the candidate and others during the course of this project elucidated the genetic basis for Craniosynostosis Adelaide Type (FGFR3 craniosynostosis). In addition, the candidate characterised FGFR2 craniosynostosis mutations to further elucidate the molecular genetic basis for that group of disorders.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Throughout the thesis, the term 'the candidate' refers to Georgina Hollway.

GEORGINA HOLLWAY

30.4.98

LIST OF PUBLICATIONS

Some of the work presented in this thesis has been published, or accepted for publication and is in press. Reprints or final manuscripts as accepted for publication of these papers are included as appendices to this thesis.

Hollway, G.E. , Phillips, H.A., Ades, L.C., Haan, E.A. and Mulley, J.C. (1995) Localisation of craniosynostosis Adelaide type to 4p16. *Human Molecular Genetics* **4**, 681-683.

Hollway, G.E., Suthers, G.K., Haan, E.A., Thompson, E., David, D.J., Gecz, J. and Mulley, J.C. (1997) Mutation detection in *FGFR2* craniosynostosis syndromes. *Human Genetics* **99**, 251-255.

Muenke, M., Gripp, K.W., McDonald-McGinn, D.M., Gaudenz, K., Whitaker, L.A., Bartlett, S.P., Markowitz, R.I., Robin, N.H., Nwokoro, N., Mulvihill, J.J., Losken, H.W., Mulliken, J.B., Guttmacher, A.E., Wilroy, R.S., Clarke, L.A., Hollway, G., Adès, L.C., Haan, E.A., Mulley, J.C., Cohen Jr, M.M., Bellus, G.A., Francomano, C.A., Moloney, D.M., Wall, S.A., Wilkie, A.O.M. and Zackai, E.H. (1997) A unique point mutation in the Fibroblast Growth Factor Receptor 3 gene (*FGFR3*) defines a new craniosynostosis syndrome. *American Journal of Human Genetics* **60**, 555-564.

Hollway, G.E., Suthers, G.K., Battese, K.M., Turner, A.M., David, D..J. and Mulley, J.C. (1998) Deafness due to Pro250Arg mutation of *FGFR3*. *The Lancet* **351**, 877-878.

Hollway, G.E. and Mulley, J.C. Polymorphic Variants within the Homeobox Gene *MSX1*. Clinical Genetics - accepted for publication.

ABBREVIATIONS

A - adenine

[α ^{32}P]dCTP - alpha-labelled deoxyadenosine triphosphate

AC - dinucleotide repeat microsatellite

ACH - achondroplasia

bp, Kb - base pairs, kilobase pairs

C - cytosine

CAT - Craniosynostosis Adelaide type

Ci, µCi - Curies, micro Curies

cM - centimorgan (the genetic distance within which there is expected to be one crossover every 100 meioses)

CEPH - Centre d'Etude du Polymorphisme Humain (Centre for the Study of Human Polymorphism)

CHRI - Child Health Research Institute, WCH, North Adelaide, South Australia

CVS - chorionic villus sample

EDTA - ethylenediaminetetra-acetic acid

FGF - fibroblast growth factor

FGFR - fibroblast growth factor receptor

G - guanine

g, mg, ug, ng, pg - gram, milligram, microgram, nanogram, picogram

HA - heteroduplex analysis

IPTG - isopropylthioβ-galactoside

JW - Jackson Weiss

JWS - Jackson Weiss syndrome

l, ml, µl - litre, millilitre, microlitre

LCL - lymphoblastoid cell line (derived from a eukaryotic cell)

lod score - the decimal logarithm of the likelihood ratio of linkage versus no linkage

M, mM - moles per litre, millimoles per litre

MDE - mutation detection enhancement

$MgCl_2$ - magnesium chloride

NaAc - sodium acetate

$NaNO_3$ - sodium nitrate

NIH - National Institutes of Health

OD260 - optical density at 260 nanometres

PCR - polymerase chain reaction

PE-SA - phycoerythrin conjugated to streptavidin

pH - negative logarithm of the hydrogen ion concentration (in moles per litre)

RFLP - restriction fragment polymorphism

rpm - revolutions per minute

RT - reverse transcriptase

RT PCR - reverse transcription polymerase chain reaction

SCS - Saethre Chotzen syndrome

SDS - sodium dodecyl sulphate

SPSS sequencing - solid phase single strand sequencing

SSCA (SSCP) - single stranded conformation analysis (polymorphism)

T - thymidine

TD - thanatophoric dysplasia

TEMED - tetramethylethylenediamine

Tris - tris[hydroxymethyl]amino methane

VNTR - variable number of tandem repeats (also known as minisatellite)

WCH - Women's and Children's Hospital, North Adelaide, South Australia

X-gal - 5-bromo-4-chloro-3-indoyl-β-galactoside

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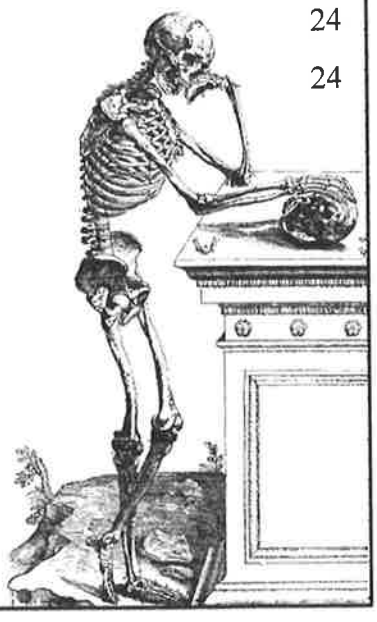
CHAPTER 1: INTRODUCTION



Preamble

This thesis describes an investigation into the molecular genetic basis of craniosynostosis. During the course of this project great progress has been made, through work done both by the candidate and other investigators, working in the field of craniosynostosis genetics.

1. CRANIOSYNOSTOSIS

1.1 Sutures and Craniosynostosis

Disruption of the normal development of the cephalic region in humans can lead to a variety of malformations. These malformations can include disruptions to cellular proliferation, cellular degeneration and cellular differentiation. Three main groups of malformations can be distinguished, cerebrocranial dysplasias (malformations of the brain and cranium), cerebro-craniofacial dysplasias (facial defects involving the brain and/or eyes and cranium) and craniofacial dysplasias (defects of the face and cranium) (Vermeij-Kerrs, 1990). In each case the malformations can be associated with abnormalities in other regions of the body. Craniosynostosis disorders are included in the third category, the craniofacial dysplasias. Craniosynostosis is the premature bridging or fusion (synostosis) of one or more sutures of the skull. Sutures are one of the three types of immovable joints (synarthroses) of the human skeletal system. Sutural joints are found only in the craniofacial complex. The cranium has five major sutures, three are paired (coronal, lambdoidal and squamosal) and two are single (sagittal and metopic) (Fig 1.1). There are many sutures on the face, those most commonly involved in premature fusion are sphenofrontal, frontoethmoidal and frontonasal sutures (Cohen, 1986b). Premature fusion of the facial sutures is much rarer than that of the cranial sutures (Cohen, 1993a).

Cranial bones originate as a series of ossification centres within the fibrous covering of the brain. The brain expands rapidly during early fetal life, and the borders of these bones are widely separated. As the brain growth slows down, the edges of the bones become closer, and the development of sutures occurs. During normal growth and development of the skull and face, the sutures are the sites of bone deposition and resorption thus allowing adjustments in size, shape and spatial arrangement of the bones. As the brain expands the

intracranial pressure on the skull bones causes separation and widening of the sutures, which causes the deposition (of osteoid) and bone formation. Cranial growth occurs perpendicular to each of the major sutures. Changes in the displacement and curvature of the skull bones during development are necessary to accomodate the expanding brain, thus normally during growth periods the sutures develop but remain patent (Cohen, 1993a). The cranial sutures normally fuse (become obliterated by bone) relatively early in adult life, in the second to third decade, following the completion of brain growth. The facial sutures, with the exception of the midpalatal suture, do not normally fuse before the seventh to eighth decades of life (Kokich, 1976).

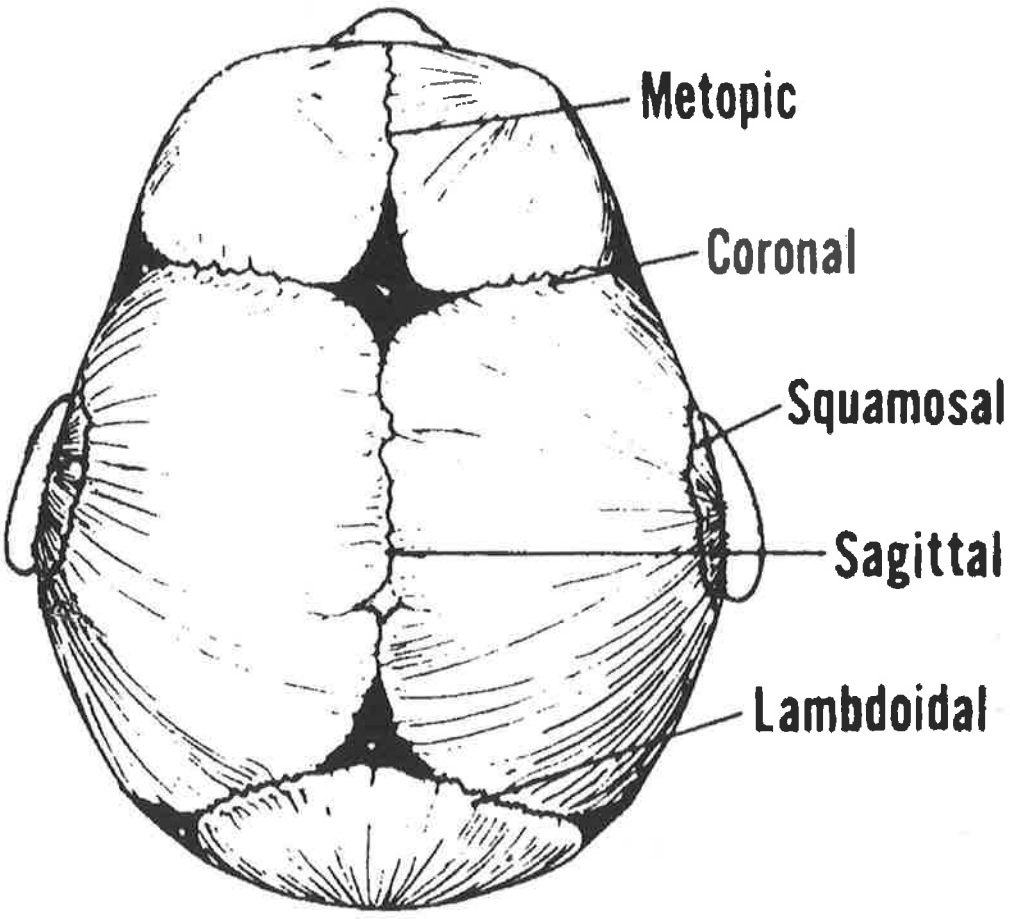


Fig 1.1: Superior view of normal infant skull showing the five major sutures. Modified from Cohen, M. 1986.

Sutures start as straight edges of bone united by a thin layer of fibrous tissue (Kokich, 1976), then interdigitations gradually develop and increase in size as fusion slowly occurs (Cohen, 1993a). The cause of suture closure is still unclear but it has been attributed to many different factors at different times, including vascular, hormonal, genetic, mechanical, local and biochemical factors (Cohen, 1993a). It is possible that there is more than one mechanism that leads to suture fusion. Cessation of growth could be involved in triggering suture closure, but there can be considerable delay between the cessation of growth and the start of suture closure. Premature fusion is known to start at any one point on a suture and move along (Cohen, 1993a).

If one or more sutures fuse prematurely, the normal displacement between the bones involved is prevented and normal growth patterns are inhibited. This causes compensatory expansion elsewhere, as the brain increases in size, and the abnormal growth pattern results in cranial and facial morphology changes (Kokich, 1986). Secondary consequences of altered head and face shape can include neurologic and ophthalmologic abnormalities. The extent and nature of head shape alterations depend on which sutures fuse prematurely, the order in which they fuse and when in development this occurs. The earlier a suture prematurely fuses, the greater the subsequent effect on craniofacial growth and development (Cohen, 1986b). Craniosynostosis can occur prenatally or postnatally (during infancy or childhood).

Simple craniosynostosis is the premature fusion of one suture, whereas in cases of compound craniosynostosis, two or more sutures are fused prematurely (Cohen, 1986a). Most cases of craniosynostosis are primary in that there is a defect which directly causes premature suture fusion. There are however some cases where craniosynostosis occurs secondarily to a known disorder, for example, in a thalassemia patient thickening of the skull leading to sutural obliteration can occur (Cohen, 1986a). Secondary craniosynostosis may be observed in cases of central nervous system malformations, metabolic disorders, hematologic disorders and others. Craniosynostoses can be divided into isolated and syndromic cases. A patient with isolated craniosynostosis has no other abnormalities except

those which occur as a result of the premature suture synostosis (such as abnormalities of the eyes and brain). Syndromic craniosynostosis is accompanied by other primary defects such as abnormalites of the limbs, ears and cardivascular system (Cohen, 1986a). The incidence of congenital heart disease among patients with coronal and/or sagittal synostosis is considerably higher than in the general population (Hunter and Rudd, 1976; 1977). Diagnostically, an unusual head shape, combined with a lack of appreciable movement at the sutures and palpable perisutural ridging points to craniosynostosis in the patient being examined (Cohen, 1986b), though physical signs are not always pronounced.

1.2 Pathogenesis of Craniosynostosis

The actual mechanisms leading to craniosynostosis are thought to be heterogeneous, for example a defect in the mesenchymal blastema or lack of growth stretch across the sutures. In some cases craniosynostosis is thought to be normal suture closure commencing too early, in other cases true sutures fail to form *ab initio*. Cohen (1993a) proposed two general pathogenetic types of craniosynostosis : primary (non physiologic) and secondary (physiologic). The primary form may result from sutural agenesis, that is, failure of suture formation *ab initio* or from compression/constraint causing the disruption of early sutural or presutural physiology. The secondary form may be metabolic, malformational or caused by some unknown triggering factor.

1.3 Etiology of Craniosynostosis

In addition to pathogenetic heterogeneity, craniosynostosis is known to be etiologically heterogeneous (Cohen, 1993a). Craniosynostosis can be caused by different factors including genetic factors (including monogenic conditions and chromosomal syndromes), metabolic disorders (such as hyperthyroidism), mucopolysaccharidoses (such as Hurler syndrome), prenatal exposure to certain teratogens (such as methotrexate, diphenylhydantoin and retinoic acid), hematologic disorders (such as sickle cell anemia) and malformations (such as microcephaly) (Cohen, 1993a). Both autosomal dominant and autosomal recessive inheritance of simple craniosynostosis has been identified, with autosomal dominant being much more common. However most cases of simple, isolated

craniosynostosis are sporadic, not familial (Hunter and Rudd 1976; 1977), in fact only 8% of coronal synostosis patients and 2% of sagittal synostosis patients represent familial cases. In simple craniosynostosis, the sagittal suture is the one most commonly involved (Cohen, 1986a). Of the craniosynostosis syndromes of known genesis, most are monogenic, with autosomal dominant, autosomal recessive and X-linked forms having been identified (Cohen, 1986c). There are also a number of autosomal and sex chromosomal syndromes with craniosynostosis as a common feature. They are mostly partial deletions (partial monosomies) or partial duplications (partial trisomies). It has also been hypothesised that human head constraint *in utero* could cause craniosynostosis in some cases (Graham *et al.* 1979) though this is difficult to test. Hunter and Rudd (1976, 1977) found a significant excess of males among cases of sagittal synostosis. They also found slightly more males with coronal synostosis than females.

Bradley *et al.* (1995) reported a study investigating parental occupations as risk factors for craniosynostosis in offspring. They found no strong associations for maternal occupation, and moderately increased risks for paternal occupations in two broad groups, agriculture and forestry, and mechanics and repairmen. They considered this may have been due to exposure to certain toxins in the work place.

Estimates of the frequency of craniosynostosis range from 0.25/1000 to 1.6/1000 with 0.4/1000 considered as probably the most accurate (Cohen, 1986b). Cases of craniosynostosis, both isolated and syndromic, have been observed in a wide range of racial groups (Cohen, 1986b).

The relationship between the premature fusion of sutures and the deformation of the cranium and cranial base has intrigued many people. In 1852 Vichow proposed that calvarial suture synostosis was a primary malformation affecting calvarial growth, and that this secondarily caused deformities of the cranial base (Cohen, 1993a). Moss (1959 and 1975 papers discussed in Cohen, 1993a) alternatively proposed that changes in the cranial base were the primary abnormalities and that they resulted secondarily in synostosis of the

overlying calvarial sutures. Since these two proposals there has been much debate as to which is correct. The relationship between the cranium and the cranial base is complex, in some cases of craniosynostosis it appears that the premature fusion of sutures is the primary abnormalities, in other cases the cranial base changes appear to be the primary abnormalities.

Mental deficiency should not be used as a diagnostic tool. The risk of mental deficiency is almost always present in cases of craniosynostosis. Also, mental retardation can be a secondary consequence of craniosynostosis or it can be due to some other primary cause such as a central nervous system defect. In one study Hunter and Rudd (1976) found 8.9% of sagittal synostosis patients were mentally retarded, but in almost half of these cases it was clearly unrelated to the synostosis. In syndromic craniosynostosis, cases of mental retardation occur in few Pfeiffer patients, some Crouzon patients, a higher proportion of Apert patients (than Crouzon) and it occurs in most Carpenter syndrome patients.

1.4 Classification of Craniosynostoses

There have been a number of attempts at classifying the craniosynostoses, the first of which appeared in 1851 by Virchow (Cohen, 1986a). Since then there have been many attempts including those by Grieg (1926), and Tessier (1981) (Cohen, 1986a). Different classification systems are useful in different applications, for example from a surgical point of view, a thorough clinical description and specification of which sutures are involved, is important. Whereas genetically, the overall pattern of anomalies and affection status of individuals is more useful, since patients with the same genetic condition may have different sutures fused (Cohen, 1986a).

1.5 Craniosynostosis Syndromes

One subgroup of craniosynostosis syndromes is the acrocephalosyndactyly (ACS) group. Disorders in this group are charaterised by the occurrence of digital anomalies in association with craniosynostosis. There is considerable phenotypic overlap between the different ACS syndromes which complicates the diagnosis of new cases (Escobar and Bixler, 1977). The symptoms in a new family could represent a new syndrome or a variation of a syndrome

previously described. Gene identification and gene localisation is beginning to clarify the situation and will eventually lead to a genetic classification system for these disorders. The autosomal dominant ACS syndromes are Apert syndrome (ACSI) (Blank, 1960), Saethre-Chotzen syndrome (ACSIII) (Pantke *et al.*, 1975), Pfeiffer syndrome (ACSV) (Pfeiffer, 1969), Jackson Weiss syndrome (Jackson *et al.*, 1976), Craniosynostosis Philadelphia type (Robin *et al.*, 1996) and possibly Robinow-Sorauf type ACS (Robinow and Sorauf, 1975). There can be considerable phenotypic overlap between and within families with these disorders, for example Cohen (1986d) believes that Robinow-Sorauf syndrome and Saethre-Chotzen syndrome are one and the same. Apert syndrome patients have craniosynostosis (causing protruding eyeballs and increased distance between the eyes) and severe symmetrical syndactyly of the hands and feet (Blank, 1960). Saethre Chotzen syndrome patients have craniosynostosis, ptosis of the eyelids, tear duct anomalies, prominent ear crura, low frontal hair line, facial asymmetry and cutaneous syndactyly of the hands and feet (Pantke *et al.*, 1975; Cohen 1975). In addition to craniosynostosis, Pfeiffer syndrome patients generally have broad thumbs and great toes and variable cutaneous syndactyly of the hands and feet (Peiffer, 1969; Cohen, 1975). The features of Jackson Weiss syndrome include craniosynostosis with hand and/or foot abnormalities (discussed in more detail in Chapter 3). The features of Craniosynostosis Boston type include sagittal craniosynostosis with syndactyly of the fingers and toes (Robin *et al.*, 1996). Cohen believes that the acrocephalosyndactylies and the acrocephalopolysyndactylies should be considered as one group, so that Carpenter syndrome (Temtamy, 1966) and Sakati syndrome are then part of this group (Cohen, 1986d). The first mapping of a gene responsible for an ACS syndrome was the localisation of Saethre-Chotzen syndrome to 7p21 by linkage (Brueton *et al.*, 1992).

Grieg cephalopolysyndactyly and Crouzon syndrome are related disorders, but are not members of the ACS group. Crouzon patients have craniosynostosis and facial features such as protruding eyeballs (ocular proptosis), but limb development is generally normal, without digital anomalies. Grieg cephalopolysyndactyly features pre- and post- axial polydactyly and syndactyly of the hands and feet but only sometimes involves craniosynostosis. Brueton *et al.* (1988) used clues from cytogenetic abnormalites to assign

the gene for Grieg cephalopolysyndactyly to 7p and Vortkamp *et al.* (1991) implicated the zinc finger protein gene, *GLI3* as the gene responsible.

Soon after the mapping of Saethre Chotzen syndrome, Warman *et al.* (1993) recognised a new autosomal dominant craniosynostosis disorder, now known as Craniosynostosis Boston type. The features of this syndrome include variable suture involvement and associated problems including headache, poor vision and seizures, and an absence of the characteristic features of the well-described syndromes (such as midfacial hypoplasia, orbital hypertelorism, hand or foot abnormalities). The gene responsible mapped to the telomeric region of 5q (Muller *et al.*, 1993), and was identified by the detection of a mutation within the highly conserved region of the homeodomain of the *MSX2* gene in all affected members of the family (Jabs *et al.*, 1993).

2. ISOLATING GENES CAUSING CRANIOSYNOSTOSIS

Knowledge of gene localisations, or preferably, identification of the genes involved in craniosynostosis would enable the development of a genetic based system of classification of the craniosynostosis syndromes. Gene identification will lead to better understanding the causes and mechanisms and possible prevention of craniosynostosis. Genes involved in craniosynostosis could be identified in cases where they are mutated and causing a recognisable phenotype. Further research on these genes (and their mutations) should lead to a better understanding of the mechanisms by which craniosynostosis occurs and of craniofacial development in general.

2.1 Functional Cloning

Since the birth of recombinant DNA technology in the late 1970s, many genes have been cloned, and the majority of these resulted from functional cloning (Collins, 1992). That is, the protein product of a disease gene was known, usually through its dysfunction in a

particular disorder (the biochemical defect causing the disease), and from this the gene encoding the protein was identified and cloned. This can be done by purification of the normal protein product and determination of part of the amino acid sequence. An example of functional cloning is glucose-6-phosphate dehydrogenase (G6PD) deficiency. To isolate cDNA clones for the gene *Gd* (responsible for G6PD deficiency), Persico *et al.* (1986) probed a cDNA library with a number of 17mers with sequences deduced from part of the peptide sequence for the gene's product, G6PD. They used the positive cDNA from this probing to screen three other cDNA libraries. After extensive sequencing of the resultant positive cDNAs they were able to assemble the cDNA sequence of the *Gd* gene.

2.2 Positional Cloning

For those diseases where the underlying biochemical defect is not known, functional cloning of the gene responsible is not possible. This is the case for the majority of monogenic disorders listed in McKusick's catalogue, Mendelian Inheritance in Man, according to Collins, (1992). For many of these disorders positional cloning may be a viable option, or possibly the candidate gene approach. The candidate gene approach involves mutation screening genes which are likely to be involved, given knowledge of the defect causing the disorder. Positional cloning involves mapping the gene responsible to a chromosome and successively narrowing of the candidate region to one Mb or less, then the candidate region is screened for transcribed sequences until the gene of interest is identified (Collins, 1992).

Gross gene rearrangements (such as deletions or translocations), even if present in only a small subset of patients, will speed up the process of gene identification (Collins 1992). These can be detected cytogenetically and smaller deletions can be detected by probing Southern blots of DNA from affected and unaffected individuals. Many successful positional cloning efforts have been helped by such rearrangements, such as the case of the identification of the type 1 neurofibromatosis gene (Wallace *et al.*, 1990). It had not been possible to find a consistent abnormality in NF1 tissues to allow functional cloning of the gene. Hence, gene identification on the basis of chromosomal map position was undertaken. Genetic linkage analysis was followed by a collaborative multipoint mapping effort. Finer

localisation was then achieved using two NF1 patients with appparently balanced translocations and these led to the identification of the NF1 gene because both translocations interrupted this gene.

One of the earliest positional cloning efforts was the identification and characterisation of the gene involved in chronic granulomatous disease (CGD) (Royer-Pokora *et al.*, 1986). The X-linked form of this disease was mapped using deletion patients and by formal linkage analysis. mRNAs were obtained from the defined region and a specific mRNA was studied in affected patients, leading to the characterisation of the gene responsible for CGD.

The refinement of a localisation and the narrowing of a candidate region is limited by the availability of markers, the number of informative meioses available in each pedigree and the number of pedigrees available with the given condition. The positional cloning approach has also been modified to the positional candidate approach (Ballabio, 1993; Collins, 1995), where the gene is localised to a chromosomal region, and previously characterised candidate genes mapped to that region are analysed for mutations in those individuals with the disease. Most genetic diseases are not localised with the precision necessary for positional cloning, either because there are insufficient affected families for a precise genetic localisation by linkage or there are no known patients with identifiable chromosomal rearrangements (Ballabio, 1993). Candidate genes can be prioritised for mutation detection in a number of ways, including by consideration of their patterns of tissue expression (and relevance to the disease under study) or knowledge of the genes responsible for similar or related disorders. The broad steps of the positional candidate cloning approach are clinical characterisation of suitable families, linkage analysis to assign and regionally localise the gene, examination of databases to identify genes which map in the same region, followed by mutation detection in likely candidate genes.

According to Orkin (1986), it is desirable to show the correct gene has been isolated by functional assays based on transfer of the normal copy of the gene in question into phenotypically abnormal cells. An example of this is the work by Puffenberger *et al.* (1994),

who mapped a susceptibility locus for Hirshsprung's disease (HSCR2) to 13q22 using a Mennonite kindred. They mapped a candidate gene, endothelin-B receptor (*EDNRB*) to this region and were able to demonstrate mutations in this gene in some Hirshsprung's disease patients. To confirm *EDNRB* as HSCR2 they transfected the mutant receptor into suitable chinese hamster ovary (CHO) cells and assessed certain responses which were greatly reduced as compared to certain controls.

2.3 Genome Scanning by Linkage Mapping

To locate a gene responsible for a particular trait with an identifiable mode of inheritance (such as autosomal dominant, autosomal recessive, X-linked recessive), one requires a suitable pedigree or collection of pedigrees with the trait segregating within, and a set of genetic markers spread throughout the genome (or along the X-chromosome) to test for linkage between the trait and the markers.

2.3.1 Pedigree Material

The success of a linkage study is in part dependent on the selection and characterisation of suitable pedigree material. The affection status of pedigree members must be determined by clinical examination, and for those in the pedigree who are not diagnosed as affected, it is important to know if they are unaffected after careful clinical examination or if their status is unknown due to incomplete clinical examination. Age of onset information for the disease is important, since individuals who are too young to have expressed the disease should be considered as of unknown affection status or assigned to an appropriate penetrance class. It is important to establish the mode of inheritance for the linkage analysis. Because of possible genetic heterogeneity, a single large pedigree is preferable to a collection of smaller pedigrees. Before commencement of the linkage study, it is advisable to determine whether the pedigree material will provide sufficient information to map the trait. For a fully penetrant simple Mendelian trait this can be achieved by calculating the maximum lod score that can be expected from a tightly linked fully informative marker. There are also more sophisticated approaches to determine the power of a pedigree to detect linkage, such as use of the simulation program SLINK (Weeks *et al.*, 1990).

2.3.2 Linkage Analysis

After the clinical characterisation of families the next step in positional cloning efforts is linkage mapping. The aim of gene mapping by linkage analysis is to assign the gene in question to a chromosome and then refine the localisation to a particular region of a chromosome. Genetic linkage is the phenomenon of the non-independent segregation of alleles at two loci in close physical proximity on the same chromosome, that is fewer recombinants occur than would be expected from independent segregation (Ott, 1991). The strength of genetic linkage is measured by the recombination fraction which is the proportion of recombinants among all of the informative meioses. Two loci that are segregating independently are genetically unlinked and have a recombination frequency (theta) between them of 0.5. Two loci are completely linked if they have a recombination frequency (theta) of 0.0 between them (Ott, 1991).

A recombination event can occur anywhere along a chromosome. The closer two loci are on a chromosome, the less likely it is that a recombination event will occur between them, and hence the lower the recombination frequency expected between them is. This is assuming that a recombination event is equally likely at any point along a chromosome. However the recombination frequency is known to increase towards the telomeres and decrease towards the centromeres (Mohrenweiser *et al.*, 1998; and references therein). Also, additional chiasma are inhibited in the vicinity of a crossover (interference). The recombination frequency in females is on average 1.5 times greater than in males (NIH/CEPH Collaborative Mapping Group, 1992).

Linkage analysis is based on the estimation of the recombination fraction between two loci. This is generally done using computer programs, such as LINKAGE (Lathrop and Lalouel, 1984), which calculate the lod scores. The lod score (Z) is the logarithm of the odds for the null hypothesis of independent segregation over the alternative hypothesis that the loci in question are linked (Morton, 1955). It is a statistical test for significance of linkage at a

specific recombination fraction, if Z is greater than or equal to 3 the linkage is significant (Ott, 1991).

2.3.3 Polymorphic Markers

In addition to suitable pedigree material, a set of polymorphic markers, covering the genome, is required for a genome-wide search by linkage analysis. A genetic marker is any polymorphic genetic entity which follows a Mendelian mode of inheritance, this includes electrophoretically detectable enzyme and protein polymorphisms (Harris and Hopkinson, 1976), restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980) and blood cell antigen polymorphisms (Race and Sanger, 1975). A marker is polymorphic when the frequency of the most common allele is 0.99 or less. Some of these polymorphisms are expressed phenotypically, such as variable antigenic properties of a blood proteins. Others such as many RFLPs have no known phenotype effects.

In 1980 Bostein *et al.* proposed the construction of a human genetic linkage map, for linkage mapping, based on the use of probes to identify polymorphisms in restriction fragment lengths after digestion of DNA with DNA sequence specific restriction endonucleases. They estimated that there would need to be linked markers about every 20cM along the map for genome scanning. Cleavage site specific restriction enzymes are endonucleases from bacteria which recognise and cleave specific nucleotide sequences in double stranded DNA (Smith, 1979). The first cleavage site specific restriction enzyme was isolated between 1968 and 1970 by Smith and colleagues when working on *Haemophilus influenzae* strain Rd (Smith, 1979). Other restriction enzymes found before this did not cleave DNA at specific sites.

Until approximately 1988 RFLPs were the genetic markers most commonly used in linkage analysis studies. Jeffreys *et al.* (1985) demonstrated the use of another group of markers, the variable number of tandem repeats (VNTRs), or minisatellite markers which were shown to be highly polymorphic, but had some disadvantages, like standard RFLPs. Polymorphic short tandem repeats (STRs), also called microsatellites, were an improvement over VNTR

markers and superseded the use of RFLPs in linkage analysis. STRs are regions of tandemly repeated DNA where the repeat unit is up to approximately 6bp in length. The STRs are found throughout mammalian genomes and specific STRs tend to be polymorphic with respect to the number of repeats tandemly arrayed (Weber and Wong, 1993). These STR polymorphisms can be rapidly analysed by PCR, using primers to the sequences flanking the repeats (Weber and Wong, 1993).

One major subclass of eukaryote tandemly repeated DNA contains very short simple sequence repeats (dC-dA)n.(dG-dT)n (Weber and May, 1989), hereafter referred to as AC repeats. Hamada and Kakunga (1982) reported the existence of an AC repeat (25 units long) within intron IV of a human cardiac muscle actin gene. They then demonstrated the widespread distribution of AC repeats in the human genome. Weber and May (1989) and Litt and Luty (1989) reported that some AC repeats within the human genome are polymorphic in length (varying between individuals) and they recognised the potential of these sequences as a vast new group of potential genetic markers. The majority of the AC repeat blocks they investigated were found to be polymorphic in length and their results were consistent with the number of tandem repeats being the variable factor. They demonstrated Mendelian inheritance in these polymorphisms. They recognised that because of the abundance of these AC repeats it was likely they would be used in the study of many human genetic diseases, and their use should lead to improved resolution of the human genetic map.

Weber and Wong (1993) investigated how frequently new mutations occur within these repeats, and they concluded that the majority of apparent new mutations occurred during or after the establishment of lymphoblastoid cell lines (LCL), used in DNA studies. For the STR polymorphisms on chromosome 19 they found an average mutation rate of 1.2×10^{-3} per locus per gamete per generation. Mutations were generally the gain or loss of a repeat unit or a pair of repeat units. Mutations were found to occur preferentially in males compared to females. Weber and Wong (1993) believed that these mutations are more likely to be caused by strand slippage than by unequal recombination between homologues.

AC repeat polymorphisms were fairly readily accepted by the linkage mapping community and are now in widespread use. This is because they have a number of advantages over the types of markers that were previously used such as RFLPs. They are relatively abundant throughout the human genome and they are generally more informative than the standard unique-sequence probe polymorphisms (Weber and May,1989). They can be quickly and easily assayed by PCR amplification and electrophoresis, and they require less DNA than that required for Southern blotting to assay an RFLP (Weber and Wong, 1993). However, dinucleotide repeats are more prone to stutter bands than tri and tetra nucleotide repeats.

In 1992 Weissenbach *et al.* published a linkage map of the human genome with 814 AC repeat polymorphisms. Generally the heterozygosity of the markers was above 70% and they covered the human genome with average gaps of 5cM. The markers were located on the basis of typing them through 8 Centre d'Etude du Polymorphisme Humain (CEPH) families. The basic set of CEPH families distributed to all CEPH collaborators are a reference panel which contains cultured LCLs from 40 families. The families are Caucasian from USA, France and Venezuela, and consist of large sibships, parents and in most cases all grandparents. CEPH provides DNA from the LCLs, to researchers (Dausset, 1990). A similar map of the human genome based on the CEPH database, containing RFLPs, VNTRs and microsatellite markers was also published in 1992 (NIH/CEPH Collaborative Mapping Group, 1992). An updated version of the PCR based map released by Weissenbach *et al.* (1992) was published by Gyapay *et al.* (1994) and Dib *et al.* (1996).

AC repeat polymorphisms are widely distributed throughout the genome, but the coverage of regions such as the telomeres and centromeres is uncertain. Weissenbach *et al.* (1992) noted that most of the gaps in their map corresponded to subtelomeric regions and that VNTRs could be found to cover these regions, if necessary.

Knowles *et al.* (1992) were concerned that using microsatellite markers in linkage analysis could lead to dramatic lod score bias, due to assigning equal marker allele frequencies for

polymorphic alleles, when used on the pedigrees typically used to study genetic disorders. According to Knowles *et al.* (1992) using equal allele frequencies when there are untyped individuals in the pedigree tends to increase the lod scores and the increase can be quite large. The practice of assigning equal marker-allele frequencies is relatively common in linkage studies because of the difficulties of comparing allele sizes to published sizes and in calculating frequencies relevant to the population from which the study pedigree comes. Freimer *et al.* (1993) used computer simulation to assess the effect on linkage analyses of using incorrect allele frequencies for polymorphic markers, in particular the use of equal frequencies for the alleles of a particular marker. They found the most common effect of incorrect marker allele frequencies was false evidence of linkage. They found that generally the use of incorrect allele frequencies does not prevent the detection of true linkage, except with very small pedigrees. Since true linkage is not missed by using equal allele frequencies, this represents a justifiable saving of time for the purposes of a genome wide search for linkage. Once linkage is suspected on the basis of lod scores near +3, exact marker allele frequencies can then be determined for those markers linked to the disease gene and exact lod scores computed.

Another problem found in large scale linkage studies using microsatellite polymorphisms is the non-amplification of some alleles. This can lead to apparent non-Mendelian inheritance of a polymorphism, or apparent non-paternity (or non-maternity). This non-amplification has been attributed to primer binding site polymorphisms and can usually be overcome by redesigning one of the primers from the associated GENBANK sequence (Phillips *et al.* 1991; Weber *et al.* 1991).

2.4 Identification of Transcribed Sequences

For projects based on positional cloning, once the region of interest has been identified, the next step is to search for transcripts in this region. For the positional candidate approach, one begins with candidate genes in the region which have already been cloned. New transcripts would only need to be identified when these candidates have been excluded.

2.4.1 Traditional Techniques

Traditionally transcripts have been identified by one of a number of means, which can be labour intensive and become impractical when applied to large regions of genomic DNA. These techniques include the use of zoo blots (Southern blots of genomic DNA from a variety of species) to search for evolutionary conserved sequences (Monaco *et al.*, 1986), because many low copy sequences conserved between species represent genes. Another technique uses the presence of a number of sites for rare-cutter enzymes to indicate the location of CpG islands. These islands are short regions of unmethylated DNA with a higher than normal proportion of CpG dinucleotides. In the human genome they are found at the 5' end of all housekeeping and widely expressed genes and are associated with 40% of tissue specific or limited expression genes (Larsen *et al.*, 1992).

Island rescue PCR can be used to amplify CpG island-associated genes (Patel *et al.*, 1991; Valdes *et al.*, 1994). In this method the DNA region of interest is digested with enzymes that target CpG islands (for example *Eag* I, *Sac* II and *Bss* HII) and vectorette linkers are ligated to the cleaved ends. PCR is conducted using a forward primer to the linker and a reverse primer to Alu repeat sequence. The resultant amplified segments are used to probe a cDNA library.

The probing of cDNA libraries with genomic DNA from the region of interest is another method which has been used to identify transcripts from a specific region. Elvin *et al.* (1990) reduced the effect of repetitive sequences in filter hybridisation by preassociating the probe DNA (large genomic DNA fragments cloned into YAC vectors) with unlabelled sheared human placental and pBR322 DNA. One of the problems with the probing of cDNA libraries is that a transcript may have a limited spatial and/or temporal distribution and thus a number of cDNA libraries need to be screened since it would be absent from some libraries.

2.4.2 Exon Trapping and cDNA Selection

Recently a number of new techniques have been developed to more efficiently identify transcribed sequences, including exon trapping and direct cDNA selection. Exon trapping was developed to identify transcribed sequences from stretches of genomic DNA by the presence of intron-exon splice sites (Auch and Reth, 1990; Duyk *et al.*, 1990 and Buckler *et al.*, 1991). The technique has been refined a number of times and improved exon trapping vectors created (Hamaguchi *et al.*, 1992 and Church *et al.*, 1994). The basic strategy involves cloning partially digested genomic DNA into an exon-trapping vector, transfecting the constructs into COS-7 cells where RNA transcripts are produced and spliced, and harvesting mature mRNA from which transcripts can be amplified by reverse transcription PCR. The method is independent of the expression level of the corresponding gene and of transcript availability *in vivo*. Not all genes have introns, though most do, so some would be missed, which is a drawback.

Coding sequences can be identified by cDNA selection by hybridising cDNA fragments to cosmid or YAC DNA from a specific region (Parimoo *et al.*, 1991; Lovett *et al.*, 1991). The cosmid or YAC DNA is Southern blotted or lysed colony DNA is immobilised onto filters, quenching agents are used to block for repetitive, ribosomal or GC rich sequences, then the filters are probed with random cDNA fragments amplified from a library by PCR. Unbound and nonspecific cDNA fragments are washed away and those selected (left bound) are amplified by PCR. This technique was successfully applied by Guo *et al.* (1993) to identify the human hepatic glycerol kinase cDNA coding sequence. The technique has also been modified to make it easier for cDNA probes to find their targets by biotinylating the genomic DNA to be screened, hybridising the biotinylated DNA with the cDNA probes in solution and then capturing the hybridised complexes using streptavidin-coated magnetic beads (Tagle *et al.*, 1993). The efficiency of cDNA selection can be improved by repeating the hybridisation with the products of the first selection, to enrich for the desired cDNAs. This method will detect genes that don't have associated CpG islands, and don't have introns, also those that are not transcribed in hybrid cell lines or have diverged between

species will not be missed. A drawback of this technique is that it requires a cDNA library with all possible mRNAs in order to identify all coding sequences, also it will pick up pseudogenes.

2.5 Mutation Detection

Mutation detection can be used to implicate or exclude candidate genes from involvement in a particular disorder. There are a number of mutation detection methods currently available, and they differ by, the size of the DNA fragments they can satisfactorally screen, whether they can detect point mutations and /or deletions and whether they give information about the location of any mutations found. No knowledge of the positions of intron/exon boundaries are required to screen cDNA and for a gene with many exons, screening cDNA rather than genomic DNA involves less effort. However genomic DNA is preferable for dominant conditions because a mutant allele may not be represented in the corresponding cDNA and RTPCR will not necessarily detect splice site mutations if these occur on the intron side of the intron/exon boundary. It is necessary to show that a sequence change found is actually a mutation, not a polymorphism or rare benign variant. In some cases a true pathogenic mutation is obvious, such as the case of a premature stop codon in a highly conserved domain or the creation or destruction of a splice site. Otherwise evidence that the variant is involved in the disease can be accumulated by showing the absence of the variant in a large number of controls, evolutionary conservation of the amino acid (implying functional significance), functional importance of the particular region to the molecule and/or carrying out site-directed mutatgenesis and *in vitro* testing.

2.5.1 Conformation Change-Based Techniques

One group of mutation detection methods is based on conformational changes caused by altered sequence. Single-strand conformation analysis (SSCA) relies on the fact that under certain conditions, single stranded DNA molecules have a defined secondary structure. This structure is related to the sequence and can be altered by a single base change. Differences in secondary structure can be detected by electrophoresis through a non-denaturing gel. Orita *et al.* (1989) investigated SSC polymorphisms (SSCPs) as a possible source of

polymorphisms for linkage work. They found that single base substitutions altered the mobility of single stranded DNA fragments when electrophoresed through a neutral polyacrylamide gel. The SSCPs they found were shown to be true polymorphisms inherited as Mendelian traits.

Since this work, SSCA has been used in many mutation detection experiments. Nigro *et al.* (1992) used multiplex PCR and SSCA to efficiently screen portions of the dystrophin gene for mutations in Duchenne and Becker muscular dystrophy patients. A number of modifications have been made to improve the detection rate of SSCA. Sarkar *et al.* (1992) showed that SSCA was possible on RNA. They found that RNA SSCA was generally superior to standard SSCA because it gave a higher detection rate, especially with larger fragments. Transcripts were produced for SSCA by performing PCR with primers containing phage promoter sequences, then transcribing these fragments. SSCA (and RNA SSCA) do not detect 100% of mutations, but they are relatively simple methods. Estimates of the proportion of mutations detected vary, but a general figure is about 80% (Cotton, 1993). The proportion of mutations detected decreases with increasing fragment size. According to Grompe (1993), SSCA detects 70-95% of mutations in PCR products 200bp or less in size, but this drops to less than 50% for PCR fragments over 400bp in size. Hayashi (1996) recommends using fragments less than 300bp in size for SSCA.

Heteroduplex analysis (HA) is similar to SSCA, and is based on the fact that a heteroduplex molecule containing a single base pair mismatch can be separated from heteroduplexes without a mismatch (Keen *et al.*, 1991). Using non-denaturing MDE gels, Keen *et al.* (1991) detected a mismatch in a 420bp PCR product. Like SSCA, HA does not detect 100% of mutations. Ganguly *et al.* (1993) modified HA to increase its efficiency by adding various mildly denaturing solvents to optimise gel conditions in terms of mismatches causing conformational changes and hence differential migration. Mismatches close to the ends of PCR products are less likely to be detected. They also found that the sequence around a mismatch (that is the sequence context) and length of the fragment are important factors in whether or not a mismatch will be detected. White *et al.* (1992) achieved a mutation

detection rate of over 80% when they investigated the HA technique. It is thought to work most efficiently for fragments 200-300bp long (Cotton, 1993). The HA and SSCA techniques indentify mutations on the basis of different principles and thus using a combination of both these techniques should lead to a mutation detection efficiency closer to 100% (White *et al.*, 1992). For example, a single base change in the loop of a hairpin structure would be expected to have little or no effect on the secondary structure, whereas a base change of this nature is more likely to be detected in a heteroduplex molecule.

Single base changes in a DNA fragment can also be detected by electrophoresis of double stranded DNA through a gradient of increasing denaturant, that is denaturing gradient gel electrophoresis (DGGE). The two strands partially dissociate and this leads to a sudden decrease in mobility, a base change can cause a different point of arrest. Fragments from 50-500bp can be screened in this way. Detection efficiency of approximately 50% is improved by the use of heteroduplex molecules (between mutant and wildtype) and by adding a GC clamp (GC rich sequence-high melting point region) to the fragment, making it a low melting point region relative to the clamp (Sheffield *et al.*, 1989). Once the conditions have been optimised for a particular mutation, it can be detected with high efficiency, thus this technique is better suited to screening for known mutations. DGGE requires a special apparatus to keep the gel temperature constant. Attempts have been made to overcome this by using temperature gradients instead of denaturant gradients. Wenkert *et al.* (1994) used temperature gradient gel electrophoresis of GC clamped PCR products to find novel mutations in the V2 vasopressin gene of patients with X-linked nephrogenic diabetes insipidus.

There are a number of drawbacks to these detection methods based on conformational changes caused by mismatches. Some of these techniques require the optimisation of the conditions separately for each fragment to be tested. This makes such a technique more suitable for testing a few fragments from many different samples (that is target particular regions where mutations are known to occur), rather than many fragments from a few individuals. The sensitivity of these techniques decreases with increasing size of the DNA

fragment, and no information is obtained about the nature and location of the mutation. Also, none of these mutation detection methods detect 100% of mutations and thus failure to detect a mutation doesn't necessarily equate to exclusion of the gene region as a candidate.

2.5.2 Cleavage of Mismatches-Based Techniques

Another group of mutation detection techniques is based on the ability of some proteins and chemicals to recognise sequence mismatches within heteroduplexes. These techniques can provide information about the location of any mutations detected.

Chemical mismatch cleavage (CMC) exploits the fact that some chemicals, such as hydroxylamine and osmium tetroxide, recognise and react with mismatched bases, and these can then be cleaved with piperidine (Cotton *et al.*, 1988). After these reactions the DNA samples are electrophoresed and if cleavage has occurred a smaller product can be detected. While this method detects 100% of mismatches and can be used to screen DNA fragments of 1-2Kb, it does require the use of dangerous chemicals (Cotton, 1993). Kilimann *et al.* (1992) used multiplex PCR and chemical cleavage to detect point mutations and polymorphisms in the dystrophin gene in a group of Duchenne muscular dystrophy patients.

A related technique has been developed which uses bacterial resolvases (a group of enzymes involved in the resolution of branched DNA intermediates that form during genetic recombination) instead of chemicals, to detect mismatches in DNA fragments. Youil *et al.* (1995) tested the ability of T4 endonuclease VII to detect mismatches in heteroduplexes. They tested eighteen single-base change mutations and only one (A toT) was not detected. T4 efficiently recognised G.T which is the mismatch generally considered to be the most thermostable. Marshal *et al* (1995) also used resolvases, and found that deletions were cleaved more efficiently than point mutations making this a suitable technique for the detection of deletions and point mutations. The use of resolvases is preferable since they are not toxic or explosive. The cleavage of mismatches techniques are the same for all

fragments to be tested (that is no repeated optimisations) and larger fragments can be tested as compared to SSCA and others.

2.5.3 Sequencing

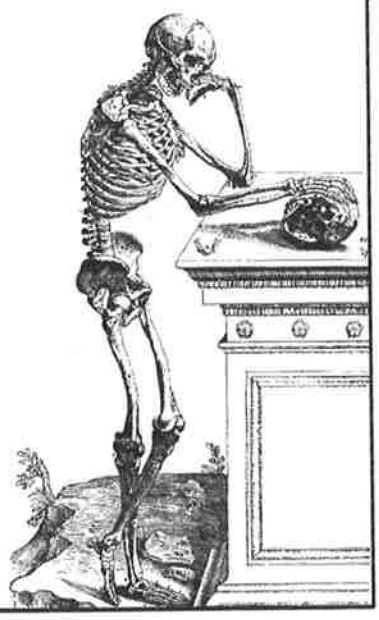
Sequencing is the ultimate and only sure way to exclude a gene (assuming 100% accurate sequence is obtained). The exact nature of the mutation normally can only be determined by sequencing. Once the sequence is determined and, for instance, a base substitution changing a restriction site recognised, other samples can be tested for the change by enzyme digest without sequencing. Various sequencing strategies include manual sequencing, and automated sequencing (such as Dye Terminator sequencing of PCR product, Dye Primer sequencing of cloned DNA fragments and Dye Terminator sequencing of single stranded template, using PRISM kits).

Once a mutation is detected in a patient, and is demonstrated to be not just a polymorphism, then it is likely that a gene causing the disorder under study has been found

3. AIMS

1) Mapping by linkage in large craniosynostosis pedigrees.
2) Identification of genes by positional candidate approach.
3) Determination of spectrum of mutations in genes responsible for craniosynostosis.
4) Examination of functional significance of mutations.

CHAPTER 2: MATERIALS AND METHODS



Introduction

This chapter contains descriptions of the material and methods used to produce the results described in the subsequent chapters, though not all methods are included here. Some methods which were used exclusively (or almost exclusively) in a particular chapter are described in the chapter where those techniques have been used to produce the results described. Linkage analysis and associated techniques are described in Chapter 3 and flow cytometry techniques are described in Chapter 6. Some techniques are described in greater detail, in the chapters in which they were used.

2.1 MATERIALS

2.1.1 SOLUTIONS AND MEDIA

All solutions were made with deionised water (dH_2O), unless otherwise specified, and were autoclaved where necessary. Solutions that were supplied as components of kits are not necessarily mentioned.

5% polyacrylamide/7M urea: made with 40% polyacrylamide solution (19:1 acrylamide:bisacrylamide) (BioRad), with final concentration 42% w/v urea and 1xTBE. This solution was filtered through 3MM Whatman paper.

4.5%, 6% and 10% polyacrylamide gels for SSCA: varying percentages of polyacrylamide (all with ratio 49:1 acrylamide:bisacrylamide), made from 40% acrylamide and 2% bisacrylamide solutions (BioRad). SSCCA gels were 5% v/v glycerol and 1xTBE.

MDE gels: For SSCA, MDE gels were 37.5% v/v and for HA gels were 50% v/v MDE, both with 0.6xTBE.

Gel Denaturing Solution: 2.5M NaCl, 0.5M NaOH

Gel Neutralising Solution: 1.5M NaCl, 0.5M Tris-HCl pH7.5

Hybridisation Solution: 5xSSPE, 1% w/v SDS, 50% v/v deionised formamide, 10% w/v dextran sulphate

20xSSC: 3M NaCl, 0.3M tri-sodium citrate, pH adjusted to 7.0 with 36% HCl

20xSSPE: 3.6M NaCl, 0.2M $NaH_2PO_4.2H_2O$, 0.02M EDTA

5xTBE: 0.45M Tris base, 0.45M Boric acid, 0.01M EDTA pH8.0

X-gal: 50mM (2% w/v) X-gal in dimethyl-formamide

2xPCR mix-high dNTP: 33mM $(NH_4)_2SO_4$, 133mM Tris HCl (pH 8.8), 0.013mM EDTA, 0.34 mg/ml BSA, 20% v/v DMSO, 3mM dATP, 3mM dCTP, 3mM dGTP, 3mMdTTP (modified from Kogan *et al.* 1987)

2xPCR mix-low dNTP: 33mM $(NH_4)_2SO_4$, 133mM Tris HCl (pH 8.8), 0.013mM EDTA, 0.34 mg/ml BSA, 20% v/v DMSO, 0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP, 0.4mMdTTP

Phenol: buffered with Tris-HCl pH 7.4

PBS (phosphate buffered saline): purchased as powder from MultiCel, Trace

10xLoading Buffer (for loading samples on agarose gels): 0.1M Tris-HCl pH8.0, 0.2M EDTA pH8.0, 2% w/v Sarcosyl, 20% w/v Ficoll 400, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol

Formamide Loading Buffer (for loading samples on acrylamide gels): 95% deionised formamide, 1mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol

LB (Luria-Bertani) broth: 0.5%w/v yeast extract, 1% w/v bactotryptone, 0.5% NaCl, pH to 7.5 with NaOH

Indicator plates: LB broth containing 1.5% bacto-agar and 100ug/ml ampicillin, with 100ul of 100mM IPTG and 20ul of 50mg/ml X-gal (2% w/v in dimethyl-formamide) added to and spread across each plate at least one hour prior to use.

2.1.2 DNA SIZE MARKERS

Puc/HpaII (Breasatec)-effective size range 60-500bp

Fragment Sizes: 501/489, 404, 331, 242, 190, 147, 111/110 and 67 bp

SPP1/EcoRI (Breasatec)-effective size range 0.5-8.5Kb

Fragment Sizes: 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 Kb

2.2 METHODS

2.2.1 DNA EXTRACTION, PURIFICATION AND QUANTIFICATION

Extraction of genomic DNA from human blood

Genomic DNA was extracted from blood samples from random blood donors and members of study families by Jean Spence, using a modified form of the method of Wyman and White (1980), including lysis with Proteinase K and SDS, phenol and chloroform extractions and precipitation with NaAc and ethanol.

Phenol/Chloroform Extractions

DNA samples were purified by phenol/chloroform extraction, based on the technique described by Sambrook *et al.* (1989). A DNA sample was mixed with an equal volume of phenol, vortexed, then centrifuged for 6 mins at 13,000rpm in an IEC MicroMax centrifuge.

The aqueous phase was then removed to a clean tube and mixed with an equal volume of chloroform, vortexed, then centrifuged as before. Again the aqueous phase was removed to a clean tube and DNA was precipitated by the addition of 1/8 volume of 2M NaAc and 3 volumes of ethanol. Following mixing and incubation at -20°C overnight, or -80°C for 1 hour, the solution was centrifuged at 13,000rpm in an IEC MicroMax centrifuge for 10 mins. The resultant pellet was washed with 70% ethanol and resuspended in water.

DNA Quantitation by Spectrophotometry

DNA was quantitated using a Cecil CE-2020 spectrophotometer, with a deuterium lamp. The absorbance at a wavelength of 260nm was measured and multiplied by the dilution factor and a constant value particular to the type of DNA being quantitated : plasmid and genomic DNA-50, primer (single stranded DNA)-30, single stranded RNA-40. The result is in ug/ml.

2.2.2 RESTRICTION ENZYME DIGESTS, AGAROSE GEL ELECTROPHORESIS AND SOUTHERN BLOTTING

Restriction Enzyme Digests and Agarose Gel Electrophoresis

For Southern Blots 10ug of genomic DNA was digested in a 50ul volume with 33-45 units of commercially supplied restriction enzyme in the appropriate restriction enzyme buffer (supplied with the enzyme), and 1x BSA (final concentration of 100ng/ul). Spermidine was added to a final concentration of 5mM. Genomic digests were left at the appropriate temperature overnight. Three ul of each digest was separated on a 0.8% agarose tester gel and if the DNA was digested, the remainder of each digest was electrophoresed on a 0.8% gel for approximately 19 hours at 12.5mA followed by 27.5mA for approximately 3 hours. The gel was then stained in ethidium bromide and photographed under UV light. Digests of PCR products or cloned products were conducted as for the genomic digests, though they were generally left at the recommended temperature for three hours, rather than overnight. In cases where small fragments were to be separated (for instance to confirm the presence of

a restriction enzyme site detected by sequencing) a higher percantage of agarose was used in the gels, up to 2%.

Transfer of Digested DNA to Membrane

Genomic gels were denatured in Denaturation Solution for 30 mins at room temperature with shaking, they were then neutralised in Neutralisation solution for 30 mins. The genomic DNA was transferred to Hybond N^+ Membrane (Amersham), by a modified form of the method of Southern (1975), using a blotting tray with 10xSSC, overnight. The wells from the gel were then marked in pencil on the filter and the filter was removed from the gel. The filter was rinsed in 0.5M NaOH for 30s and then in 0.2M Tris pH7.5/2xSSC for 1 min, both with manual shaking. Filters were air dried at room temperature on 3MM Whatman paper.

Radioactive Probe Preparation and Filter Hybridisation

Radioactive probes were prepared using a DuPont NEN labelling kit, using a modified form of the procedure used by Feinberg and Vogelstein (1983). DNA to be labelled was purified using Prep-a Gene or Qiaquick and quantified. Purified DNA (50-100ng) was diluted to 12ul with water and heated (96°C) for 8 mins, briefly centrifuged, then kept on ice. The DNA was mixed with 6ul 5xextension buffer, 6ul triphosphate mix, 5ul [α^{32}P]dCTP and 1ul Klenow fragment and briefly centrifuged, then incubated at 37°C for 10-15mins. The probe was placed on ice, then just prior to addition to the hybridisation bottle, it was heated to 96°C for 5 mins. Filters to be probed were prewet with 5xSSPE, then placed in a hybridisation bottle and incubated at 42°C with 10ml Hybridisation Solution plus 100ul freshly boiled salmon sperm DNA, for at least 30mins. The denatured probe was then added to the hybridisation bottle, and incubated at 42°C with rotation in a Hybaid Mini Oven Mk II, for approximately 16 hours. The filters were then washed at 65°C, twice in 2xSSC/1%SDS for 15 mins, then twice in 0.1xSSC/0.1%SDS for 30mins and once in 0.05xSSC/0.1%SDS for 15mins (Sambrook *et al.*, 1989). The filters were then autoradiographed.

Removing bound Probe from Filters (to allow filter reuse)

Filters were incubated at 42°C, with shaking, in 0.4M NaOH for 30mins, then 0.2M Tris pH7.5/0.1xSSC/0.1% SDS for 30 mins. If radioactive counts were still detectable on the filters, this procedure was repeated.

2.2.3 CLONING AND SYNTHESIS OF DNA FRAGMENTS

Preparation of Competent Cells

XL1-Blue (Stratagene) competent cells were prepared by adding 20ml LB, 5ul tetracyclin (50mg/ml) plus 400ul of an overnight culture of XL1-Blue cells to a 50ml tube. This was left at 37°C for approximately 3.5 hours with shaking (agitation). The cells were then centrifuged 15 minutes at 2,250rpm in a Jouan Plasma R1000 centrifuge, at 4°C. The supernatant was then discarded and the cells resuspended in 2ml ice cold LB broth with 10% w/v PEG 3350, 5%v/v DMSO and 50mM $MgCl_2$ (Chung *et al.*, 1989). The competent cells were snap frozen in liquid Nitrogen, then stored in 300ul aliquots at -80°C.

Ligations

PCR products to be cloned were ligated to the pGEM-T vector using a modified version of the technique described by the manufacturers (Promega). One ul of a 100ul PCR was mixed with 1ul of T4 DNA ligase, 1ul of T4 DNA ligase buffer, 1ul of pGEM-T vector and 6ul of water, and incubated at 16°C for at least 3 hours.

Transformations

Recombinant vectors were cloned into competent XL1blue cells, using a technique modified from a technique described by Maniatis *et al.* (1982). Two ul of ligation was combined with 50ul of competent cells and gently mixed. This was incubated on ice for 20mins, then heat shocked at 42°C for 45-50 seconds. After a further 2 mins on ice, 1.4ml of LB was added, mixing was achieved by inversion and the mixtures spent 1hr at 37°C with shaking. One hundred ul from each transformation was plated on Ampicillin/X-gal/IPTG indicator plates and incubated at 37°C overnight. Blue colonies represent cells with nonrecombinant

plasmid, and white colonies represent cells with recombinant plasmid; white colonies were selected for further study.

Plasmid Preparations

Plasmid preparations were conducted using a modified form of the technique described by Manitatis *et al.* (1982). Five ml of LB broth with 5ul of 100mg/ml Ampicillin was seeded with a single colony and grown overnight at 37°C with shaking. A glycerol stock was made by combining 500ul of the resultant culture, with 500ul of glycerol. The remaining 4,500ul was centrifuged at 1,800rpm in a Jouan Plasma R1000 centrifuge for 15 mins at 4°C. Plasmid DNA was recovered and purified from the resultant pellet using the rpm kit (Rapid Pure Miniprep-BIO-101)-according to the manufacturer's instructions.

2.2.4 PCRs AND ACRYLAMIDE GEL ELECTROPHORESIS

All PCRs were performed using a Perkin Elmer Cetus DNA Thermal Cycler (model 480), and all reactions were overlaid with paraffin before cycling commenced. All PCRs were conducted using thermostable DNA polymerase (Kogan *et al.*, 1987).

Non radioactive PCR

Product to label for use as a probe, or digestion to confirm a mutation or for a number of other uses, were produced using non radioactive PCR. Generally 100ul reactions were used, with 2xPCR mix-low dNTP or Boehringer Mannheim Buffer. The reactions were scaled up from the AC and SSCA reactions, but without radioactivity.

AC Repeat Markers

The polymorphic AC regions were amplified in reactions of 10ul volume with 100ng of genomic DNA, 5ul 2xPCR mix, 1.0 unit of *Taq* polymerase (Boehringer Mannheim or Gibco BRL), 150ng of each primer, [α^{32}P]dCTP, 0.1ul of 1M b-mecaptoethanol (equivalent to a final concentration of 10mM), and $MgCl_2$ to varying final concentrations. Initially the PCR mix used had a concentration of 30mM for each dNTP (2xPCR mix-high dNTP).

When this mix was used each primer pair was tested with a range of $MgCl_2$ concentrations in reactions that were the same as above, but twice the volume, and no [α^{32}P]dNTP was added. The most appropriate $MgCl_2$ concentration was selected on the basis of band intensity, and lack of nonspecific bands, when the cold reactions were electrophoresed on 0.8% agarose gels. The radioactive reactions were then conducted at the selected $MgCl_2$ concentration, (generally in the range 4.5 to 7.5mM) with 0.5ul [α^{32}P]dCTP (5uCi). Later in the project, the 2xPCR mix used had all dNTPs at concentration of 400uM (2xPCR mix-low dNTP). When this PCR mix was used, cold optimization was not necessary, and all reactions were conducted at a final $MgCl_2$ concentration of 1.5mM and 0.2ul [α^{32}P]dCTP (2uCi) was used per reaction. Later in the project, two markers were put in the same PCR and were thus amplified and electrophoresed together. For multiplexing, markers were selected that were close in size, but such that the smallest allele of one marker was at least 10bp bigger than the largest allele of the other marker.

The thermal cycling program used was file 21: PCR conditions were 10 cycles of 94°C for 1 minute, 60°C for 1.5 minutes and 72°C for 1.5 minutes, followed by 25 cycles of 94°C for 1 minute, 55°C for 1.5 minutes and 72°C for 1.5 minutes, and finally 10 minutes at 72°C.

Following cycling, 30ul of formamide loading buffer was added to each reaction. The reactions were then heated to 100°C for approximately 10 minutes, then loaded on a pre-electrophoresed 5% denaturing (7M urea) polyacrylamide gel. Acrylamide gels were prepared and electrophoresed using BioRad Sequencing Cell Equipment (BIORAD SEQUI-GEN SEQUENCING CELL apparatus and a BIORAD Model 3000Xi computer controlled electrophoresis power supply). The glass plates (42cmx50cm) were washed with detergent, rinsed with water and ethanol, then dried. Gelslick was applied to the back plate according to the manufacturer's instructions (FMC bioproducts). The plates were clamped together with 0.4mm spacers between and sealed across the bottom using 50ml 5% polyacrylamide (set with 250ul TEMED and 250ul 25%w/v APS). Once the seal was polymerised, the main gel was poured using 80ml 5% polyacrylamide (set with 80ul TEMED and 80ul 25%w/v APS). Shark's teeth combs were inserted (inverted to form an even edge) and the gel was

left for at least 2 hours to set. Gels were electrophoresed at approximately 1,800 to 2,100V, aiming to maintain a gel temperature of approximately 50°C. Time of electrophoresis varied between 3 and 8 hours according to the size of the product.

The gels were blotted onto 3MM Whatmann paper and the blotted gels were dried on a BIORAD model 583 gel dryer for 1-2 hours. The dried gels were generally autoradiographed for 1 to 5 days at -70°C. X ray films were developed using an automatic developer in the Department of Radiology, WCH. The autoradiographs were examined and the genotypes recorded.

Primers/Oligonucleotides

Primers for the amplification of and/or sequencing of candidate genes were designed where ever possible to have approximately 50% GC content, and an annealing temperature of approximately 60°C, calculated by 2x(A+T)+4x(G+C) (Suggs *et al.*, 1981). Inter and intra strand complementarity was avoided.

Primers to candidate genes were checked for homology to non-target DNA by applying a BLAST search to the proposed primer sequence, prior to synthesis (Altschul *et al.*, 1990). Most oligomers (for the amplification of AC repeats and also those for candidate genes) were synthesised on an Applied Biosystems Model 391 PCR-MATE EP DNA synthesizer, or a Beckman 1000 DNA synthesizer, according to the manufacturer's instructions, by Kathy Holman, Annette Osborn, Shirley Richardson, Kathy Friend or Jean Spence.

Following synthesis, the oligomers were cleaved from the columns using ammonium hydroxide. One ml of ammonium hydroxide was added to the column via a one ml syringe, after a 1.6ml polyproylene tube (with an extra "O"ring fitted) was attached to the other end of the column. The ammonium hydroxide was left to sit on the column for 30 minutes then ammonium hydroxide was flushed and left to sit on the column for a further 30 minutes. The solution was then sealed in the polypropylene tubes and left overnight at 55°C. The following day the oligomers were (deprotected/purified) by the method of Sawadago and

Van Dyke (1991). The ammonium hydroxide solution was cooled to room temperature then transferred to a 50ml tube. After the addition of 10ml of butanol, the solution was vortexed and centrifuged at 3,000rpm in a Juan Plasma R1000 centrifuge for 45 minutes. The supernatant was discarded and the oligomer pellet was resuspended in 200ul of water, and the butanol extraction was repeated. The pellet was then air dried, resuspended in 100ul of water and quantitated using a Cecil CE 2020 spectrophotometer.

Towards the end of the project, a small number of oligomers were synthesized and purified externally by AMRAD. The biotinylated primers were also purchased from Gibco BRL Life Technologies.

2.2.5 MUTATION DETECTION

The nomenclature used to refer to mutations and polymorphisms in Chapters 4 to 9 depends on whether the DNA sequence or protein change is being described. When referring to changes in the DNA sequence the gene symbol is shown in italics and the base change at a certain nucleotide is shown, for example C749G of *FGFR3*. When referring to the change in the corresponding protein the protein symbol is shown in normal type and the mutation is described using the codon and the amino acid abbreviations (three letter code), for example Pro250Arg of FGFR3.

Single Stranded Conformation Analysis

Single stranded conformation analysis (SSCA) was conducted using a modified form of the method used by Richards and Friend (1991). PCRs were conducted as for the AC repeat markers using 2xPCR mix-low dNTP and the thermal cycling file 21. Samples were prepared for SSCA by diluting the radioactive PCR samples 1 to 1 with formamide loading buffer, the samples were then heat denatured (10 minutes at 100°C) then placed on ice, and immediately loaded.

The SSCA gels were a modified form of those described in Richards and Friend (1991), they were prepared as for the AC repeat gels, except with a 49:1 ratio of acrylamide to bis acrylamide, and containing 5% glycerol and no urea. Well forming combs were used for SSCA gels. Gels were prepared with varying percentages of polyacrylamide, including 4.5%, 6% and 10%. Gels were electrophoresed using the same equipment as for the AC repeat gels, at 600V or 700V for between 12 and 36 hours, depending on the size of the fragment being tested and the polyacrylamide percentage of the gel. SSCA gels were electrophoresed in 1xTBE. MDE (FMC bioproducts) gels were prepared from a 2xconcentrate stock solution to give a final concentration of 37.5% MDE, and a final concentration of 0.6xTBE. MDE gels were electrophoresed using the same equipment, in 0.6xTBE, for approximately 16 hours at 700V. Gels were dried and autoradiographed as for the AC repeat gels except exposure time varied between 30 mins and 48 hours.

Heteroduplex Analysis

Heteroduplex analysis was conducted by a modified form of the method published by Keen *et al.* (1991). Samples were prepared for heteroduplex analysis by radioactive PCR reactions as for AC repeat analysis with 2xPCR mix-low dNTP, using thermal cycling file 21, unless otherwise specified. Following cycling, the reactions were heated to 100°C for 10 minutes, then slowly cooled to room temperature (30-60 minutes). Following cooling, 3ul of reaction was added to 3ul of MDE gel loading dye (Hydrolink). Three ul of each reaction dye mix was loaded on a 50% MDE gel, with a TBE concentration of 0.6x. Gels were run at approximately 800V, in 0.6xTBE for approximately 16 hours for a 300bp fragment. Gels were prepared, dried and autoradiographed as for AC gels except 1mm spacers were used and exposure time varied between 30mins and 48 hours.

2.2.6 SEQUENCING

Manual Sequencing

Manual sequencing by the dideoxy chain termination method (Sanger et al., 1977) was performed using the Exo(-) *Pfu* Cyclist DNA Sequencing kit, according to manufacturer's

instructions (Stratagene). Three ul of the appropriate ddNTP was added to each of four termination tubes (kept on ice) then the following mix was prepared:

60-120ng template

1pmol primer

4ul 10xsequencing buffer

1ul ^{32}PdCTP (10uCi)

1ul Exo$^-$ *Pfu* DNA polymerase (2.5U)

4ul DMSO

and water to bring volume to 30ul

Seven ul of this mix was added to each of the four termination tubes for a particular primer/template combination. The reactions were heated to 95°C for 5 minutes, then cycled through 95°C for 30 seconds, 60°C for 30 seconds and 70°C for 60 seconds for 30 cycles. Following cycling 5ul of stopdye mix was added to each reaction, they were heated to 100°C for 5 minutes, then 3ul was loaded onto a 5% sequencing gel. These gels were prepared as for the AC gels, except 0.25mm wedge spacers and 6% acrylamide were. Gels were electrophoresed in 1xTBE at approximately 22,000V for between 1 and 4 hours depending on the region of sequence required. Following electrophoresis, the gels were blotted, dried and autoradiographed as for the AC gels.

Automated Dye Terminator Sequencing

Automated Dye Terminator Sequencing was originally done with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (with AmpliTaq), and later in the project the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS was used (that is, when the new AmpliTaq DNA polymerase FS became available, it became the sequencing kit of choice). Both kits were purchased from ABI PRISM, Perkin Elmer Corporation. Before the Ready Reaction Mix was available, reaction mix was prepared according to the manufacturer's instructions, by combining 4ul of 5x TACS buffer, 1ul dNTP mix, 1ul of each of DyeDeoxy A, C, G and T terminator, 0.5ul of AmpliTaq DNA polymerase and water per reaction. Nine point five ul of premix (prepared as above, or the Ready Reaction Mix) was combined with 100-200ng of purified PCR fragment and 3.2pmol

primer. The reactions were added to the thermal cycler at 96°C and cycled through 28 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Before the availability of the FS polymerase reactions were treated with a chloroform and two phenol/chloroform extractions following cycling, then precipitated by 15ul of 2M NaAc pH4.5 and 300ul 100% ethanol (immediately centrifuged for 15mins in an Eppendorf or an IEC MicroMax microcentrifuge at 13,000rpm) and then rinsed with 70% ethanol, recentrifuged and dried. Reactions done with the FS polymerase were precipitated with 50ul of 100% ethanol and 2ul 3M NaAc pH5.2, following 10 minutes on ice, the reactions were centrifuged at 13,000rpm for 20 minutes then rinsed with 70% ethanol, recentrifuged and dried.

Dye Primer Sequencing

Dye primer sequencing was initially conducted using the PRISM Ready Reaction Dye Primer Sequencing Kit, then, when it became available, the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit With AmpliTaq DNA polymerase, FS was used. For both kits, four reactions were prepared, according to manufacturer's instructions, for each sample to be sequenced. The A and C reactions were prepared by combining four ul of d/ddNTP mix with 1ul of DNA template, the G and T reactions were prepared by combining 8ul of the appropriate d/ddNTP mix with 2ul of DNA template. The template was at a concentration of between 0.2 and 0.25ug/ul for the first kit, and between 0.1 and 0.15ug/ul for the later kit. The reactions were subjected to 15 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 70°C for 1 minute, followed by 15 cycles of 95°C for 30 seconds and 70°C for 1 minute. Following cycling the reactions were precipitated by the addition of 100ul of 100% ethanol and 3ul of 2M NaAc pH4.5. The reactions were left on ice for 15 minutes, then centrifuged at 13,000rpm for 20 minutes. They were rinsed with 250ul of 70% ethanol, then dried.

Dye Terminator Sequencing on Single Stranded PCR Products

Single stranded PCR products were produced and Dye Terminator Sequencing was conducted on the single stranded PCR Products according to the instructions in the Solid-Phase DNA Sequencing chapter of the Dynal technical handbook: Biomagnetic Techniques

in Molecular Biology. For each sample to be treated, 10ul(100ug) of Dynabeads were prepared by washing with 20ul of 2x binding and washing buffer, twice. The Dynabeads were then resuspended in 40ul of Binding and Washing buffer. PCRs were conducted with an excess of the non-biotinylated primer. The PCR products were immobolised onto Dynabeads by adding 40ul of PCR product to 40ul of pre-washed Dynabeads and leaving at room temperature for 15 mins, on a rotating wheel (gentle tipping), (with some additional gentle tapping). The tube was then placed in the Magnetic Particle Concentrator (MPC), and the supernatant was removed using a pipette. The beads were washed with 40ul of 2x Binding and Washing Buffer and the supernatant removed using the MPC. The beads were then resuspended in 8ul of 0.100M NaOH (freshly prepared or frozen for not more than 3 months), and left at room temperature for 5 mins. Using the MPC the the supernatant was removed to a clean tube and and the dynabeads were washed once each with 50ul of 0.100M NaOH, 40ul of 1x Binding and Washing Buffer, and 50ul of TE buffer. The beads were then resuspended in water to give a final concentration suitable for dye terminator sequencing. The PCR reactions were quantitated before commencing the Dynal procedure. The beads or eluted strands were then included in dye terminator sequencing reactions as per normal.

Following precipitation and drying all automated sequencing reactions were stored at 4°C in darkened containers for up to 5 days before electrophoresis. Sequencing samples were electrophoresed by Dr J Gecz on an ABI 373A DNA Sequencer, or on the same machine when it was relocated to the Australian Genome Reseach Facility in Brisbane.

Template Preparation for Dye Terminator Sequencing

Templates were prepared for dye terminator sequencing using the Prep-a-Gene (BioRad) or QIAquick (Qiagen) purification kits, according to manufacturer's instructions.

Prep-a-Gene: Purifications were conducted, according to the manufacturer's instructions (BioRad), by adding the appropriate volume of binding buffer then matrix to each sample, the samples were then left on ice for 10 minutes with occasional mixing, then centrifuged at 13,000rpm for 20 seconds. The samples were then washed twice with 500ul of binding

buffer, then three times with 500ul of wash buffer. Following the last centrifugation, all liquid was removed, and the samples were left to dry at room temperature. Each sample was then resuspended in 10ul of water, and left at room temperature for 10 minutes, followed by 10 minutes at 37°C. Following a two minute centrifugation at 13,000rpm, the supernatant (containing the purified DNA) was transferred to a new tube, then the centrifugation and the transfer were repeated to ensure minimal matrix contamination of the DNA sample.

QIAquick:

Ten ul of a 100ul PCR reaction were electrophoresed on agarose, to confirm that the PCR had worked, then the remaining volume of the PCR was removed from under oil and purified according to the instructions provided by the manufacturers (Qiagen). The PCR product was mixed with a 5xvolume of buffer PB. This mixture was added to a kit supplied column, and spun for 1 minute at 13,000rpm. The collection tube was drained, and 750ul of buffer PE was then centrifuged through the column for 1 minute. The collection tube was drained, then the column was centrifuged for a further 1 minute. The column was then placed in a clean flip top eppendorf tube, 50ul water was added to the column, and the column was centrifuged for 1 minute and the purified sample collected in the clean tube.

2.2.7 CELL CULTURE AND RNA ISOLATION

Cell Culture

The lymphoblast cell lines from CEPH/Utah pedigree 1345 were revived and maintained by Sharon Lane.

RNA Isolation and Transcription

All RNA manipulations were performed using gloves and where possible equipment exclusively for RNA work. Disposable centrifuge tubes were specifically prepared for RNA work and filtered Gilson pipette tips were used.

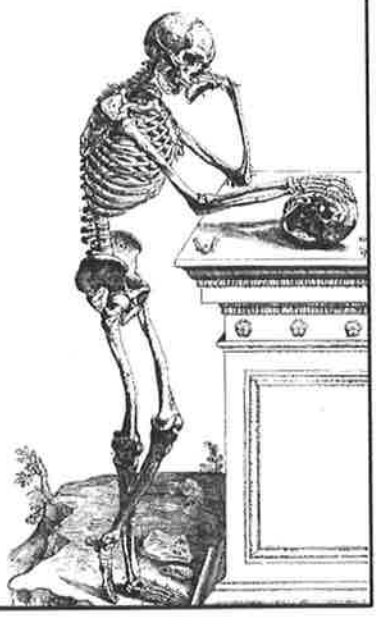
Total RNA was isolated from lymphoblast and fibroblast cells using Trizol (Gibco BRL) according to the manufacturers instructions. For every $1x10^7$ cells, 1ml of TRIZOL was added to the cell pellet, and immediately vortexed. (Generally worked with $1\text{-}4x10^7$ cells). The subsequent lump-free solution was split amongst a number of screw-top eppendorf tubes, and to each 1/10 volume of chloroform was added. Following 15s of vigorous shaking, and 5mins on ice, the tubes were centrifuged at 4°C (in Hettisch at 80%) for 15 mins. The upper, acqueous phase was removed to clean tubes and an equal volume of isopropanol was added to each. Following vortexing, the tubes were left at 4°C for 1 to 20 hours. The tubes were then centrifuged at 4°C (in Hettisch at 80%) for 15 mins. The supernatant was discarded and the tubes were rinsed with 75% ethanol, and air dried. Each tube was resuspended in 50ul of 1mM EDTA and the samples were combined and vortexed. One tenth of volume of 2M NaCl and 2 volumes of 100% ethanol were then added and following vortexing the tubes were left at -20°C for 1 hour. The tubes were centrifuged at 4°C (in a Hettisch Mikro Rapid /K Centrifuge at 80%) for 15 mins and then the pellets were rinsed with 75% ethanol. The mass of the pellets were estimated by eye and then the pellets were air dried. The pellets were resuspended in a volume of 1mM EDTA equal to the mass of pellet estimate, vortexed, then stored at -20°C for imminent use.

mRNA was isolated from lymphoblast cells using the mRNA Direct kit from Dynal, according to the manufacturer's instructions. One ml of lysis binding buffer was added to each of 4 tubes, and the PBS-washed cells were resuspended by pipetting up and down. The viscosity of the resultant solution was reduced by forcing it through a 21 guage needle. Half of the lysate was added to 250ul beads and after 5 mins at room temp the tube was placed in the MPC and the supernatant removed. The other half of the lysate was then added and following 5 mins at room temp the supernatant was removed using the MPC. One ml of wash buffer with LiDs was added and mixed for 5 mins, then removed following 5 mins in the MPC. The beads were then washed, in this way, 3 times with wash buffer (without LiDS). Twenty ul of elution solution was then added and the tube incubated at 65°C for at least 2 mins. The solution was then separated from the beads using the MPC, and stored at -20°C.

The beads were regenerated for subsequent use by resuspending them in 200ul of reconditioning solution, transferring to a new tube, shaking, 2 mins incubation at 65°C, and following 30s in MPC the supernatant was removed. This was procedure repeated twice without the 65°C incubation. The beads were then resuspended in 200ul of storage buffer, mixed then the supernatant removed using the MPC. This procedure was repeated twice, then the regenerated beads were stored at 4°C.

First strand cDNA was synthesised from the RNA preparations using Superscript reverse transriptase (SUPERSCRIPT Rnase H⁻ Reverse Transcriptase, Gibco BRL). To 1.5ul total RNA was added 1ul random hexamer (for random primed cDNA), 8.5ul of sterile distilled water (DEPC treated), and the solution was centrifuged briefly. Following 10mins at 70°C, brief ice incubation, and another brief centrifugation, 4ul of 5x1st strand buffer, 2ul 0.1M DTT and 1ul mixed dNTP's (pH neutral, 10mM of each) were added. This solution was gently vortexed and briefly centrifuged, then incubated at 42°C for 2 mins. One ul (200units) of Superscript reverse transcriptase was added, the solution was mixed gently, then incubated at 42°C for 1hour, then the tubes stored at -20°C. PCRs were conducted on 1ul of cDNA. Positive controls for PCRs on the cDNA were provided using primers to the housekeeping gene Esterase D.

CHAPTER 3: LINKAGE MAPPING OF A CRANIOSYNOSTOSIS SYNDROME



3.1 SUMMARY

Linkage mapping was undertaken to determine the chromosomal location of the gene responsible for an autosomal dominant craniosynostosis syndrome segregating in a large South Australian kindred. Candidate regions on chromosomes 5 and 7 were excluded by others, then exclusion mapping was conducted by testing markers spread at approximately 20cM intervals throughout the genome until linkage to chromosome 4 was detected. A maximum two-point lod score of 6.2 at a recombination frequency of zero with marker D4S412 demonstrated linkage. The gene responsible for the craniosynostosis in this family was localised to chromosome 4p16 telomeric to marker D4S394 (Hollway *et al.*, 1995, Appendix 3.1). No craniosynostosis was known to map to this region; hence this study delineated a new disorder named Craniosynostosis Adelaide Type. Positional candidate genes from the region are *FGFR3*, *MSX1* and *ZNF141*.

3.2 INTRODUCTION

The first two craniofacial syndromes to be mapped were Saethre Chotzen syndrome and Greig cephalopolysyndactyly. Saethre Chotzen syndrome was localised by linkage mapping to distal chromosome 7p (in the 7p21 region) which confirmed earlier associations between chromosomal abnormalities involving 7p and craniosynostosis (Brueton *et al.*, 1992). Howard *et al.* (1997) and El Ghouzzi *et al.* (1997) found various mutations of the *TWIST* gene (including deletions, missense and nonsense mutations) in some cases of Saethre Chotzen syndrome. The *TWIST* gene codes for a basic helix-loop-helix transcription factor, and the human gene was cloned and the genomic sequence determined by Wang *et al.* (1997). Howard *et al.* (1997) found evidence in *Drosophila melanogaster* that Twist may affect the transcription of the FGFRs. Translocations involving breakpoints near (but not interrupting the coding sequence of) the *TWIST* gene have also been reported in some cases of Saethre Chotzen syndrome (Krebs *et al.*, 1997; Rose *et al.*, 1997).

Greig cephalopolysyndactyly was also assigned to 7p (in the 7p13 region); Tommerup and Nielsen (1983) found a reciprocal translocation between chromosomes 3 and 7 to be associated with Greig cephalopolysyndactyly, (t(3;7)(p21.1;p13)). Later, Brueton *et al.*

(1988) demonstrated linkage between markers in 7p12-13 and Greig cephalopolysyndactyly and Pettigrew *et al.* (1991) confirmed the importance of this region when they reported a Greig cephalopolysyndactyly patient with a *de novo* interstitial deletion of chromosome 7p with breakpoints located at p13 and p14. Vortkamp *et al.* (1991) implicated the zinc finger protein gene, *GLI3*, as the gene responsible for Greig cephalopolysyndactyly.

Warman *et al.* (1993) described a new autosomal dominant craniosynostosis syndrome which is now known as craniosynostosis Boston type, and was mapped to the long arm of chromosome 5 (5q34-qter) (Muller *et al.*, 1993). Craniosynostosis Boston type was found to be caused by a mutation in the human *MSX2* gene (Jabs *et al.*, 1993).

For the cause of the craniosynostosis syndrome being studied in this project, candidate regions of the genome were those regions where craniosynostosis syndromes had already been mapped, such as 5q34-qter and 7p13. Other candidate regions included all sites where homeobox and zinc finger genes had been mapped, since these genes have been shown to play important roles in the regulation of development (for example Krumlauf, 1994 and Wilkinson *et al.*, 1989). It was planned to use linkage analysis to determine the chromosomal location of the gene causing craniosynostosis in this large South Australian kindred and refine the localisation as a prelude to gene identification. After the main candidate regions were excluded by linkage, a genome-wide scan was commenced and linkage to chromosome 4p was detected. During the course of this project considerable progress was made toward the genetic mapping and delineatiation of other craniosynostosis syndromes.

3.3 MATERIALS AND METHODS

Clinical Description

DNA was extracted from blood collected from 35 individuals of a large pedigree with a clinically characterised craniosynostosis syndrome (Appendix 3.2 and Fig. 3.1). Family members were clinically and radiologically examined by Dr Eric Haan and Dr Lesley Ades, clinical geneticists from the Department of Medical Genetics, WCH. In summary, facial

asymmetry was found in all clinically affected individuals. Pedigree members IV-16 and IV-19 had no facial asymmetry, appeared clinically unaffected and were diagnosed as affected purely on the basis of radiological findings, demonstrating the importance of radiology in characterisation of the family members. Other clinical features seen in affected family members included a broad forehead, turricephaly, craniosynostosis (which when present involved the coronal sutures exclusively) and deafness. The deafness component of the phenotype is further explored in a second large family, the subject of Chapter 9. Clinical features less commonly observed included low anterior hairline, hypertelorism, ptosis, strabismus, nasal beaking, maxillary hypoplasia, mandibular prognathism, mental retardation (ranging from borderline to moderate) and brachydactyly of the hands and or feet. Features of Jackson Weiss syndrome which were absent from the affected members of this pedigree included prominent ear crura, cutaneous syndactyly and broad great toes (Appendix 3.2 - Ades *et al.*, 1994). Although the diagnosis of Jackson Weiss syndrome was the most likely, the possibility existed that this was a previously undescribed syndrome.

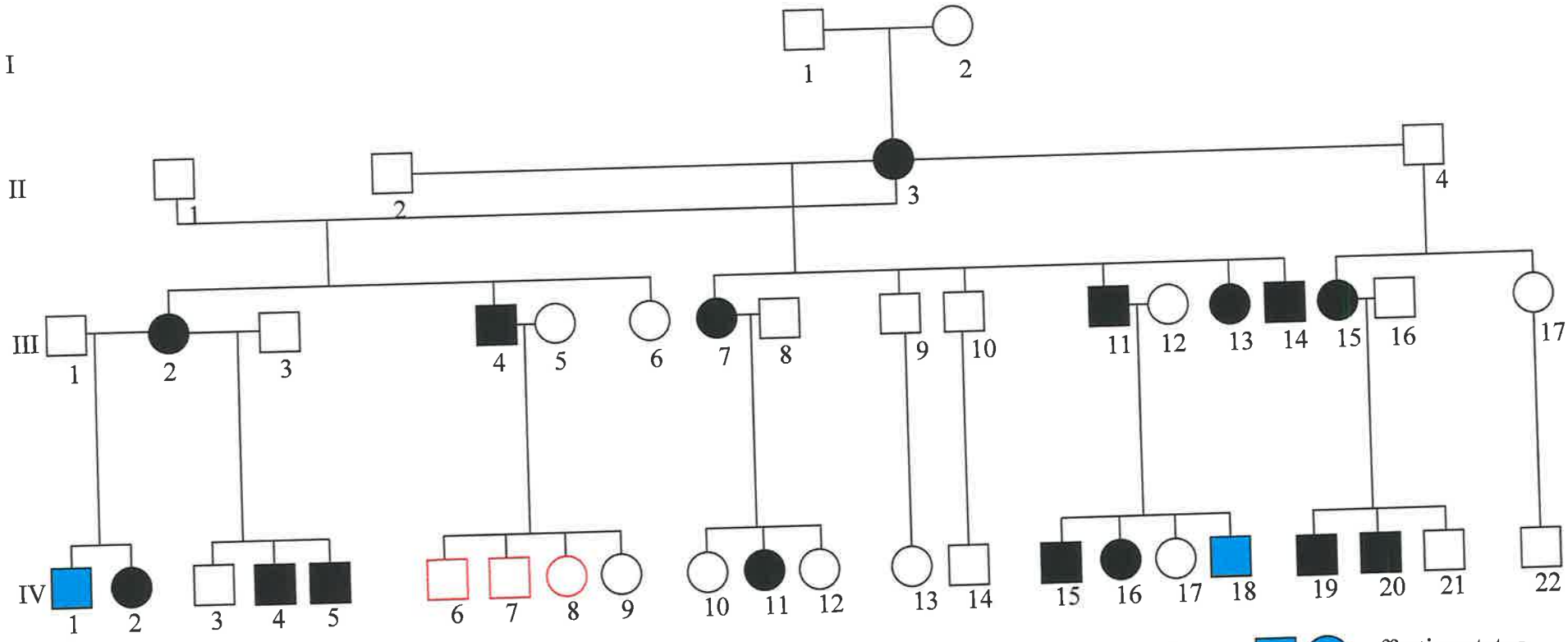


Fig 3.1 : Pedigree of a large South Australian family segregating an autosomal dominant craniosynostosis syndrome. -affection status unknown, -90% penetrance. DNA was obtained from all pedigree members except I-1, I-2, II-1, II-2, III-1, III-3, III-8, III-16, IV-13 and IV-14.

Laboratory DNA numbers for those pedigree members from whom DNA was obtained.

II-3	3217	III-6	3352	III-12	3257	IV-1	3358	IV-6	3234	IV-11	3215	IV-18	3362
II-4	3252	III-7	3212	III-13	3214	IV-2	3353	IV-7	3235	IV-12	3216	IV-19	3238
III-2	3159	III-9	3327	III-14	3213	IV-3	3332	IV-8	3236	IV-15	3360	IV-20	3211
III-4	3232	III-10	3228	III-15	3209	IV-4	3160	IV-9	3237	IV-16	3229	IV-21	3210
III-5	3233	III-11	3256	III-17	3254	IV-5	3161	IV-10	3253	IV-17	3361	IV-22	3255

The most common radiological feature of the hands was middle phalangeal hypoplasia of index, ring and little fingers. Less common radiological features were coned epiphyses and carpal bone segmentation abnormalities. The most common radiologic features of the feet were congenital fusion of the cuboid and calcaneum, also phalangeal fusion and hallux vagus. Less common features of the feet were coned epiphyses and variable patterns of other tarsal and/or metatarsophalangeal fusions (Appendix 3.2 - Ades *et al.*, 1994).

Prior to the commencement of genotyping, an hypothetical marker with close linkage to the disease gene was analysed to confirm that detection of linkage was feasible in this pedigree. This resulted in a maximum lod score of 6.62 at a recombination fraction of zero (Fig 3.3) and thus it was concluded that it should be possible to map a disease causing locus in this pedigree. This was not unexpected given the large size of the pedigree and the reasonably high number of affected individuals within the pedigree. Alternatively the SLINK simulation program (Weeks *et al.*, 1990) could have been used; however, this was unnecessary given that there was no doubt that this pedigree was of sufficient size to detect linkage.

Genotyping

Polymorphic AC repeat markers were chosen approximately every 20cM along the autosomes, such that the disease gene would never be more than 10cM from a marker locus. Markers were primarily selected from the maps of Weissenbach *et. al.* (1992) and then Gyapay *et al.* (1994). The primer pairs of each chosen marker were prepared and the polymorphic AC regions amplified as described in Chapter 2.

Linkage Analysis

The craniosynostosis syndrome was analysed as an autosomal dominant trait with 100% penetrance in all individuals who were clinically and radiographically examined. Three individuals (IV-6 (3234), IV-7 (3235) and IV-8 (3236), Fig 3.1) were clinically examined and appeared normal, but no radiographs could be obtained. They were coded as unaffected, with 90% penetrance at the disease locus to take into account the possibility that they may have features of the disorder detectable only by radiology.

III-5 (3352) was unavailable for clinical examination but radiographs of the hands and feet were normal therefore they were coded as unaffected since there were no obligate carriers without radiological abnormalities in this pedigree (Ades *et al.*, 1994). IV-3 (3332) was not available for clinical or radiographic assessment, and IV-18 (3362) was assessed clinically and radiologically but affectation status was unclear due to young age. These two individuals were coded as unknown affection status for the linkage analysis.

The data were analysed assuming the frequency of the affected allele to be 0.0001. When a marker had more than 7 alleles it was recoded down to 7 alleles to overcome memory constraints associated with computer analysis. The analysis was further simplified by the use of equal allele frequencies for the different alleles of each particular marker. When linkage was detected, marker alleles were identifed (from CEPH individual 134702) and exact allele frequencies were used to compute accurate lod scores. Exact allele frequencies were used in the analysis of markers D4S412, D4S432 and D4S394, equal allele frequencies were used for the other markers analysed. When there are multiple missing family members lod scores can be affected by the incorrect specification of marker allele frequencies however generally the detection of true linkage is not prevented (Freimer *et al.*, 1993).

The genotyping data were originally analysed according to the pedigree given in Fig 3.1, however widespread non-paternity was consistently found among the children of 3217. Most of the fathers were not available, however non-paternity was inferred when sibships were found to have more than four marker alleles for each of several marker loci. To overcome this problem the pedigree was redrawn so that many of the individuals in the third generation of the pedigree had separate fathers, and these fathers were coded as unaffected (Fig 3.2). New genotyping data were analysed according to this pedigree and pre-existing data were reanalysed with the new pedigree arrangement. This reduced the power of the analysis to detect linkage; however the risk of not detecting linkage because of using an incorrect pedigree needed to be addressed. The power of the redrawn pedigree remained sufficient to detect linkage.

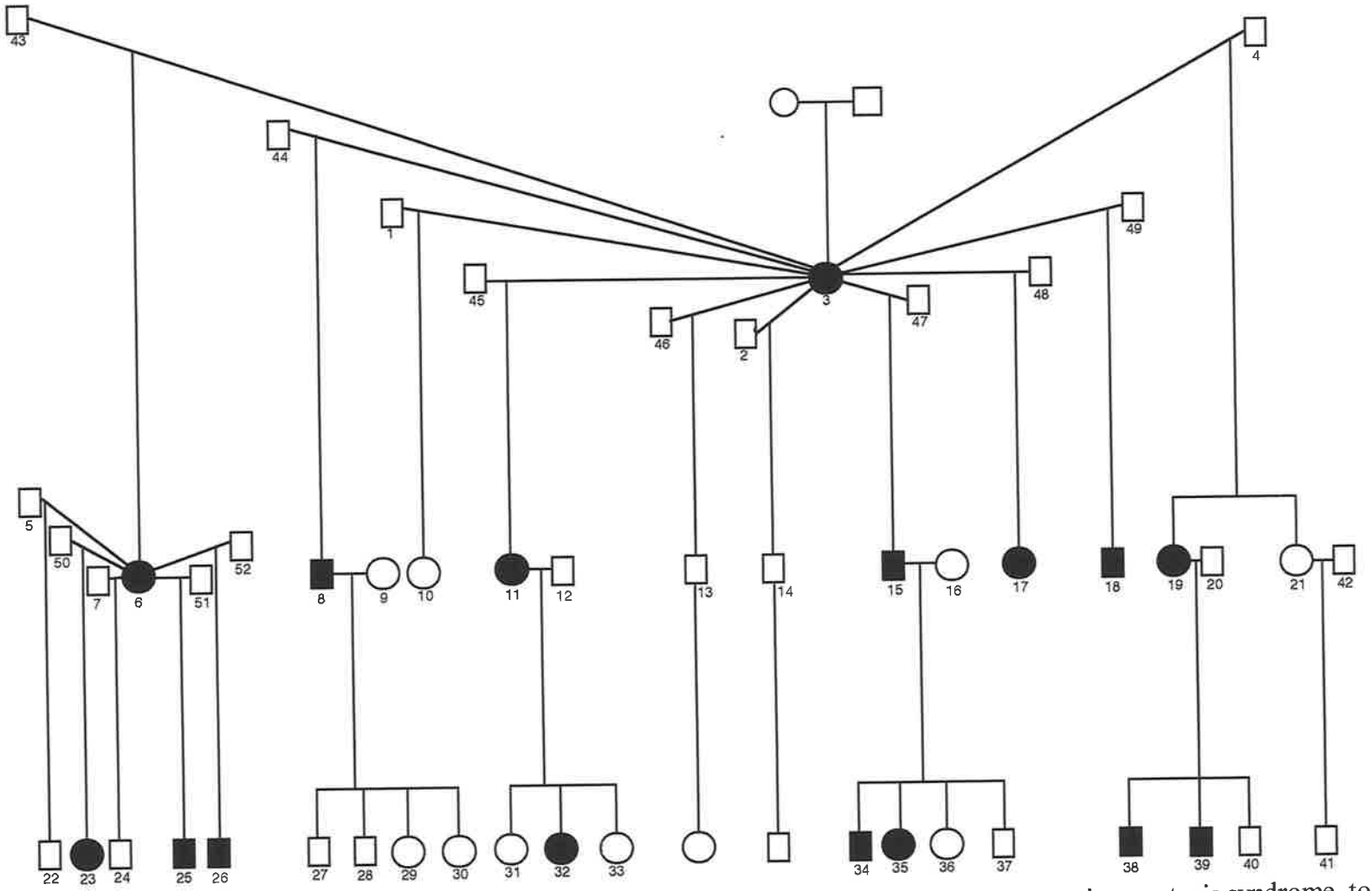
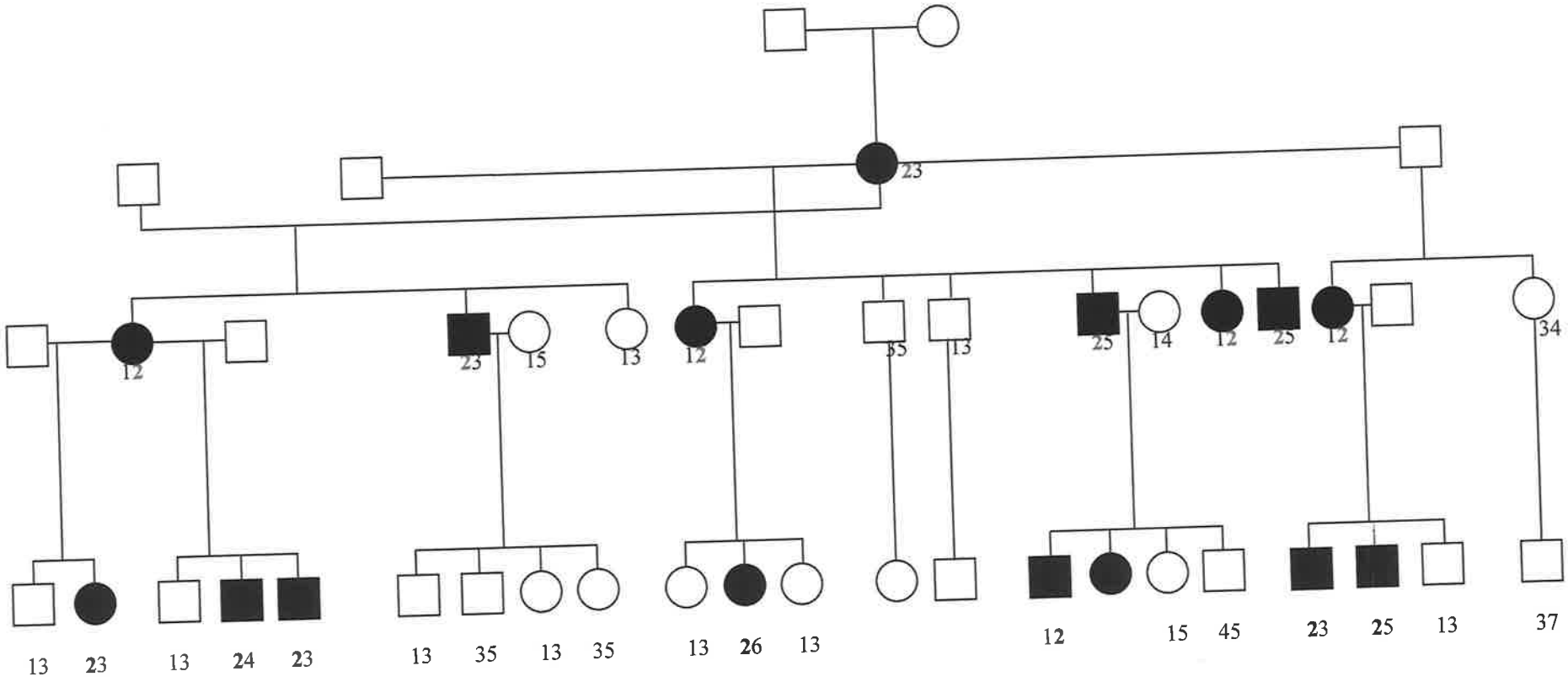


Fig 3.2 : Redrawn pedigree of a large South Australian family segregating an autosomal dominant craniosynostosis syndrome, to account for non-paternity.

Fig 3.3 : Genotyping of an hypothetical marker with close linkage to the disease gene, and the resultant lod scores.

Recombination Fraction (theta)	0.0	0.01	0.05	0.1	0.2	0.3	0.4
Lod Score	6.62	6.51	6.07	5.50	4.25	2.87	1.35



The genotypes generated by the markers tested were analysed by two-point linkage analyses (testing for linkage between the marker and a disease locus) using the MLINK program of the LINKAGE program package Version 5.1 (Lathrop and Lalouel, 1984). This LINKAGE program package is writen in pascal and is used for linkage analysis and genetic risk calculation with an arbitrary number of loci. MLINK, a program within the package, can be used for the calculation of lod scores or location scores and for risk analysis. The program has the capacity to interpret disease phenotypes as presence/absence of a trait, affection status or as quantitative measurements. Multiple alleles at marker loci and incomplete penetrance at the disease locus can be specified.

The MLINK program which calculates the lod scores is supported by a number of programs which are used to enter the data. Pedigree and phenotype information and the marker results to be analysed are entered by creating an infile with the pedigree data in the form: pedigree identification (ID) number, individual ID number, father ID number, mother ID number, sex, affection status, liability class (to cater for different penetrance classes, such as variable age of onset or different level of clinical investigations) and results for the markers to be tested. This is then converted to a PEDFILE (different form), using the program MAKEPED. The DATAFILE is created or modified using the menudriven program PREPLINK. The DATAFILE contains information specifying the number of loci, descriptions of the loci (number of alleles and the frequency of each) and the recombination parameters to be used in the analysis.

3.4 RESULTS

Before commencing the linkage mapping, cytogenetic analysis was conducted (by the cytogenetics section of the Department of Cytogenetics and Molecular Genetics, WCH) on four affected pedigree members (III-14 (3213), IV-16 (3229), III-11 (3256) and IV-15 (3360)). This did not detect any translocation or microscopically detectable deletion associated with the disease as all had normal karyotypes.

Regions on chromosomes 1, 5, 7, 14, 15, 16 and 19 were excluded by Dr Lesley Ades and Isa Senga (Appendix 3.2 and Senga, 1993-Honours thesis). A systematic scan of the rest of the genome was then commenced, using microsatellite markers spaced at approximately 20cM intervals along the autosomes. Two hundred and thirty polymorphic markers were examined (and the majority of the genome excluded) prior to the detection of linkage on chromosome 4p (Appendix 3.3). When linkage was initially detected, the localisation was defined as distal of marker D4S394. A recombination event occured in IV-10 (3253) with marker D4S394 to give this proximal boundary. The distal boundary of the initial localisation was the 4p telomere since a distal recombinant could not be found. The markers initially tested within this region were HD, D4S412, D4S432 and D4S394. The maximum lod score was 6.20 with marker D4S412 (Table 3.1). Later, more markers were tested as they became available: D4S3038, D4S2936, *FGFR3* (exon 6) polymorphism (Chapter 5), D4S1614, D4S3034, D4S2925, D4S3023, *MSX1* polymorphisms (Chapter 4) and D4S431. An example of the results of AC repeat marker electrophoresis is shown in Fig. 3.4. The lod scores resulting from chromosome 4 AC repeat marker analysis are shown in Table 3.1. The haplotypes of the pedigree members resulting from the chromosome 4 markers tested are shown in Fig. 3.5.

Fig 3.4 : AC Repeat Marker D4S431, on Craniosynostosis Adelaide Type family

Lane loadings:
1:III-2, 2:IV-15, 3:IV-5, 4:III-15, 5:IV-21, 6:IV-20, 7:III-7, 8:III-14, 9:III-13, 10:IV-11, 11:IV-12, 12:II-3, 13:III-10, 14:IV-16, 15:III-4, 16:III-5, 17:IV-6, 18:IV-7, 19:IV-8, 20:IV-17, 21:IV-19, 22:II-4, 23:IV-10, 24:III-17, 25:IV-22, 26:III-11, 27:III-12, 28:III-9, 29:IV-18, 30:IV-3, 31:III-6, 32:IV-2, 33:IV-1, 34:IV-9, 35:IV-4

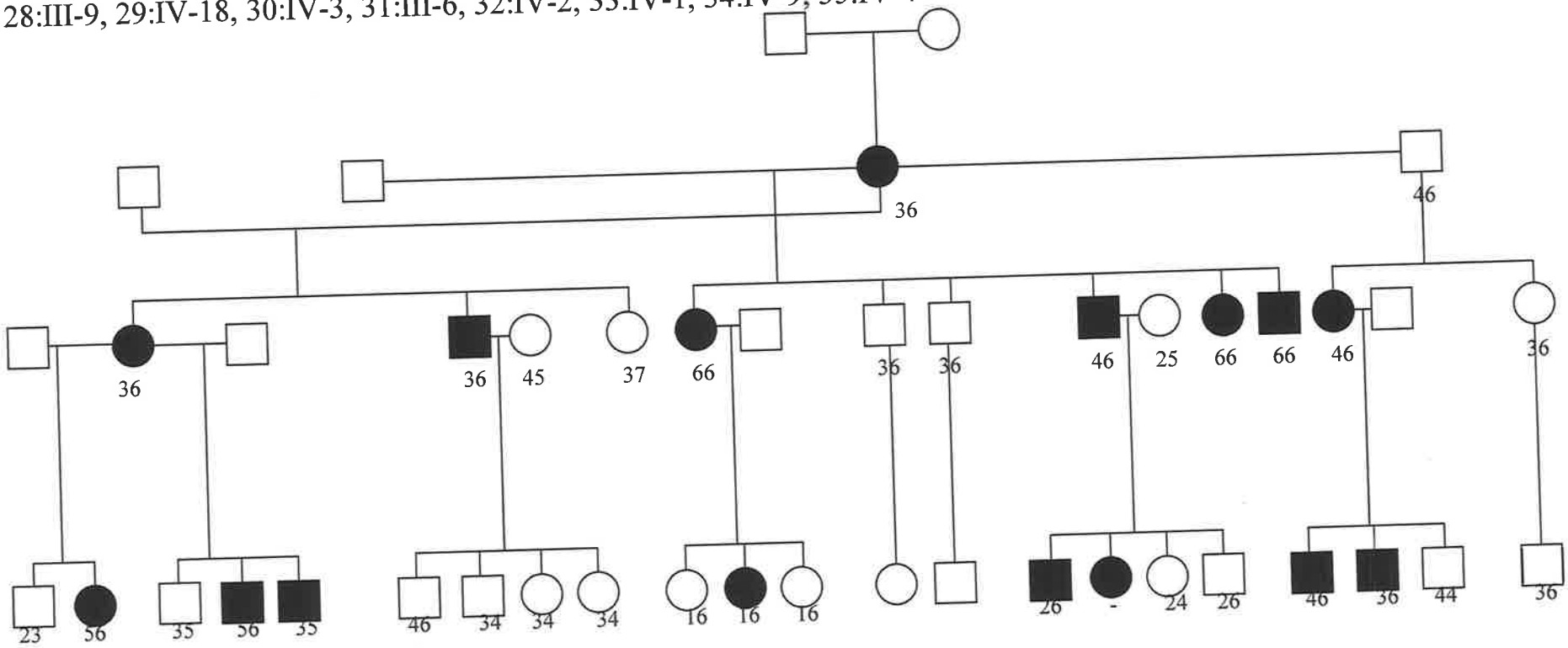


Table 3.1 : Lod scores for chromosome 4 markers.

θ	0.0	0.01	0.05	0.1	0.2	0.3	0.4
Marker							
HD*	3.65	3.63	3.49	3.26	2.68	1.95	1.07
D4S3038	3.49	3.44	3.23	2.95	2.36	1.68	0.91
D4S2936[#]	5.55	5.46	5.09	4.61	3.56	2.39	1.10
FGFR3	1.78	1.75	1.64	1.49	1.18	0.83	0.44
D4S1614	2.39	2.38	2.32	2.19	1.82	1.34	0.74
D4S3034	3.65	3.62	3.46	3.21	2.58	1.79	0.86
D4S412	6.20	6.13	5.82	5.36	4.30	3.02	1.52
2cM							
D4S432	2.21	2.21	2.17	2.06	1.73	1.28	0.71
D4S2925	-	1.34	2.44	2.64	2.37	1.74	0.86
1cM							
D4S3023[$]	4.46	4.39	4.10	3.73	2.92	2.01	0.96
Ex1 1st	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deletion	1.68	1.66	1.56	1.42	1.13	0.81	0.44
Ex2 1st	0.55	0.53	0.46	0.37	0.22	0.1	0.02
(AC)n	0.64	0.64	0.63	0.61	0.52	0.40	0.22
D4S431	-	1.95	2.46	2.46	2.05	1.40	0.62
3cM							
D4S394	-	2.94	3.33	3.24	2.67	1.87	0.89

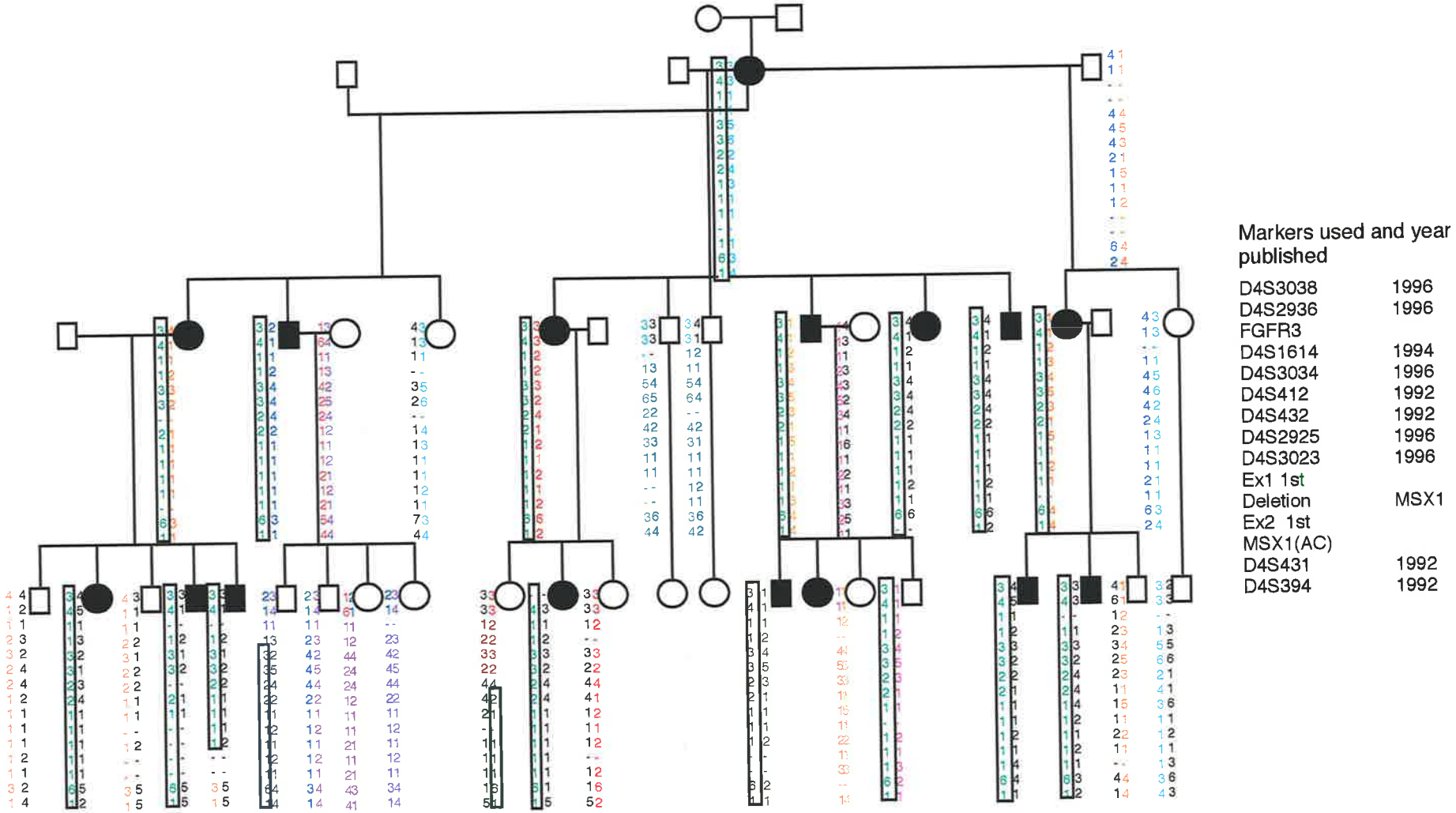
*the trinucleotide repeat responsible for Huntington's disease

[#]D4S2936-D4S1614=3cM

[$]D4S3023-D4S431=4cM

Ex1 1st, Deletion, Ex2 1st and (AC)n are polymorphisms within the MSX1 gene (Chapter 4).

Fig 3.5 : Chromosome 4p haplotypes in large South Australian family segregating an autosomal dominant craniosynostosis syndrome.



Markers used and year published	
D4S3038	1996
D4S2936	1996
FGFR3	
D4S1614	1994
D4S3034	1996
D4S412	1992
D4S432	1992
D4S2925	1996
D4S3023	1996
Ex1 1st Deletion	MSX1
Ex2 1st MSX1(AC)	
D4S431	1992
D4S394	1992

Multipoint analysis was not necessary since the high lod score clearly indicated linkage, and the majority of the rest of the genome had been excluded.

3.5 DISCUSSION

Regions on chromosomes 1, 16 and 19 were excluded by Dr Lesley Ades (Appendix 3.2). These regions were chosen because some of them were considered to be gene-rich and others because makers were readily available in these regions at the time. The candidate regions of 7p (Saethre-Chotzen syndrome) and 5qter (Craniosynostosis Boston type) were also excluded (Appendix 3.2). Isa Senga then examined additional microsatellite markers from chromosomes 5, 7, 15 and 19 to complete the exclusion of these chromosomes, and then excluded the gene from chromosome 14 (Isa Senga-Honours thesis 1993). Chromosome 7 was analysed because it has zinc finger and homeobox genes, and in addition, both Saethre Chotzen and Greig cephalopolysyndactyly had been mapped to this chromosome (Brueton *et al.*, 1992 and Brueton *et al.*, 1988). Chromosome 19 was examined because it has many of the known zinc finger loci (Lichter *et al.*, 1992). Markers on chromosome 15 were analysed because there had been reports of an association between craniosynostosis and this region. Pederson (1976) reported a patient with craniosynostosis (clover leaf skull anomaly), hydrocephalus, many malformations and severe mental retardation, who had partial trisomy of chromosome 15, as a result of an unbalanced 12/15 translocation. Pederson (1976) proposed that there may be an association between the craniosynostosis and the partial trisomy 15. Van Allen *et al.* (1992) reported complex craniosynostosis (involving sagittal, metopic, lambdoid and coronal sutures) and other congenital abnormalities in an infant with duplication of 15q and deletion of 2q. Considering their patient and the report of Pederson (1976), Van Allen *et al.* (1992) proposed there may be a gene critical for correct synostosis of the sutures on chromosome 15. Chromosome 14 was analysed because of the presence of zinc finger and homeobox genes on this chromosome.

Following the exclusion of the main candidate regions (the known sites of craniosynostosis genes), the remaining candidate regions for the disorder included all chromosomal regions

containing known zinc finger and homeobox genes since they had been shown to play significant roles in the regulation of development. Either these regions could have been specifically targetted or a systematic scan of the rest of the genome undertaken. There were a number of homeobox and zinc finger genes mapped at this time, zinc finger genes had been mapped to chromosomes 1, 2, 3, 5, 7, 8, 10, 11, 12, 16, 17, 18, 19, 20, 22, X and Y (Lichter *et al.*, 1992) and homeobox gene clusters were known to map to chromosomes 2, 7, 12 and 17 (Boncinelli *et al.*, 1988), plus additional isolated homeobox genes on other chromosomes. Also, difficulties can be encountered when trying to select markers on a linkage map to correspond to the locations of mapped genes (however, this problem should diminish with the advent of future comprehensive maps incorporating radiation hybrid data). Because of these factors, it was decided to proceed with a systematic scan of the rest of the genome. Also if linkage was not detected at the candidate loci it would have then been necessary to fill in the non-excluded regions to complete a genome wide scan, as there was no guarantee that zinc finger or homeobox genes would be involved.

Linkage to marker D4S412 (on chromosome 4p) was detected after the majority of the genome had been excluded. The gene was initially localised to 4p, distal to marker D4S394, a region of at least 12cM (Gyapay *et al.*, 1994). Included in this initial localisation were the candidate genes *MSX1*, *FGFR3* and *ZNF141*. *MSX1* is a member of the same homeobox family as *MSX2*, responsible for Craniosynostosis Boston type. *ZNF141* is a zinc finger gene which maps to 4p16.3/D4S90 and was a candidate gene for Wolf-Hirschhorn syndrome (Tommerup *et al.*, 1993). *FGFR3* became a candidate after the initial localisation was determined, as gene family members *FGFR1* and *FGFR2* were implicated in other craniosynostosis disorders during the course of the present study (Chapter 5).

Later as more markers became available, with the publication of Gyapay *et al.* 1994 and Dib *et al.* 1996, and were tested, the haplotypes became clearer (Fig 3.5). The *MSX1* and *FGFR3* polymorphisms described in chapters 4 and 5 respectively, were also used. IV-10

(3253) has a recombination event between the 2 main candidate genes *MSX1* and *FGFR3*. However the clinicians then became unsure as to whether or not IV-10(3253) was unaffected (they considered there to be a slight chance that this individual was affected, considering the very variable phenotype of this disorder). Thus, the recombination could not safely be used to exclude *MSX1* as a candidate gene. Individual IV-6 (3234) has a recombination event between the two candidate genes, however this could not be used to exclude any candidate genes since this person was clinically normal, but they could not be radiographed, so their diagnosis was not certain. Another individual, IV-5 (3161), has a recombination event with marker D4S431, so the localisation initially published is not in question.

Just prior to the detection of linkage the majority of the genome had been scanned (Appendix 3.3). At this point there was a concern that the initial genome scan would fail to detect linkage. Undetected non-paternity was ruled out since the pedigree had been redrawn. To accomodate the possibility that the disorder was non-penetrant in a proportion of family members with the gene, it was planned to reanalyse the data, with less than 100% penetrance for the individuals examined both clinically and radiologically. Plans were also made to analyse the lod score data using the EXCLUDE program (Edwards, 1989) in order to highlight regions of the genome not adequately excluded, due to reduced informativeness of some of the markers used in the genome screen or excessive gaps between informative markers. However in the late stages of the genome screen linkage was detected and the contingencies just discussed did not need to be invoked.

When this mapping project commenced there were a number of possible candidate regions to test for linkage, the sites of craniosynostosis disorders that had already been mapped. The strong association between craniosynostosis and FGFR genes that has recently been uncovered, was not known then. Following the exclusion of the candidate regions, a systematic linkage analysis of the remaining portions of the genome was commenced. Since this time strategies have been published to target gene rich regions of the genome in

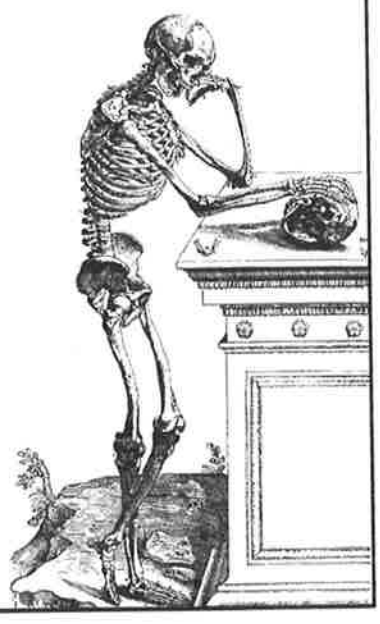
linkage mapping studies, to make such studies more efficient. Because of the non-random distribution of genes throughout the genome Antonarakis (1994) proposed that in order to speed up linkage mapping projects the GC-rich/gene-rich portions of the genome should be targeted first. Then if this does not bring success, proceed with a systematic scan. In principle this is how the Craniosynostosis Adelaide Type mapping project was conducted, when the most likely candidate regions were excluded, a systematic scan was commenced. Antonarakis (1994) also noted that selecting markers to screen particular areas can be difficult because DNA segments are not precisely localised to chromosomal bands, as yet. Inglehearn (1997) published two sets of markers to conduct intelligent linkage analysis. Following on from the work of Antonarakis, the set A markers screen one tenth of the genetic map, yet screen over one quarter of the localised ESTs (approximately). Set B screens some additional gene dense regions. Had this information been available at the time of the Craniosynostosis Adelaide Type linkage mapping project, these sets of markers could have been used following the exclusion of the candidate regions. Another option available today is automated genotyping, which would have been much faster than the manual methods used in this study.

The syndrome present in this pedigree was renamed Craniosynostosis Adelaide type (CAT) since it mapped to a different location from JWS, which was by this time mapped to chromosome 10q23-q26 (Li *et al.*, 1994), and phenotypic differences between this family and JWS had been noted (Ades *et al.*, 1994). This family was originally reported as having JWS (Ades *et al.*, 1994), however even then differences were noted in phenotype, it was concluded at the time that the features of the affected individuals were more like JWS than any of the other craniosynostosis syndromes described. Some features present in the Craniosynostosis Adelaide Type family that are generally absent from JWS were low frontal hairline, dental malocclusion, impaired hearing and short stature. There were also features absent from the Craniosynostosis Adelaide Type family that are sometimes seen in JWS, these include flat nasal bridge, cleft palate, cutaneous syndactyly, broad great toes, varus deformities of the great toes and occasionally large thumbs with varus deformities (Ades *et al.*, 1994).

The original diagnosis of JWS was based on radiological changes in the feet that have been reported in JWS. In spite of this there were still differences in the radiological abnormalities of the hands and feet and Ades *et al.* (1994) described abnormalities not previously reported in JWS individuals. They concluded the radiological examination may not have been done in some cases and noted its importance since some individuals with radiological abnormalities appeared clinically normal.

In hindsight it is clear that these differences represented the first description of the phenotype of a new syndrome. Craniosynostosis Adelaide type is considered to be a syndrome since in addition to craniosynostosis there are hand and foot abnormalities, mental retardation and hearing defects variously present in affected individuals. Further documentation of this syndrome from a large collection of families is given in Chapter 5. This is now recognised as a relatively common craniosynostosis syndrome among patients attending craniofacial clinics.

CHAPTER 4: MUTATION SEARCH IN *MSX1*



4.1 SUMMARY

Craniosynostosis Adelaide Type was mapped to 4p16 by linkage and within the localisation were three candidate genes *MSX1*, *FGFR3* and *ZNF141*. *MSX1* and *ZNF141* were considered candidate genes because both homeobox and zinc finger proteins have been shown to be important in the regulation of development. Further evidence to support *MSX1* being a candidate gene included the fact that *MSX1*$^{-}$ (null) mice have developmental abnormalities including cleft secondary palate, failure of tooth development and skull abnormalities, and *MSX2* (also from the *MSX* gene family) has been shown to be mutated in Craniosynostosis Boston Type. Mutation detection was conducted on *MSX1* in some affected and unaffected members of the CAT family. This resulted in the detection of three polymorphisms, but no mutations. The polymorphisms detected were a C539G base change in exon 1, an 11bp deletion in intron 1 and an heteroduplex change in exon 2 (Appendix 4.1). The focus of mutation screening then shifted to FGFR3 (Chapter 5). A mutation detection screen of *ZNF141* was planned, but was abandoned when a mutation in *FGFR3* was implicated as the cause of Craniosynostosis Adelaide Type.

4.2 INTRODUCTION

There is considerable evidence accumulating which indicates that homeobox genes and zinc finger genes play important roles in the regulation of development in animals.

Homebox Genes

Homeobox genes code for proteins with a homeodomain, which is a 60 amino acid conserved DNA-binding domain, coded for by a conserved region called the homeobox. This domain is assumed to have arisen early in evolution (estimated to be at least one billion years ago) because it is present in the proteins of fungi, plants and animals and insects (Scott, 1992) and there appears to be strong evolutionary pressure for the conservation of these sequences (Gehring *et al.*, 1994). The homeodomain was originally discovered in *D. melanogaster* homeotic genes, where mutations lead to the replacement of one body structure with a different body structure (a homeotic mutation), and this implies a defect in a regulatory gene (Scott, 1992).

Genes of the homeobox family are involved in the genetic control of development, especially the control of the anterior-posterior body plan. Asymmetry is established during early embryogenesis, to provide cells with specific identities along the anterior-posterior (A-P) axis. The genetic system used to accomplish this in *D. melanogaster* has been found to be conserved across many species, including vertebrates, to the extent that the *Pax-6*-equivalent gene from mouse (called *Sey*) can be used to induce ectopic eyes in *D. melanogaster* (Gehring, 1996). Homeobox genes have been shown to be involved in limb, skeletal, craniofacial and central nervous system development.

The homeobox genes have been divided into various classes on the basis of amino acid similarities of the homeodomain and flanking protein sequence. An example of this is the *Antp* class, the homeodomains from the members of this class share at least 60% identity with the version of the homeodomain that is present in the *D. melanogaster* gene, *Antennapedia*, and meet a number of other criteria relating to the amino acid sequence of the homeodomain and surrounds (Akam, 1989). The *Antp* class includes the *Hox* genes, which are clustered in four unlinked gene complexes in the human genome on chromosomes 2, 7, 12 and 17 (Boncinelli *et al.*, 1988). The *Hox* genes are also clustered in zebrafish, Xenopus and chicken (Akam, 1989), in addition many homeotic genes are clustered in complexes in insects and in mammals (Scott, 1992). In fact *Hox* genes are organised into complexes in all vertebrates so far examined, hence Graham *et al.* (1989) believe that there is strong evidence for a common ancestral origin of these complexes. The four *Hox* clusters are now known as A, B, C and D, and the *Hox* genes are numbered 1 - 13 within each cluster, thus each *Hox* gene is referred to by a letter which identifies which cluster it comes from, and a number which identifies which gene in the cluster (Scott, 1992). There are approximately ten genes in each cluster and they all lie in the same transcriptional orientation (Krumlauf *et al.*, 1993). In *D. melanogaster* there are two separate but adjacent complexes of homeobox genes, the *Antennapedia* (*ANT-C*) and *Bithorax* (*BX-C*) complexes and the genes of these complexes are related to the Hox genes of vertebrates (Boncinelli *et al.*, 1988; Graham *et al.*, 1989; Duboule and Dolle, 1989).

Lewis (1978) noted that the order of genes along the chromosome in the *D. melanogaster* Bithorax gene complex (*BX-C*) corresponded to their domains of expression along the anterior-posterior axis of the organism. This phenomenon was subsequently found in the Hox complexes of a number of other organisms.

Dolle *et al.* (1989) suggested the timing of expression of genes of the *Hox5* complex in the murine limb bud may be related to their position in the complex. Graham *et al.* (1989) concluded that the relative domain of expression of the gene along the anteroposterior axis of the embryo is related to the position of gene in the *Hox2* cluster.

In fact, a relationship between the chromosomal arrangement, temporal sequence of activation, and spatial order of expresssion has been shown, and this is conserved in species ranging from *D. melanogaster* to mammals (for example, Izpisua-Belmonte *et al.* (1991) showed the conservation between mouse and *D. melanogaster*). According to Akam (1989), one explanation for the correspondence between the *HOM* and *HOX* clusters is that they are truly homologous, derived from an ancestral cluster of homeobox genes, present in a common ancestor of insects and vertebrates. According to Graham *et al.* (1989) the conservation in expression and organisation of the mouse and *D. melanogaster* homeobox genes may be due to *cis*-acting regulatory elements distributed along a cluster, so that if the cluster was broken up, the expression of the genes would not be regulated correctly.

Graham *et al.* (1989) examined the expression of 7 members of the *Hox2* complex (now known as the *HoxB* complex, Scott, 1992), and compared aspects of structure and expression with other mouse and *D. melanogaster* complexes. They found the domain of expression of a gene of *HoxB* in the developing mouse embryo was analogous to its position in the *HoxB* gene cluster, that is, the more 3' the chromosomal location of the gene, the more anterior the boundary of expression of the gene in the central nervous system (with respect to the anterior-posterior axis). Graham *et al.* (1989) compared sequences and found significant similarities between many of the mouse homeodomains in genes of different clusters (that is corresponding genes (subfamilies) in different

complexes), and they could assign a corresponding *D. melanogaster* gene to many of the subfamilies. They also found that if the mouse and *D. melanogaster* subfamilies were aligned, the order of the *D. melanogaster* genes and most closely related mouse genes, along the respective chromosomes, was identical. This fact, in combination with the relationship between physical gene order, and domain expression argues in favour of a common ancestral origin of these homeobox gene complexes.

Izipsua-Belmonte *et al.* (1991) also studied the expression of some murine homeobox genes, namely the novel genes *Hox4.6* and *Hox4.7* (now *HoxD6* and *Hox D7*) and found that both the timing and location of expression of these genes correlated with the physical order of the genes along the chromosome.

Experiments disrupting the expression of various *Hox* genes have demonstrated the importance of homeobox genes to the developing limbs, skeleton, craniofacial region and central nervous system. Dolle *et al.* (1989) showed the *Hox5* complex of murine homeobox genes are important in the development of murine limbs. Morgan *et al.* (1992) also demonstrated the importance of homeobox genes to limb development in vertebrates. They showed that altering the expression of *Hox4.6* in the developing chick limb causes conversions between digits. They concluded that this gene is involved in specifying fate along the anterior/posterior axis of the developing limb. Balling *et al.* (1989) and Kessel *et al.* (1990) demonstrated the importance of the *Hox1.1* homeobox gene to normal craniofacial and skeletal development in mice. They used the chicken beta-actin promoter to ectopically express *Hox1.1* in mice and the resultant transgenic mice died soon after birth with craniofacial and skeletal abnormalities. The importance of homeobox genes to skeletal development has also been demonstrated in experiments by Le Mouellic *et al.* (1992), who created mice with a null mutation for *Hox3.1*. The homozygous null mice died a few days after birth and had skeletal abnormalities.

Homeobox genes are also involved in the development of the nervous system. Graham *et al.* (1989) used *in situ* hybridisation to show that seven members of the *Hox2* complex are differentially expressed in the central and peripheral nervous system. Lufkin *et al.* (1991) disrupted the *Hox1.6* gene by homologous recombination and introduced the

mutated gene into the mouse germline. The mice homozygous for the mutated gene died at birth and certain cranial nerves and ganglia were missing and they had multiple malformations of the inner ear and bones of the skull. *HOX1.6* (now known as *HOXA1* (Scott, 1992)) is on human chromosome 7 at 7p14-21 (Bucan *et al.*, 1986). This may be considered as a candidate gene for any craniosynostosis syndromes mapping to 7p14-21.

These and other experiments show that the disruption of a homeobox gene can cause problems in more than one cell or tissue type which is indicative of a role in developmental regulation, thus homeobox genes have been shown to be important in the normal regulation of vertebrate embryogenesis.

Recently, naturally occurring mutations have been identified in the triplet repeat regions of two *Hox* genes, the murine *Hoxa13* gene, and the human *HOXD13* gene. Muragaki *et al.* (1996) identified small repeat expansions of a region coding for alanine repeats in the human homeobox gene *HOXD13*, in the affected individuals of three synpolydactyly pedigrees. Synpolydactyly is generally considered to be an autosomal dominantly inherited trait with webbing and duplications of some digits. The expansion occurs in a region that has not been conserved during evolution. Mortlock *et al.* (1996) found a 50bp deletion in exon one of the murine *Hoxa*13 gene of hypodactyly mice. Hypodactyly is a semidominant condition; heterozygous hypodactyly mice are normal except for a shortened digit I on all four limbs (they are fertile), while homozygous hypodactyly mice usually die *in utero.* Those that survive until birth have a single digit on each limb and usually survive until adulthood, but are infertile. The 50bp deletion they found creates a frameshift after the 25th codon, and thus has the potential to produce a protein that lacks most of the normal amino acid sequence.

Another class of homeobox proteins are those coded for by the *msh*-like genes. In vertebrates these are the *Msx* genes and they are related to the *Drosophila* gene muscle-segment homeobox (*msh*) (Davidson, 1995). The human genes *MSX1* and *MSX2* are members of this class. The mouse gene *Msx1* was previously known as *Hox7* and *Hox7.1*, and *Msx2* was previously known as *Hox8*. However, neither *Msx1* nor *Msx2* are members of the Hox class of homeobox genes, they are members of the msh-like genes. It was agreed to name *Hox7* and *Hox8, Msx1* and *Msx2* respectively, since they

are most closely related to the *D. melanogaster msh* gene (Scott, 1992), and the name Hox should only be used for vertebrate genes related to the *D. melanogaster ANT-C* and *BX-C* gene clusters. The members of the *msh* gene family have been grouped into three subclasses those with most similarity to *Msx1*, *Msx2* or the invertebrate *msh* gene, and these groupings are based on amino acid sequence of the homeodomain (Bell *et al.*, 1993). *Msx1* and *Msx2* are generally expressed in a position-related rather than cell-type-specific manner, and their expression patterns often overlap. *Msx1* and *Msx2* are expressed in overlapping, but not identical, patterns during embryogenesis and organogenesis (Davidson, 1995).

Zinc Finger Genes

Zinc finger genes are another group of genes which have been shown to be important in developmental regulation. Zinc finger genes code for proteins with zinc finger motifs. Zinc finger proteins were first described in *Xenopus laevis,* when Miller *et al.* (1985) found repetitive zinc-binding domains in a protein transcription factor. They found nine tandem similar units in the amino acid sequence, each unit was approximately 30 residues and contained two invariant pairs of cysteines and histidines, which are the most common ligands for zinc. The repeated units each included a tetrahedral arrangement of zinc ligands, and were thought to be DNA-binding fingers. Wilkinson *et al.* (1989) showed molecular evidence for segmentation in the development of the central nervous system, they found that the zinc finger gene *Krox*-20 was expressed in specific domains of the developing mouse brain and central nervous system.

There are a number of factors which led to *MSX1* being considered as a strong candidate for Craniosynostosis Adelaide Type. Mice lacking *Msx1* function, *Msx1*$^{-}$ homozygous mice, die in the immediate postnatal period and have developmental abnormalities including cleft secondary palate, failure of tooth development and skull abnormalities (Satokata and Maas, 1994). Satokata and Maas (1994) suggested that *MSX1* may be involved in human craniofacial development. Also *MSX2*, another member of the *msh*-like family, was implicated in the disorder Craniosynostosis Boston type (Jabs *et al.*, 1993). Liu *et al.* (1995) confirmed the importance of this gene to craniofacial development when they engineered the Craniosynostosis Boston Type mutation in the mouse *Msx2* gene and expressed this in the developing skulls of transgenic mice. These

mice were found to have craniosynostosis. Overexpression of the wildtype *Msx2* gene was also found to be capable of causing craniosynostosis in transgenic mice. This led Ma *et al.* (1996) to investigate whether the mutant phenotype was caused by the enhancement of normal *Msx2* activity. Ma *et al.* (1996) found that the mutant *Msx2* bound with higher affinity than the wildtype *Msx2* to a set of known *Msx2* target sequences, but that site specificity was minimally or not at all affected by the mutation. The increased affinity was found to be due to a reduced dissociation rate of the mutant Msx2-DNA complex.

The features of Craniosynostosis Boston Type are variable in their expression and include sutural involvement and cranial abnormalities ranging from fronto-orbital recession to clover-leaf skull deformity. Some of the affected individuals also had severe headaches, poor vision and seizures. No hand or foot abnormalities were clinically apparent, however short first metatarsals were noted on some radiographs (Warman *et al.*, 1993). This is similar to Craniosynostosis Adelaide Type, where some abnormalities were only detected radiologically, and there is also variation in phenotype within the CAT family.

4.3 MATERIALS AND METHODS

Genomic DNA from two affected members: 3212 (III-7) and 3214 (III-13) and one unaffected member: 3254 (III-17) of the Craniosynostosis Adelaide Type pedigree and one random blood donor sample, were tested for rearrangement and/or deletion of the *MSX1* gene, by probing a genomic Southern blot with labelled *MSX1* exon sequence probes. Each exon was amplified whole by PCR, purified using Qiaquick then radioactively labelled as described in Chapter 2. The enzymes used to digest the DNA for the Southern blot were *Eco* RI, *Pst* I and *Hind* III.

The *MSX1* gene was screened for point mutations by Single Strand Conformation Analysis (SSCA). PCR amplification was undertaken using the conditions described for AC repeat markers (Chapter 2), except the thermal cycling file used was 30x(94°C-30s, 60°C-1min, 72°C-1min), followed by 72°C for 5mins. Initially the two exons were amplified by PCR, whole using the primers 2079 and 2080 for exon 1 and primers 2081 and 2082 for exon 2, (Fig. 4.1). The whole exon amplicons were analysed on 4.5% and

10% acrylamide gels. The exons were then digested into sizes more suitable for SSCA, ideally less than 300bp (Hayashi, 1996). The PCR products from both exons were digested with *Not* I, because this enzyme cuts each exon once only (sequence from Hewitt *et al.*, 1991). The products were electrophoresed on 4.5% and 10% gels. Primers 2141, 2142, 2143 and 2144, (Fig. 4.1) were then designed in order to analyse the exons one half at a time. Primer sequences and product sizes are shown in Table 4.1. These products were analysed on 6% SSCP, MDE and heteroduplex gels.

Fig. 4.1: Genomic Sequence of the human *MSX1* gene from Hewitt *et al.* (1991). Primer sequences are underlined, polymorphic deletion shown in blue type, *Not* I restriction sites shown in red type.

exon1

```
   1  CCCTGTGGTCCCCTGCACCTCCGCCGTGCCCTGCCCTGCGTGCCCCCAGGCCCAGCGCTC  60

  61  CGGGCGAGTCCCCAGGAGCGCGGCCCAATGGATCGGCTCCGGCCCGCCCCCTCGCGCGCT 120

 121  GATTGCCGCCGCCCCCCGCTGGCCTCGCCTTATTAGCAAGTTCTCTGGGGAGGCGCGGTA 180

 181  GGGCCCGGAGCCGGCGAGTGCTCCCGGGAAACATGCTGCCAGCGCGGCTGGCAGGCAAAC 240

 241  CGGAGGCCAGGGCCCAGTACGCCGGAGCTGGCCTGCTGGGGAGGGGCGGGCAGGCGCGCG 300

 301  CGGGAGGCGTGCCCGCCAGGGCCCCGGGCGCTCGCAGAGGCCGGCCGCGCTCCCAGCCCG 360
                                2079
 361  CCCGGAGCCCATGCCCGGCGGCTGGCCAGTGCTGCGGCAGAAGGGGGGGCCCGGCTCTGC 420

 421  ATGGCCCCGGCTGCTGACATGACTTCTTTGCCACTCGGTGTCAAAGTGGAGGACTCCGCC 480
                          MetThrSerLeuProLeuGlyValLysValGluAspSerAla

 481  TTCGGCAAGCCGGCGGGGGGAGGCGCGGGCCAGGCCCCCAGCGCCGCGGCGGCCACGGCA 540
      PheGlyLysProAlaGlyGlyGlyAlaGlyGlnAlaProSerAlaAlaAlaAlaThrAla
                                                        2143
 541  GCCGCCATGGGCACAGACGAGGAGGGGGCCAAGCCCAAAGTGTCCCCTTCGCTCCTGCCC 600
      AlaAlaMetGlyThrAspGluGluGlyAlaLysproLysValSerProSerLeuLeuPro
                                2144
 601  TTCAGCGTGGAGGCGCTCATGGCCGACCACAGGAAGCCGGGGGGCCAAGGAGAGCGCCCTG 660
      PheSerValGluAlaLeuMetAlaAspHisArgLysProGlyAlaLysGluSerAlaLeu

 661  GCGCCCTCCGAGGGCGTGCAGGCGGCGGGTGGCTCGGCGCAGCCACTGGCGTCCCGGCCG 720
      AlaProSerGluGlyValGlnAlaAlaGlyGlySerAlaGlnProLeuAlaSerArgPro

 721  GGGTCGCTGGGAGCCCCGGACGCGCCCTCTTCGCCGCGGCCGCTCGGCCATTTCTCGGTG 780
      GlySerLeuGlyAlaProAspAlaProSerSerProArgProLeuGlyHisPheSerVal
                              2988
 781  GGGGGACTCCTCAAGCTGCCAGAAGATGCGCTCGTCAAAGCCGAGAGCCCCGAGAAGCCC 840
      GlyGlyLeuLeuLysLeuProGluAspAlaLeuValLysAlaGluSerProGluLysPro

 841  GAGAGGACCCCGTGGATGCAGAGCCCCCGCTTCTCCCCGCCGCCGGCCAGGTAGTAGCCA 900
      GluArgThrProTrpMetGlnSerProArgPheSerProProProAlaAr
                        2080
 901  GAACCCAGGCGCAGAGGGAGGGGGCCGGTGTGGGGGCGGGTGGGGTGTGGGACCCGAGGG 960

 961  CTCCTGGTGGCCTCCGCGCCTGCGTACCTGCAGCCGGTGCTAGGGAGCCGTGGGCTGCAA 1020
                                                    MSX1ex1bio
1021  GGCCGGGTCTTCGCCTCCCTCCACTCCCACCCAGGAAGAAGGTTCCAGACCTCCTCGCCT 1080

1081  TGGCCCAGAGACGCTGCGGGTGGGAGTTAACGGATAGGACACCGATGTCTGGGCACCCTG 1140

1141  TCCTCCTGCCCCCACCAAACGACCTCAGGGGTCCATGATCCCTCATCTGATCCCAAACTC 1200

1201  TGTTTCATCGGCTTCACCCCAGCGGATGAATGTGTGTGGTGCGGTATCTTCCCTGCACCC 1260

1261  GGAGTTTCACTTTCTCGCAGTAGGAGCTGGTGACCCCCAGCCCCTCTTCCCTTTCAAGTA 1320
```

Fig. 4.1 continued

exon 2

1321 CCTCTTTGCCTAGAGGTTCCGAAGCTCCTACAGAATTC----700bp-------TCTGCG 1380

1381 CTGCTCATCTCTGGTCTGGTCTGCGGGAACTACTCCTAGAATCCCGTAGGAGCGAAGTGT 1440

1441 TCCGGGGAAAGTGTAGAATTTGATTTGGATTCTATGCCACAAAACTGCCTAGCCCCACAC 1500

1501 TGAAGCACTCCGTGGGCACTGATAAATGTTTGGCCAACCGGTAAAACTAAATGTGCCCTT 1560

1561 GGGCTGGGCGCAGGGCCTCTTTCTGCATGTTCGTCAACTGTATTAACATCCACCTTTCCT 1620

1621 CTGGATGGCCCTGGGAGGAGGCCCGCCATGAAGGCCTTCCTAAGCCGCCGGGCAGCACAA 1680

1681 AGGTGATTTCACATCTTCCCAGCTGAAAATGTTTAGGCCTAAGATGTGGACATCGAGCCT 1740

1741 TCAACGTGGGTATTTTTCTCCTGGAATCTTAGTTTCTTCATTTGCAAAAAGTAGACAGGA 1800

1801 ACTTCTCCCCTGCGGGTTGCAATGGGAATTGGAGAAAATATATTTCAAGTGCCTTGCGCG 1860

1861 ATGCCCGGCACCGAGGCACTTGGCGGCACTCAATATCTGGTATTGTTTGGCTATTATTAC 1920

2081

1921 TACTTGTTGGGCTGATCATGCTCCAATGCTTCTCTCTTAACCCTTGCTTTTTTTTCTTTC 1980

3616

1981 GGCCCCTCAGGCGGCTGAGCCCCCCAGCCTGCACCCTCCGCAAACACAAGACGAACCGTA 2040

gArgLeuSerProProAlaCysThrLeuArgLysHisLysThrAsnArgL

3583

2041 AGCCGCGGACGCCCTTCACCACCGCGCAGCTGCTGGCGCTGGAGCGCAAGTTCCGCCAGA 2100

ysProArgThrProPheThrThrAlaGlnLeuLeuAlaLeuGluArgLysPheArgGlnL

3200 T

2101 AGCAGTACCTGTCCATCGCCGAGCGCGCGGAGTTCTCCAGCTCGCTCAGCCTCACTGAGA 2160

ysGlnTyrLeuSerIleAlaGluArgAlaGluPheSerSerSerLeuSerLeuThrGluT

2141 2142 3148

2161 CGCAGGTGAAGATATGGTTCCAGAACCGCCGCGCCAAGGCAAAGAGACTACAAGAGGCAG 2220

hrGlnValLysIleTrpPheGlnAsnArgArgAlaLysAlaLysArgLeuGlnGluAlaG

2221 AGCTGGAGAAGCTGAAGATGGCCGCCAAGCCCATGCTGCCACCGGCTGCCTTCGGCCTCT 2280

luLeuGluLysLeuLysMetAlaAlaLysProMetLeuProProAlaAlaPheGlyLeuS

2281 CCTTCCCTCTCGGCGGCCCCGCAGCTGTAGCGGCCGCGGCGGGTGCCTCGCTCTACGGTG 2340

erPheProLeuGlyGlyProAlaAlaValAlaAlaAlaAlaGlyAlaSerLeuTyrGlyA

2341 CCTCTGGCCCCTTCCAGCGCGCCGCGCTGCCTGTGGCGCCCGTGGGACTCTACACGGCCC 2400

laSerGlyProPheGlnArgAlaAlaLeuProValAlaProValGlyLeuTyrThrAlaH

2401 ATGTGGGCTACAGCATGTACCACCTGACATAGAGGGTCCCAGGTCCGCCCACCTGTGGGG 2460

isValGlyTyrSerMetTyrHisLeuThr

2082

2461 CCAGCCGATTCCTCCAGCCCTGGTGCTGTACCCCCGGACGTGCTCCCCTGCTCGGCACCG 2520

MSX1ex2bio

2521 CCAGCCGCCTTCCCTTTAACCCTCACACTGCTCCAGTTTCACCTCTTTGCTCCCTGAGTT 2580

2581 CACTCTCCGAAGTCTGATCCCTGCCAAAAAGTGGCTGGAAGAGTCCCTTAGTACTCTTCT 2640

2641 AGCATTTAGAGATCTACCCTCTCGAGTTAAAAGATGGGGAAACTGAGGGCAGAGAGGTTA 2700

Table 4.1: Primer sequences for primers designed to amplify the *MSX1* gene, and some product sizes.

	Primer Sequences	Product Size
Exon 1, first half (2079&2144)	F:TGG CCA GTG CTG CGG CAG AAG R:TTC CTG TGG TCG GCC ATG AGC	262bp
Exon 1, second half (2143&2080)	F:TTC GCT CCT GCC CTT CAG CGT R:TCC CTC TGC GCC TGG GTT C	332bp
Exon 2, first half (2081&2142*)	F:CTT GTT GGG CTG ATC ATG CTC C R:(GCC) TCT TGT AGT CTC TTT GCC	296bp
Exon 2, second half (2141&2082)	F:GAT ATG GTT CCA GAA CCG C R:AGC ACC AGG GCT GGA GGA ATC	315bp
MSX1ex1bio	R:bio AGG TCT GGA ACC TTC TTC CTG	
MSX1ex2bio	R:bio CTG GAG CAG TGT GAG GGT TAA AG	
2988	F: ATG CGC TCG TCA AAG CCG AGA G	

*Primer 2142 was later redesigned to include an additional three bp, which are bracketed, and this new primer was 3148.

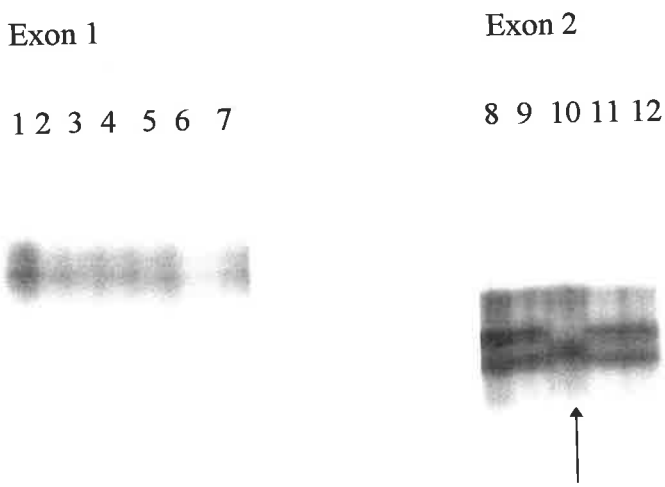
Attempts were made to sequence *MSX1* by manual sequencing, dye terminator sequencing, dye primer sequencing and solid phase single strand sequencing, conducted as described in Chapter 2.

4.4 RESULTS

No consistent differences between the affected individuals and the unaffected individuals were detected by Southern blot analysis with either exonic probe.

There were no consistent differences found between the affected and the unaffected individuals by SSCA of the whole exon products. One different SSCA pattern was observed, in a random blood bank sample, BB727 (Fig 4.2).

Fig 4.2: Whole exon MSX1 PCR products electrophoresed on 4.5% SSCA gel. Gel loadings - Lane 1: III-12 (3257), 2: III-15 (3209), 3: IV-15 (3360), 4: IV-17 (3361), 5: IV-19 (3238), 6: IV-20 (3211), 7: IV-21 (3210), 8: IV-15 (3360), 9: IV-17 (3361), 10: BB727, 11: BB770, 12: BB765. Different SSCA pattern indicated with an arrow.



There were no consistent differences detected between the affected and unaffected samples tested by SSCA on digested exon PCR products. One different exon 2 SSCA pattern was observed in a random blood donor control sample, BB727 (Fig. 4.3).

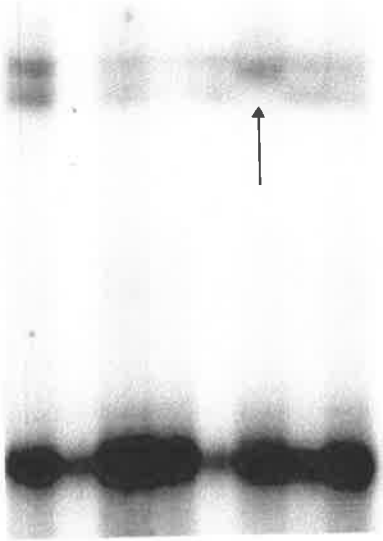
Fig 4.3: Digested exons of MSX1, electrophoresed on 4.5% SSCA gel. Gel loadings - Lane 1: II-3 (3217), 2: II-4 (3252), 3: III-11 (3256), 4: III-12 (3257), 5: III-15 (3209), 6: IV-15 (3360), 7: IV-17 (3361), 8: IV-19 (3238), 9: IV-20 (3211), 10: IV-21 (3210), 11: IV-18 (3362), 12: BB727, 13: BB770, 14: BB765. Different SSCA pattern indicated by an arrow.

Exon 1

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Exon 2

3 4 6 7 8 12 13 14



When the exons were analysed by SSCA one exon half at a time, a change was found in some individuals in the exon 1 first half product on 37.5% MDE gel (Fig 4.4), no other changes were observed. This SSCA change was analysed in the Craniosynostosis Adelaide Type family, and did not segregate with the syndrome (genotypes shown in Fig. 3.5). This change was shown to be a polymorphism, it occurred in some DNA samples from random blood bank donors and Mendelian inheritance was demonstrated in CEPH families 1345, 1334 and 66 (Appendix 4.1; Fig 4.5). The half exon products were also screened by heteroduplex analysis. By this technique, a difference was detected in some individuals in the first half of exon 2 (Appendix 4.1; Fig 4.6). This change did not segregate with affection status in the Craniosynostosis Adelaide Type family (genotypes shown in Fig 3.5), was detected in DNA samples from random blood donors and Mendelian inheritance was demonstrated in CEPH families 1345 and 1334 (Appendix 4.1; Fig 4.7).

Fig 4.4 MSX1 exon 1 first half electrophoresed on 37.5% MDE gel. Gel loadings - Lane 1: 3212, 2: 3256, 3: 3257, 4: 3254 and 5: BB820. Different SSCA pattern indicated by an arrow.

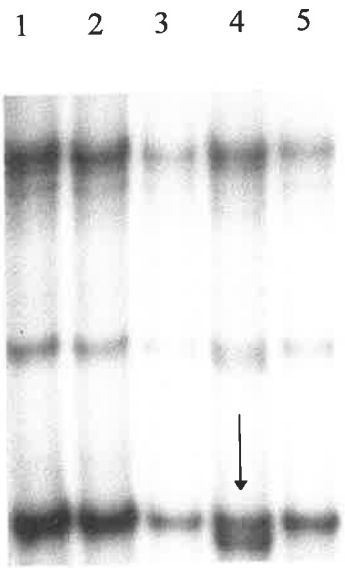


Fig 4.5: *MSX1* exon 1 first half SSCA polymorphism in CEPH family 1344. (Nomenclature is as given in Appendix 4.1)

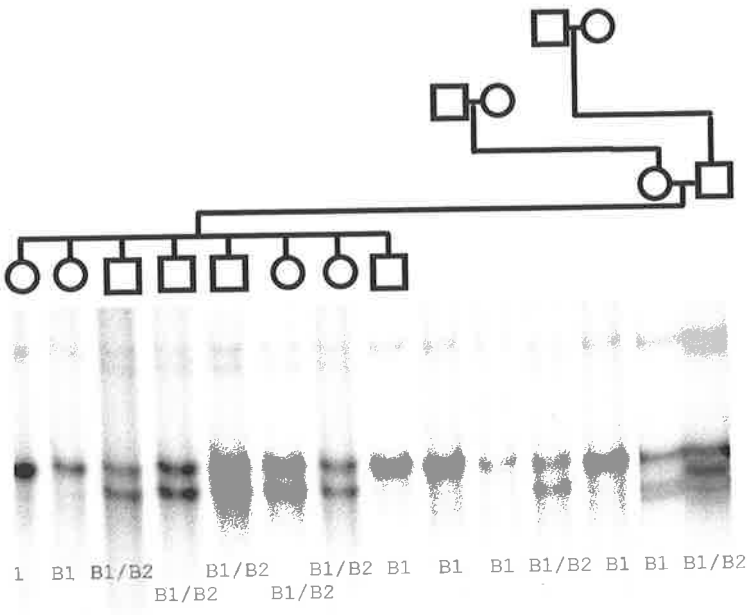


Fig. 4.6: *MSX1* half exon products electrophoresed on Heteroduplex gel. Gel Loadings - lanes 1, 6, 11 and 16: 3256, lanes 2,7,12 and 17: 3257, lanes 3, 8, 13 and 18: 3211, lanes 4, 9, 14 and 19: 3210, lanes 5, 10 and 15: BB832. Sample with heteroduplex band indicated by arrow.

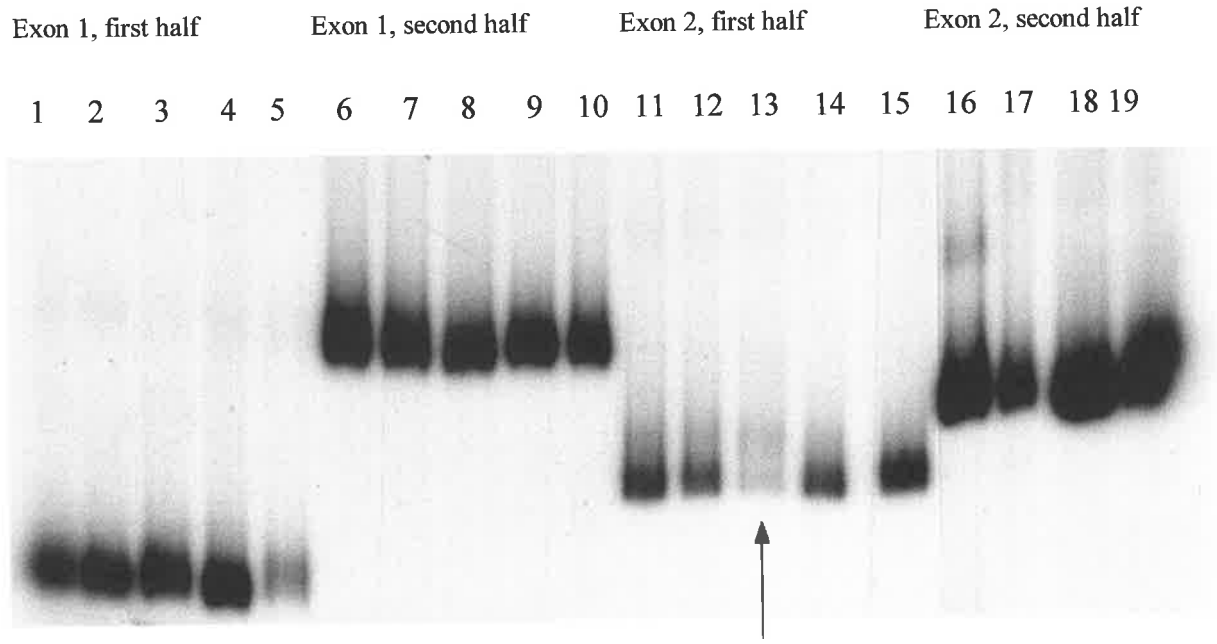
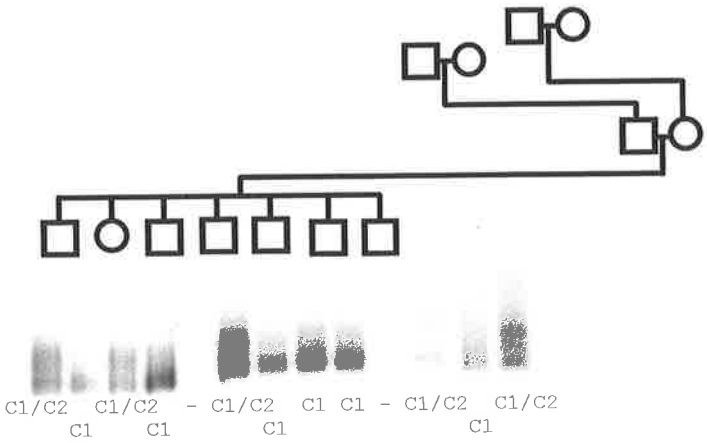


Fig. 4.7: *MSX1* exon 2 first half heteroduplex polymorphism in CEPH family 1334. Allele nomenclature is as given in Appendix 4.1.



SSCA does not detect 100% of base changes, thus the *MSX1* gene could not be excluded as a candidate on the basis of the negative SSCA and HA gel findings. Several attempts were made to sequence the *MSX1* gene for mutation detection purposes. Manual sequencing produced reasonable sequence for the majority of the *MSX1* coding sequence using the primers 2079-2082 and 2141-2144 on affected and unaffected individuals. There were a number of sequence changes seen inconsistently, and the sequencing was not of high enough standard to detect mutations.

Dye terminator sequencing was also attempted on *MSX1* exon 1 using the same primers as for the manual sequencing. This did not produce high quality sequencing results, and could not be used for mutation detection.

Dye Primer sequencing was also attempted on the *MSX1* coding sequence. Ten clones of exon 2 from the affected pedigree member, 3215 (IV-11), were sequenced in both directions, and reasonable sequence resulted, but a number of sequence differences each time made mutation detection difficult (an example of the sequence quality is shown in Fig. 4.8).

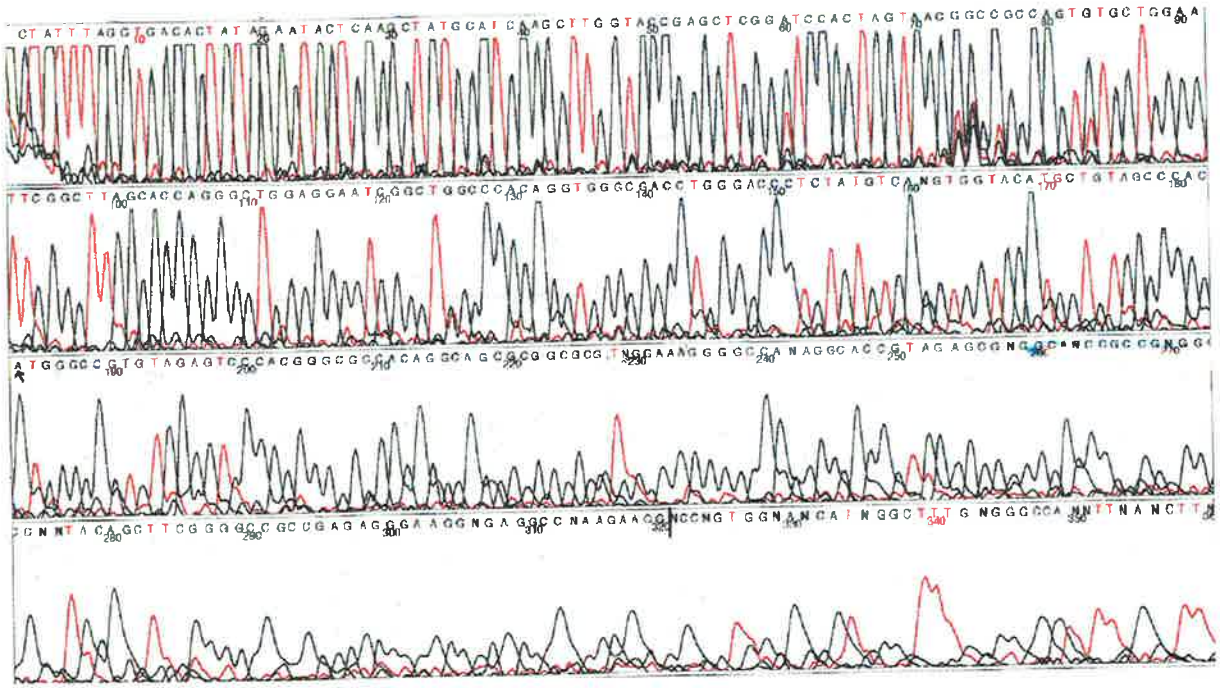


Fig. 4.8: Chromatogram of reverse Dye Primer sequence of clone C8 of exon 2 of patient sample 3215.

At this time high quality dye primer and dye terminator sequencing was being obtained, by the candidate, from the *FGFR2* gene (Chapter 7), thus it was concluded that the difficulties encountered with obtaining high standard sequencing from *MSX1* may have been due to some possible secondary structure associated with the gene. A number of options were then considered to try to improve the quality of the sequence being produced. Dye Terminator sequencing on single stranded template was then attempted. Primers MSX1ex1bio and MSX1ex2bio (Fig. 4.1) were designed to be outside the existing reverse primers for the respective exons and were manufactured with biotin at their 5' end. Solid phase single strand sequencing (SPSS sequencing) was then attempted on the *MSX1* coding sequence from affected samples 3214 (III-13), 3212 (III-7), 3211 (IV-20), 3238 (IV-19) and 3256 (III-11) and unaffected samples 3210 (IV-19), 3254 (III-17) and 3255 (IV-22). The majority of exon 2 was sequenced (first half-Fig. 4.9, second half-results not shown), before the focus of mutation screening shifted to the *FGFR3* gene.

During the SPSS sequencing an eleven bp deletion was detected in an affected individual, 3214 (III-13) compared to an unaffected individual, 3210 (IV-19) and the published sequence. The deletion was initially found when sequencing the solid phase (non-coding) strand with primer 2143. The deletion was confirmed in sequence of the eluted (coding) strand using the primer MSX1ex1bio. The eleven bp (927-937 in Hewitt *et al.*, 1991) was found to be deleted from another affected individual 3212 (III-7). A new primer was then designed, 2988 (Fig. 4.1), and this was used with the reverse primer MSX1ex1bio in radioactive PCRs. The labelled PCR products were then electrophoresed on 5% denaturing gels (as for AC repeat markers), and the eleven bp difference could be readily detected. The other members of the Craniosynostosis Adelaide Type pedigree were analysed and the deletion was not found to segregate with affection status (genotypes shown in Fig. 3.6). A series of 120 control chromosomes were then analysed (using radioactive PCR and AC gels) for the deletion and it was found in both heterozygous and homozygous forms in various individuals. The stable inheritance of the deletion was demonstrated using CEPH families 1345 and 66 (Fig 4.10). It was concluded the presence or absence of this eleven bp region represented a stably inherited two allele polymorphism (Appendix 4.1).

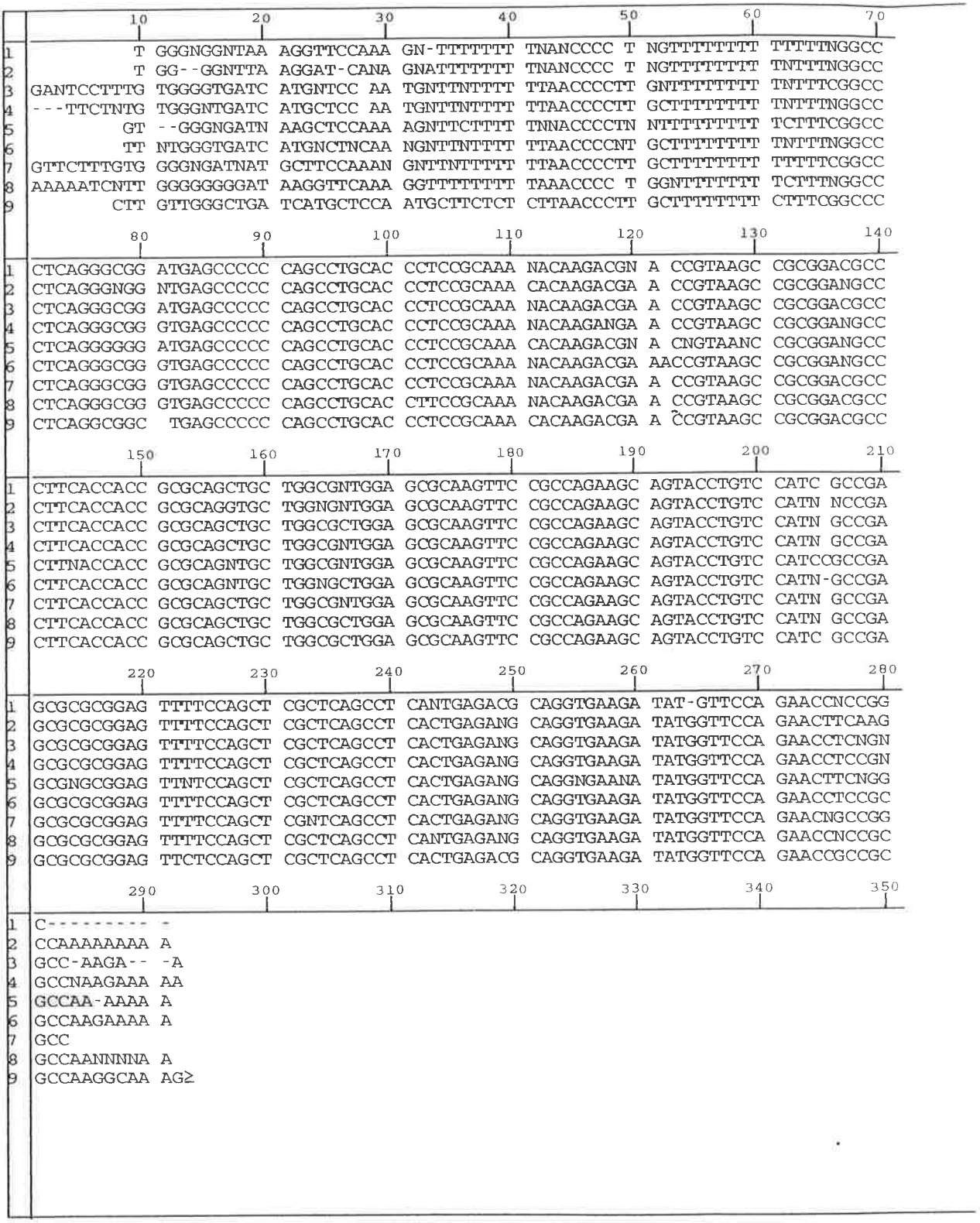
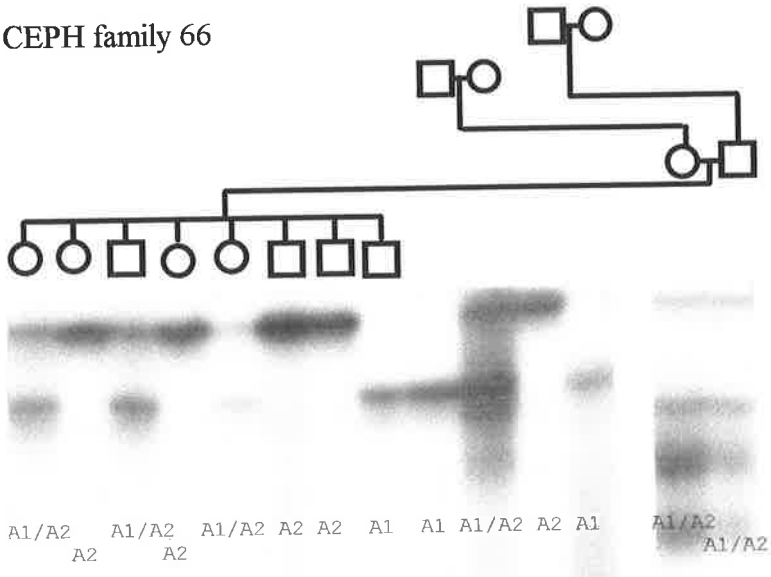


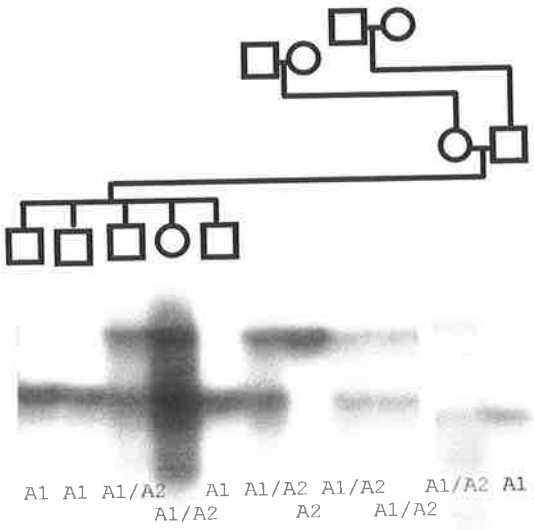
Fig. 4.9: SSPS sequencing of the first half of MSX1 exon 2. 1-3214, 2-3211, 3-3254, 4-3238, 5-3256, 6-3255, 7-3210, 8-3214, 9-published sequence from Hewitt *et al.* (1991)

Fig 4.10: *MSX1* intron 1 deletion polymorphism in CEPH families 1345 and 66. Allele nomenclature is as given in Appendix 4.1.

CEPH family 66



CEPH family 1345



4.5 DISCUSSION

MSX1 was a candidate gene for Craniosynostosis Adelaide Type because it has been demonstrated that homeobox genes are important in the regulation of development, and in particular *MSX2* (also from the *MSX* family of homeobox genes) has been implicated in the Craniosynostosis syndrome - Boston type (Jabs *et al.*, 1993) Also, *Msx1*-null mice have developmental abnormalities including skull anomalies (Satokata and Maas, 1994).

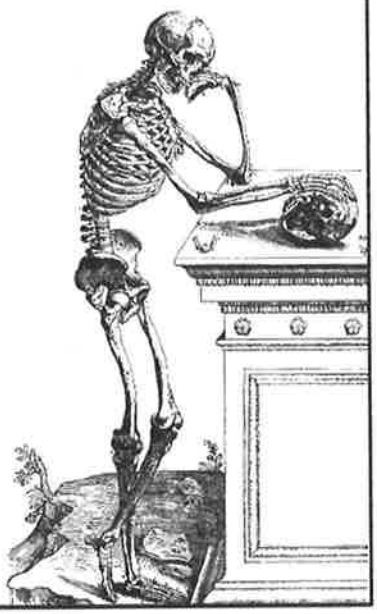
The *MSX1* gene was screened for mutations in affected members of the CAT pedigree by SSCA, HA and sequencing. No mutations were found, however three polymorphisms were identified (Appendix 4.1). Although screening with a combination of SSCA and HA is reported to increase the percentage of mutations detected, the detection rate is not 100% (White *et al.*, 1992). The gene could not be excluded of the basis of negative SSCA and HA results. The majority of the gene was sequenced.

One of the polymorphisms detected was an eleven bp deletion in intron one of *MSX1*. This was demonstrated to be a normal variant present in the general population, and inherited in a Mendelian fashion, thus was not the mutation responsible for Craniosynostosis Adelaide Type. In spite of the fact that the presence/absence of the deletion could not be correlated with craniosynostosis affection status, it was considered a possibility that the deletion may contribute to the phenotype and this may only be evident when in combination with a mutation. Such a mutation could be another genetic change in the *MSX1* gene, or possibly in some other gene, the product of which interacts with the *MSX1* gene product. In fact, all three *MSX1* polymorphisms detected in this study may not cause a phenotype when in isolation, however in combination with an *MSX1* mutation, or mutation of a gene whose product is in the same pathway as MSX1, they may cause a change to some aspect of the phenotype. The deletion may cause the second exon of the gene to be skipped in some transcripts, this could be investigated by Northern blot analysis, but this was considered to be outside the focus of this project, which was to investigate the cause of Craniosynostosis Adelaide Type.

MSX1 has also been considered as a candidate for a number of other disorders. *MSX1* and *MSX2* were both considered as candidate genes for hypodontia, congenital lack of some teeth, but were excluded by linkage (Nieminen *et al.*, 1995). *MSX1* was found to

be mutated in individuals affected by another dentition disorder, selective tooth agenesis (Vastardis *et al.*, 1996), thus it may be considered a candidate for other dentition disorders mapping to 4p16. The three intragenic *MSX1* polymorphisms identified in this study may be used to exclude the *MSX1* gene as a candidate in future studies, by demonstrating recombination between *MSX1* and the disease gene in question.

CHAPTER 5: MUTATION DETECTION IN *FGFR3*



5.1 SUMMARY

FGFR3 was considered as a candidate gene for Craniosynostosis Adelaide Type because 1) both *FGFR3* and Craniosynostosis Adelaide Type map to the chromosome 4p16 region ,
2) its spatial and temporal expression pattern is consistent with involvment, and
3) during the course of the present study other members of the *FGFR* gene family were implicated in other craniosynostosis syndromes, after Craniosynostosis Adelaide Type was mapped to 4p16.

To search for mutations as evidence for involvement of *FGFR3* in Craniosynostosis Adelaide Type , SSCA and RT-PCR SSCA were conducted on DNA and RNA from affected members of the CAT family. Two polymorphisms were found in the *FGFR3* gene (in exons 7 and 9), while searching for mutations. During the course of this work, the Pro250Arg mutation of FGFR3 was found by another group, studying a different set of patients (Bellus *et al.*, 1996) and subsequently found in a collaborative study to be the causal mutation of CAT (Appendix 5.1). The Pro250Arg mutation was shown to segregate with the craniosynostosis syndrome in the CAT family. This recurrent mutation has subsequently been found in numerous craniosynostosis patients (Chapter 9).

5.2 INTRODUCTION

Growth Factors

Polypeptide growth factors and cytokines are partly responsible for the regulation of a number of cellular processes including cell growth, differentiation, migration and apoptosis. An array of cellular responses can be triggered by growth factors, including stimulation of ion exchange and transport of glucose and amino acids (Ullrich and Schlessinger, 1990). These growth factors and cytokines are unable to pass through the membrane of a cell, therefore to perform their regulatory functions, they must transduce signals into the cell. This is achieved by binding to specific receptors at the cell surface (Heldin, 1995).

Fibroblast Growth Factors

The Fibroblast Growth Factors (FGFs) are a family of structurally related proteins which are involved in a wide variety of cellular processes. The first FGF isolated was from bovine pituitary gland and was found to have a mitogenic (proliferative) effect on fibroblast cells (Gospodarowicz, 1975). There were thought to be at least 9 genes coding for FGFs in mammals (Mason, 1994) and recently more have been isolated. Yamasaki *et al.* (1996) isolated an FGF-related cDNA, from rat embryos, by the use of degenerate primers, and this was named *FGF10*. Smallwood *et al.* (1996) isolated four new members of the FGF family and referred to them as Fibroblast Growth Factor Homologous Factors (FHFs). The chromosomal locations of the genes for two of these factors were reported by Verdier *et al.* (1997), who referred to them as *FGF11* (17p12) and *FGF12* (3q28-q29), and proposed that the other two FHFs found by Smallwood *et al.* (1996) be referred to as *FGF13* (X chromosome) and *FGF14* (chromosome 13). Mason (1994) considered that the *FGF* genes probably arose from a common ancestral gene since they are structurally related. There also appears to have been some degree of conservation of these genes throughout vertebrate evolution since amphibian homologues of some of these genes have been found (Wilkie *et al.*, 1995a).

The FGFs vary in their signal peptides. FGF 3, 4, 5, 6, 7, 8 and 10 appear to be secreted proteins with signal sequences, but it is thought that some may be released from cells by a novel mechanism (rather than through the Endoplasmic Reticulum-Golgi apparatus) (Basilico and Moscatelli, 1992 and references therein; Tanaka *et al.*, 1992; Mason, 1994 and Yamasaki *et al.*, 1996). FGF1, 2 and 9 do not have "classical" leader sequences for secretion (Mason, 1994). It had been thought that they may only be released from damaged cells, that is, it was thought that releasing growth factors from dead or damaged cells might be a rescue mechanism (since growth factors may be required when there are damaged cells to be repaired). But it is now thought that they may be released by some novel secretory mechanism. Mignatti *et al.* (1992) found evidence of FGF2 being externalised by a mechanism of exocytosis independent of the ER-Golgi complex pathway. Also, the four FHFs (FGF11-14) reported by Smallwood *et al.*(1996) lack classical signal sequences, and so they may also be released from cells via a secretory pathway not involving the ER-Golgi.

FGFs have been shown to be involved in a number of cellular activities *in vivo*, and they are also capable of causing a number of reactions *in vitro*, including stimulation of cell proliferation. They have been shown to be mitogenic for a variety of cell types including cells of mesodermal and neuroectodermal origin and other ectodermal cell types, and some endodermal derivatives (Mason, 1994). FGFs can also modulate cell motility, differentiation, extension of neurites and survival, *in vitro*. *In vivo* they have been shown to be involved in embryonic and fetal development and responses to wound healing (Mason, 1994). They are also angiogenic *in vivo* (Folkman and Klagsbrun, 1987), meaning they are associated with capillary formation, which often follows events such as wound healing, tumour growth and diabetic retinopathy. Hence they are an important family of molecules involved in diverse fields, including normal physiological processes.

The FGFs have distinct patterns of expression in space and time, they are expressed at certain times of development (including embryonic development) and some are also expressed in adult organisms. There is considerable variation among the FGFs in their expression patterns, some of which have been determined from *in situ* hybridisation studies and the roles of some of these genes have been investigated by gene disruption in animal models. Coffin *et al.* (1995) derived mice overexpressing FGF2 and found bone was primarily affected. The affected mice had skeletal abnormalities including shortening and flattening of bones and moderate macrocephaly, suggesting FGF2 plays an important role in bone morphogenesis. The isoform profiles differed between tissues, but were qualitatively similar for the murine Fgf2 and the human (transgenic) FGF2, inferring that the tissue-specific regulatory mechanisms are conserved between the species.

Mansour *et al.* (1993) derived and investigated mice with a targeted disruption of Fgf3. The homozygous mutants often died early in the postnatal period and few survived to weaning. Fertility was demonstrated in the survivors. The mutants had abnormally short, curly or kinked tails (abnormally shaped and/or fused vertebrae were found). Inner ear defects were also often found in the homozygous mutant mice. They found variation of the inner ear defects between individuals and between the two ears of a single embryo. This couldn't be attributed to non-uniform genetic background, or to leakiness of the disrupted Fgfr3. They suggested there may be parallel signalling pathways, and that utilisation of such pathways in an individual is mosaic.

Niswander and Martin (1992) investigated Fgf4 expression during mouse development and found it is expressed at specific times and in specific embryonic cell types. Fgf4 was shown to be expressed in the epiblast (which gives rise to all of the embryonic cell lineages) at the late blastocyst stage and this continues until gastrulation with progressive restriction. Fgf4 expression is then localised to specific cell populations including the apical ectodermal ridge of the developing limb bud, and the tooth bud.

Haub and Goldfarb (1991) detected Fgf5 RNA in the developing mouse embryo by *in situ* hybridisation. They found its expression to be regulated in time, tissue type and position within the tissue. Fgf5 RNA was found in some skeletal muscle groups and cranial ganglia, but not in others.

Herbert *et al.* (1991) specifically looked at murine Fgf5 in gastrulation (when embryonic ectoderm cells form ectoderm, mesoderm and endoderm) by mRNA localisation studies. They found Fgf5 expression increases dramatically just before gastrulation, in cells which form the three layers, and ceases when the three layers are almost formed. From this they hypothesised that Fgf5 stimulates cells to form the embryonic germ layers or makes them able to respond to other signals.

Hauschka *et al.* (1986) investigated growth factors in bovine bone powder, where a variety are present. They concluded that the BDGF-1.1 they found had properties consistent with it being FGF1. They also found many other growth factors.

Different isoforms can be produced from FGF genes by the use of alternative initiation codons for translation and also by alternative splicing. Using a human hepatoma cell line SK-HEP-1, Florkiewicz and Sommer (1989) found at least three immunoreactive forms of FGF2 that bind to heparin. These forms could be synthesised *in vitro* from a single cDNA size (thus excluding alternative splicing). The cDNA contains only one putative ATG translation initiation site, and using *in vivo* expression of mutations they showed the synthesis of the larger FGF2 forms is initiated at non-AUG translational initiation codons (at CTG codons). Alternative splicing has also been shown to occur for some *FGF* genes. An example of this is the *FGF8* gene, where alternative splicing produces at least seven isoforms (Tanaka *et al.*, 1992; Crossley and Martin, 1995).

Molecules Which Interact With FGFs

There are three types of molecules known to interact with the FGFs, a cysteine-rich receptor (CFR), the fibroblast growth factor receptors (the FGFRs) and heparan sulphate oligosaccharides.

Burrus *et al.* (1992) reported the cDNA cloning and biochemical characterisation of the cysteine-rich FGF receptor (CFR), which was found to be a membrane spanning receptor with no known homologies within the chicken genome, but all vertebrate species examined appeared to have DNA sequences with significant homology to CFR. They reported CFR as high affinity receptor for the FGFs, of unknown function at the time. Zhou *et al.* (1997) identified an FGF binding site in CFR and suggested that binding of FGFRs and CFR by FGFs may be mutually exclusive since similar regions of FGF2 interact with both receptor types. Zuber *et al.* (1997) suggested CFR may be involved in the intracellular trafficking of FGF and the regulation of cellular responses to FGF. This was because CFR was found to localise subcellularly (within the Golgi apparatus) and is capable of altering intracellular levels of FGFs.

Fibroblast Growth Factor Receptors (FGFRs)

The Fibroblast Growth Factor Receptors (FGFRs) are a family of high affinity receptors to the FGFs. They are receptor tyrosine kinases, which are one group of cell surface receptors. Signalling as a result of receptor tyrosine kinases is involved in both cellular proliferation and differentiation. There have been studies which demonstrated a particular receptor can lead to proliferation or differentiation depending upon the cell type in which it is expressed (Marshall, 1995). These receptors have an extracellular ligand binding domain, a cytoplasmic domain, and a hydrophobic transmembrane region separating the extra and intracellular regions. These receptors have been divided into subclasses. Subclass IV contains those receptors with three (or two depending on splicing) immunoglobulin-like repeats in the extracellular domain, and they also have a hydrophilic sequence inserted in the intracellular tyrosine kinase domain. The fibroblast growth factor receptors are members of subclass IV (Ullrich and Schlessinger, 1990, and references therein).

The binding of a ligand to the extracellular region of a receptor tyrosine kinase causes a conformational alteration of the extracellular domain which induces receptor dimerization and this leads to activation of the intracellular kinase function, which initiates a signalling cascade. Receptor tyrosine kinases catalyse the phosphorylation of tyrosine residues both within their own polypeptide chains and on other substrates. Following autophosphorylation of the kinase domain, proteins containing the Src homology 2 domain (SH2) are then recruited to the tyrosine residues on the receptor which are now phosphorylated (Marshall, 1995). This then leads to activation of the signalling molecule (the SH2 domain containing protein) by a number of possible mechanisms including tyrosine phosphorylation and conformational changes (Marshall, 1995). According to Heldin (1995), there is also some evidence indicating that several components of intracellular signal transduction pathways are also regulated by dimerization. According to Perrimon (1993), the receptor protein-tyrosine kinases seem to activate a common set of molecules. Following ligand binding and subsequent signalling, the receptors are internalised and some types of receptors are degraded, while some other types are recycled back to the cell surface (Ullrich and Schlessinger, 1990).

There are currently 4 genes in the *FGFR* family. The first FGFR was isolated by Lee *et al.* (1989). They isolated a 130kDa protein on the basis of it specifically binding to bFGF. Partial amino acid sequence was obtained and this was used to isolate a cDNA clone of the chicken bFGF receptor. They also isolated a cDNA from a human cDNA library. The FGFRs vary in their expression patterns, for example, Deng *et al.* (1994) investigated the role of FGFR1 in early embryonic development and from their results they found that Fgfr1 is required for two essential functions during mouse embryogenesis, proper embryonic cell proliferation and pattern formation. Alternative splicing is known to occur in some FGFRs (FGFR3-Chapter 6, FGFR2-Chapter 7). Alternative splicing leads to isoforms with 2 or 3 Ig-like loops in the extracellular domain, and Hou *et al.* (1992) found that these forms have qualitatively similar ligand-binding properties, but that they are structurally and functionally distinct. They showed that if one of the two cysteine residues which form putative disulphide bonds, in loop II or loop III, are substituted, then the formation of a ligand binding site in both isoforms is inhibited. They proposed that loops II and III form a common ligand-binding site in both

isoforms, and that ligand-affinity in the three loop isoform is modified by the interaction of loop I.

Interaction Between FGFs and FGFRs

The interaction between FGF and FGFR is mediated by heparin. Yayon *et al.* (1991) demonstrated that mutant cells expressing FGFRs, but lacking heparan sulphate proteoglycans, do not bind FGF2. They also showed that binding could be restored by the addition of heparin or heparan sulphate to the binding medium. They suggested that the tertiary structure of FGF2 may be significantly changed by binding cell surface or soluble forms of heparin, and that this may be essential for binding to FGFRs. Ornitz *et al.* (1992) further showed that FGF to receptor binding has an absolute requirement for heparin, and no other cell surface molecules are required, that is, heparin, FGF and FGFR form a stable complex. Mach *et al.* (1993) demonstrated 14-15 molecules of aFGF can bind to a 16-kDa heparin chain (approximately ten of these are at high affinity sites). This high density, high affinity binding of FGF may act as a concentrated, stabilised store of the FGF, which could then be released in response to the correct stimuli.

FGFR3

FGFR3 was the third member of the FGFR family of tyrosine kinase receptors to be isolated (Keegan *et al.*, 1991a). This was by screening a cDNA library with the chicken v-*sea* gene (a receptor like tyrosine kinase) under low stringency conditions. Sequencing of a resultant product revealed domains which are present in other FGFRs. The transmembrane region of the gene is longer in the FGFRs, compared to other tyrosine kinases and this provides a means of distinguishing this class of growth factor receptors.

The sequence homology between the newly isolated FGFR3 and FGFR1 (then known as FGFR/*flg*) varied between the various domains, the extracellular domain I showed the greatest divergence between FGFR3 and FGFR1 genes (22% amino acid identity), whereas extracellular domains II and III were highly conserved. Keegan *et al.* (1991a) demonstrated that their clone encoded a 125kDa transmembrane glycoprotein, by assaying transient expression in COS cells. Because amino acid similarity doesn't necessarily imply similar binding properties of corresponding proteins, they expressed the

protein in *Xenopus* oocytes and demonstrated that the FGFR3 protein is capable of being activated by FGF1 and FGF2, but they did not determine which ligands it interacts with *in vivo*. On finding this third fibroblast growth factor receptor, they suggested that the receptor previously known as FGFR/*flg* be called FGFR1 and the *bek* encoded protein be called FGFR2, since they seem to belong to a family of fibroblast growth factor receptors.

FGFR3 as a Candidate Gene for Craniosynostosis Adelaide Type

*FGFR*3 was considered a candidate gene for Craniosynostosis Adelaide Type because it has been mapped to 4p16.3 (Thompson *et al.*, 1991), within the gene localisation for Craniosynostosis Adelaide Type determined by linkage mapping. The FGFs and FGFRs are known to play important roles in development as demonstrated by their expression patterns during development and also by the effects of disruption or overexpression in mouse models. More specifically, the expression pattern of *Fgfr3* in the developing mouse includes the developing nervous system, cartilage rudiments of developing bone, the lens of the eye, and the developing cohlear of the inner ear, in particular high expression is found in the differentiating hair cells and the underlying support cells of the cochlear duct (Peters *et al.*, 1993). This is consistent with an hypothesis that defective FGFR3 causes Craniosynostosis Adelaide Type.

*FGFR*3 became a more likely candidate gene as the project progressed and mutations of other *FGFR* genes were implicated in other craniosynostosis syndromes. During the linkage study on the CAT family, Crouzon syndrome was mapped to 10q (Preston *et al.*, 1994) and following the mapping of CAT in August 1994, linkage of JWS to 10q23-q26 was also reported (Li *et al.*, 1994). Shortly after, it was shown that mutations in *FGFR2* cause Crouzon syndrome (Reardon *et al.*, 1994-[September *Nature Genetics*]). JWS was then found to be caused by mutations in the same gene (Jabs *et al.*, 1994-[November *Nature Genetics*]). Pfeiffer syndrome was linked to the centromeric region of chromosome 8 (Robin *et al.*, 1994) and mutations were then reported in *FGFR1* (Muenke *et al.*, 1994). The findings of mutations in the *FGFR1* and *FGFR2* genes in other craniosynostosis syndromes reinforced the hypothesis of *FGFR3* as a plausible candidate gene for CAT.

5.3 MATERIALS AND METHODS

Southern Blotting and Probing

Labelled probe was stripped from the Southern blot used in Chapter 4 and this filter was reprobed with the *FGFR3* probe 65796 (purchased from the The American *Type Culture* Collection), as described in Chapter 2.

SSCA of Genomic DNA

The genomic sequence of *FGFR3* was unpublished (during mutation detection work on this gene, carried out in the present study). Primers to amplify some exonic sequences of the *FGFR3* gene were kindly provided by Dr E W Jabs (Johns Hopkins University, Baltimore). The primer sequences were supplied with the primers, but their exact locations (in relation to the intron/exon boundaries) were not known to the candidate during this work.

The products (exons 5-9 and 11-18) were amplified using the 2xPCR mix-low dNTP and the same reaction conditions as for amplifying the AC repeat markers (Chapter 2), except 0.5ul of each primer at a concentration of 10uM were used. The thermal cycling conditions used are shown in Table 5.1.

Whole exon products, for exons 5-9, were analysed on 4.5%, 6%, 10% SSCA and MDE gels. The primer pairs were used to amplify products from at least four affected and four unaffected members of the Craniosynostosis Adelaide Type family, plus four unrelated control samples. If any different banding patterns were detected, then the whole Craniosynostosis Adelaide Type family was tested. The product sizes for primer pairs sent to cover exons 11-18 were too large to be effective in SSCA, since Hayashi (1996) recommends fragments of less than 300bp. The sequence was not available, thus designing nested primers was not an option. The following restriction enzymes were used to digest non-radioactive PCR products from each of the primer pairs : *Ava* I, *Bam* HI, *Eco* RI, *Hind* III, *Msp* I, *Not* I, *Pst* I, *Pvu* II, *Sac* I, *Sfc* I and *Sma* I. The aim in each case was to find an enzyme that would cut a particular product into fragments of size in the range 200-300bp, without producing any small fragments, and ideally without producing fragments that were very close in size, since analysis of SSCA patterns would be difficult. The enzymes selected were exons11/12 - *Msp* I, exons13/14/15 - *Ava* I,

exons17/18 - *Pvu* II, exon16 - *Sac* I, and exon13 - *Ava* I. Radioactive PCR products were then produced using the respective primer pairs, and the conditions described. Nine ul of unpurified radioactive PCR product was digested in a 20ul reaction in 1x the NEB-recommended restriction buffer with 1-10units of enzyme, and BSA to a final concentration of 100ng/ul was also added to the *Sac* I digests. The digests were incubated at the temperature recommended by the manufacturers for ~3-16 hours. Each primer pair was used to test at least four affected and two unaffected members of the CAT family, plus two unrelated control samples. The digested samples were analysed on 4.5%, 6% and 10% SSCA and 37.5% MDE gels.

Table 5.1

Exon	Product Size (bp)	PCR File
5	215	35*(94°C-30s, 66 °C -1min, 72 °C -1min)
6	215	35*(94 °C -30s, 66 °C -1min, 7 °C 2-1min)
7	285	35*(94 °C-1min, 63 °C-1min, 72 °C-1min)
8	259	35*(94 °C-1min, 70 °C-1min, 72 °C -1min)
9	314	35*(94- °C 1min, 70 °C-1min, 72 °C -1min)
11/12	578	file 21
13,14,15	~850	file 21
17,18	~650	file 21
16	~700	file 21
13	~650	file 21

[Exon numbering is that originally sent by Dr E W Jabs]

RT PCR SSCA

A fibroblast cell line T96/25 was established (by Sharon Lane, Department of Cytogenetics and Molecular Genetics) from a skin sample taken from 3161 (IV-5 in Fig. 3.1, Chapter 3). Total RNA was extracted from T96/25 and also from the lymphoblastoid cell lines from CEPH individuals 134505, 134506 and 134522, for use as controls (Chapter 2). The RNA was reverse-transcribed into cDNA (Chapter 2), which was used as template for PCR using the primers published by Prinos *et al.* (1995) (Table

5.2). Extensive non-radioactive optimisation testing was undertaken to determine which PCR and thermal cycling conditions were necessary to amplify regions from the *FGFR3* cDNA. The products of all reverse transcription reactions were tested by using primers to the housekeeping gene, Esterase D (ESD) to amplify a product, to test the presence and quality of the cDNA. A reaction was also conducted on each RNA sample to check for genomic DNA contamination A non-template control was included in any PCRs of cDNA, to ensure there was no contamination. The non-radioactive conditions tested included, exactly those described in Prinos *et al.* (1995), and file 21 with 2xPCR mix-low dNTP (AC marker conditions). For regions RT1, RT2 and RT8 both 2x PCR-low dNTP and BMB conditions were tried with file 22, with 55°C annealing temperature. File 22: 35x(94°C -1min, annealing temperature-1min, 72 °C -1min). For regions RT3, RT4 and RT5 file 22 was used with 63°C annealing temperature using 2xPCR mix-low dNTP. Reactions using 2xPCR mix-high dNTP (as described in Chapter 2) were conducted using file 22 with annealing temperatures of 57°C for RT8, 63°C for RT5 and 60°C for RT6. Potentially suitable annealing temperatures were selected on the basis of calculating the annealing temperature of the primers and deducting 1-3°C.

An attempt was made to amplify RT1 and RT2 by 2-round nested PCR. In the first round, the RT1 forward primer was used with the RT2 reverse primer. One ul of the first round reaction was used as template in the second round reactions which were conducted separately, that is the RT1 and RT2 primers were used in separate reactions.

Some mRNA was extracted from the cell lines of CEPH individuals 134505, 134506 and 134522 and from the cell line T96/25, cDNA was produced from the mRNA and used as template for PCRs. The primers for RT1 and RT2 were remade and tested on the cDNA from the mRNA. The primer sequences given in Prinos *et al.* (1995) were compared to the published cDNA sequence of FGFR3 (Keegan *et al.*, 1991a). Primer pairs RT5 and RT7 were tested on a fetal cDNA sample (a kind gift from Dr J Gecz).

Regions RT3 and RT4 were amplified from T96/25, 134505, 134506 and 134522 using 2xPCR mix-low dNTP with [α^{32}P]dCTP and thermal cycling file 22 with annealing temperature of 63°C. These products were electrophoresed on 10% SSCA and 37.5% MDE gels.

Table 5.2 : Primers for amplification of *FGFR3* from cDNA, from Prinos *et al.* (1995), and ESD primers, from Kuss (1996)-PhD thesis.

Primer Region	Primer Sequences	Product Size (bp)
RT1	f:GGT CAA GGA TGG CAC AGG GC r:CCA GCA CGT CCA GCG TGT AC	518
RT2	f: TCC CCG CAC CGG CCC ATC CT r:CTT GAG CAC GGT AAC GTA GG	184
RT3	f:ACG GCG GGC GCT AAC ACC AC r:CTG GCA GCA CCA CCA GCC AC	144
RT4	f:AGG AGC TGG TGG AGG CTG A r:GGA GAT CTT GTG CAC GGT GG	164
RT5	f:ACC CTG GTG CGC ATC GCA A r:GGT CCG ACA GGT CCT TGT CA	259
RT6	f:ACT GAC AAG GAC CTG TCG GAC C r:TTG CAG GTG TCG AAG GAG TAG TC	205
RT7	f:TTC GAC ACC TGC AAG CCG CC r:CCT CAG GCG CCA TCC ACT TC	258
RT8	f:AAG AAG ACA ACC AAC GGC CG r:CAG CAC TCC CGG ATG ATC AT	249
ESD	f:GGA GCT TCC CCA ACT CAT AAA TGC C r:GCA TGA TGT CTG ATG TGG TCA GTA A	

Testing for Pro250Arg mutation

Exon 7 (191bp exon, plus 150bp flanking intron sequence) was amplified using the primers EX7F: 5'-CGG CAG TGA CGG TGG TGG TGA-3' and EX7R: 5'-CCA AAT CCT CAC GCA ACC C-3' (Bellus *et al.*, 1996). The products were amplified in a final concentration of 1x BMB, 10% DMSO with 1ul EX7F (at 10uM), 1ul Ex7R(at 10uM), 0.5ul dNTPs (10mM of each nucleotide), 0.2ul BMTaq (at 5 units/ul) and 700ng template. The PCRs were initially denatured for 5mins at 94°C, followed by 35 cycles of (94 °C -30s, 60 °C -45s, 72 °C -45s), then 10mins at 72 °C . Fifteen ul of unpurified PCR product was digested with 4 units of *Nci* I, in a final concentration of 1x the recommended NEB buffer at 37 °C for between 3 and 16 hours. The digests were electrophoresed on 2% agarose gels. Wildtype DNA is digested into fragments of 218bp and 123bp, whereas the C749G mutation creates an *Nci* I site, thus digest leads to products of 218bp, 151bp, 123bp and 67bp.

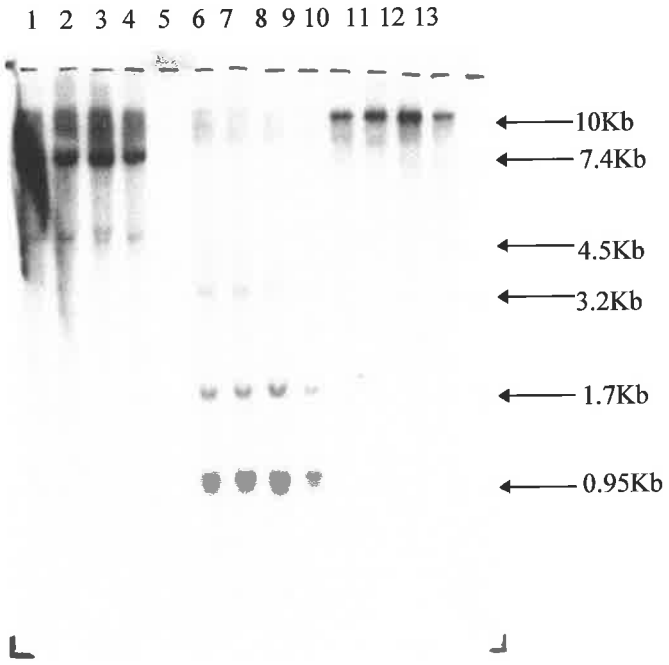
5.4 RESULTS

A Southern blot of genomic DNA from 3214 (affected), 3254 (unaffected), 3212 (affected) and unrelated control (BB894), each singly digested with *Eco* RI, *Pst* I and *Hind* III, was probed with the *FGFR3* probe 65796. No gross rearrangements of the *FGFR3* gene were detected (Fig. 5.1).

Fig. 5.1: Testing Craniosynostosis Adelaide Type samples for gross rearrangements of the *FGFR3* gene. Gel loadings: Lanes 1,6 and 10: III-13 (3214) (affected), lanes 2, 7 and 11: III-17 (3254) (unaffected), lanes 3, 8 and 12: III-7 (3212) (affected) and lanes 4, 9 and 13: unrelated control. Lanes 1-4 were digested with *Eco* RI, lanes 6-9 were digested with *Pst* I and lanes 10-13 were digested with *Hind* III. Lane 5: SPP1/*Eco*RI which did not hybridise to the *FGFR3* probe.

Fig. 5.1A: Ethidium Bromide stained genomic digests in agarose gel.

Fig. 5.1B: Genomic southern blot probed with *FGFR3* probe. Approximate fragment sizes are shown.



The products of PCR from exons 5-9 were tested by SSCA on 4.5%, 6%, 10% and MDE gels. Some altered banding patterns were seen, however there were no consistent differences between the affected and unaffected individuals tested for any of these exons (Table 5.3).

Table 5.3 : Changes observed in SSCA screen of exons 5-9.

Exon	Changes Observed
5	3353 and BB821 appeared different on 6% gel
6	polymorphism seen (3256, 3254 and BB821 have extra band)
8	differences seen in unaffected and control samples on 4.5%, 6% and 10% gels
9	sample 3353 appeared different to the other samples on MDE gel

The products of PCR from exons 11-18 were digested, then tested on 4.5%, 6% and 10% SSCA and 37.5% MDE gels. One affected sample, 3353, was found to have an altered SSCA pattern for exons 16 and 17/18. However the other affected samples appeared identical to the unaffected samples, that is, no consistent differences between the affected and unaffected samples tested, were found (Fig. 5.2). These regions were not analysed further.

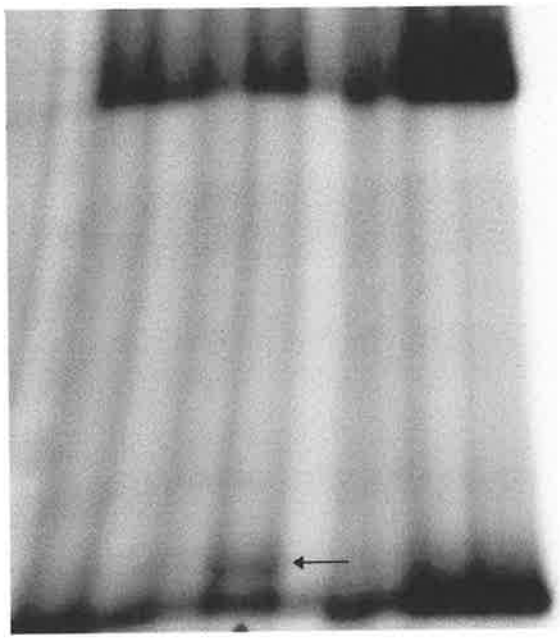
Fig. 5.2: SSCA of *FGFR3* exons 16 and 17/18. Lanes, 1: 3214, 2: 3256, 3: 3254, 4: 3353, 5: 3332, 6: 3211, 7: BB821, 8: BB826. Arrows indicate extra bands seen in sample 3353 only.

Fig. 5.2A: SSCA of FGFR3 exon 16, single stranded DNA.

1 2 3 4 5 6 7

Fig. 5.2B: SSCA of exon 17/18, double stranded DNA.

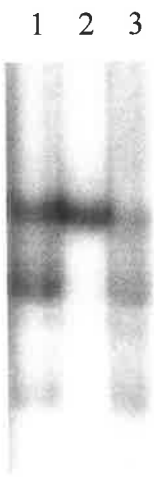
1 2 3 4 5 6 7 8



The exact regions of the gene covered by these primers were not known to the candidate. In addition digested products were not the optimal target for mutation detection by SSCA since some very small fragments may have been produced, and thus would not have been screened. As a consequence, this SSCA screen was not considered adequate and RT PCR was considered as the next step. Prinos *et al.* (1995) published primer sequences enabling amplification of 80% of the coding region of the gene from cDNA in eight fragments. Use of these primers thus seemed a suitable further screen of the *FGFR3* gene. A variety of non-radioactive PCR and thermal cycling conditions were tried, however product was successfully amplified using the RT3 and RT4 primers only. Radioactive products were then amplified using these primers and the same conditions with the addition of [α^{32}P]dCTP. These products were electrophoresed on 10% SSCA and 37.5% MDE gels. Some variability in banding patterns was observed, however there was always at least one control sample giving the same banding pattern as T96/25-from an affected member of the Craniosynostosis Adelaide Type family (Fig 5.3). Despite extensive optimisation, the regions of *FGFR3* covered by the other primers, were not

successfully amplified. The reason for the failure of amplification of the primer pairs other than RT3 and RT4 is not known.

Fig. 5.3: SSCA of RT3 cDNA on 10% SSCA gel. Lanes, 1: T96/25, 2: CEPH sample 134505, 3: CEPH sample 134506.



Because the RT3 and RT4 primers had successfully been used to produce bands, it was considered unlikely that the problem was the absence or poor quality of the cDNA. One possibility was a problem with the primers being used, the primer sequences were checked against the published cDNA sequence (Keegan *et al.*, 1991a) and two discrepancies were noted: there was an A missing from the primer RT5f and a C to G change in primer RT8r. The RT5 primers were successfully used to amplify a product from a fetal cDNA sample. This suggested that possibly a low level of *FGFR3* transcripts present in adult cDNA samples, or that the quality of the cDNA was causing the PCR failure from the T96/25 and CEPH control cDNA templates. However the fact that product had been successfully amplified from the RT3 and RT4 regions did not support this. The use of mRNA as template was attempted in case the failure to amplify was due to low copy number, however this did not improve the results. Various options were being considered (including the possibility that the RT3 and RT4 products were not the correct products, in spite of the bands being of the expected sizes) when the candidate and supervisor, Dr J Mulley, were contacted by Dr E Zackai working in the group of Prof M Muenke, and were informed that they had detected a recurrent mutation in the *FGFR3* gene which was likely to be the cause of craniosynostosis in the Craniosynostosis Adelaide Type family.

In parallel to use of the Prinos *et al.* (1995) primers, attempts were made to obtain the genomic sequence of *FGFR3*. This was eventually obtained from Dr A Winterpacht (Children's Hospital, University of Mainz), enabling primers to be designed to amplify the coding regions and intron/exon boundaries of the gene. However, this work and the RT PCR SSCA work was halted when contact was made by Dr Zackai and Prof Muenke. The candidate then tested all available members of the Craniosynostosis Adelaide Type family (with primers supplied by Muenke *et al.*) by *Nci*I digest for the C749G mutation of *FGFR3*. The mutation was found in all pedigree members coded as affected for linkage analysis (Chapter 3) and in none of the pedigree members coded as unaffected, including IV-6 (3234), IV-7 (3235) and IV-8 (3236) (Fig 3.1). These three family members were analysed as unaffected with 90% penetrance because of incomplete phenotype information (although they appeared clinically normal, radiographs could not be obtained). Two pedigree members (IV-3 (3332) and IV-18 (3362)) were coded as affection status unknown for the linkage analysis in Chapter 3. IV-3, who was not available for clinical or radiological examination, was negative for the mutation, while IV-18, whose affection status was unclear due to young age, was positive for the mutation. Pedigree member III-5 (3352) was unavailable for clinical examination but radiographs of the hands and feet were normal, so they were coded as unaffected and did not have the mutation. The segregation of the Pro250Arg mutation in the CAT family is shown in Fig. 5.4 and was published in the collaborative study (Appendix 5.1 - where the Craniosynostosis Adelaide Type family is family 12 in Fig. 1).

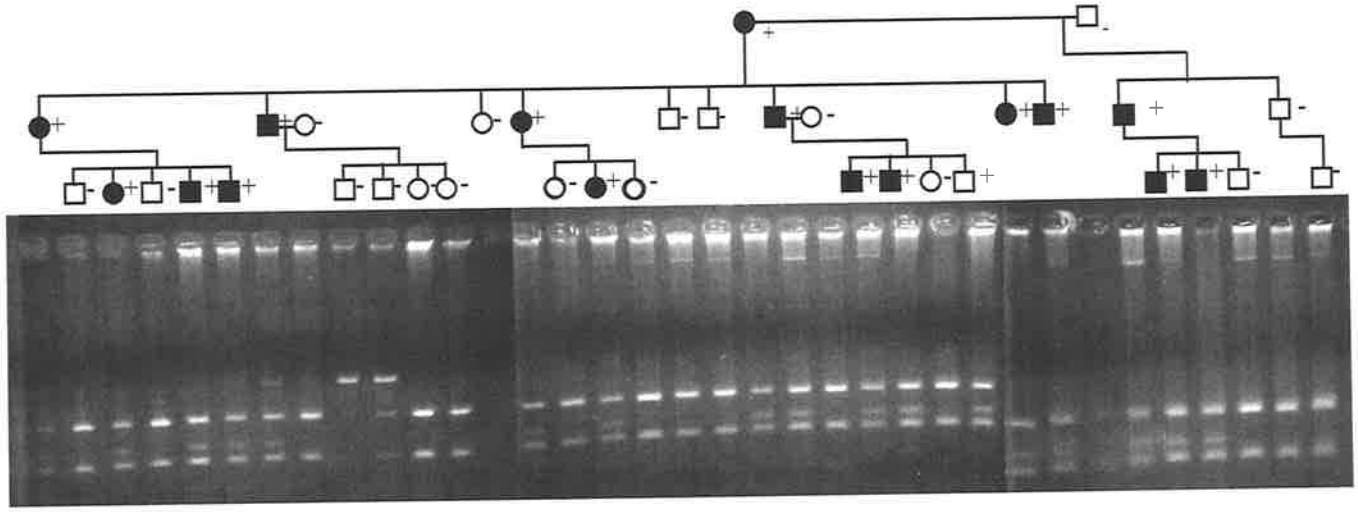


Fig. 5.4 B: *Nci* I digests of samples with (1) and without (2) the Pro250Arg mutation of FGFR3.

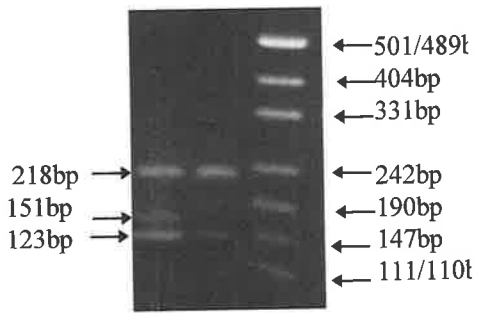


Fig. 5.4. A: *Nci* I digests of *FGFR3* exon7 from members of the Craniosynostosis Adelaide type family.
+ indicates presence of Pro250Arg mutation of FGFR3, - indicates absence of Pro250Arg mutation of FGFR3.
Samples which failed to amplify or digest were repeated and the results of these are indicated by the + or - signs.
See Fig. 5.4B for an enlargement which shows two digests with greater clarity.

Following the demonstration, by *Nci* I enzyme digestion, that the mutation causing Craniosynostosis Adelaide Type is in exon 7 of *FGFR3*, some of the SSCA screening of *FGFR3* was repeated. There was some doubt surrounding the numbering of the exon primers originally sent by Dr E W Jabs (see Discussion), thus exons 6-9 were to be retested. There was insufficient primer solution to conduct any further reactions for exon 9, so this exon was not included in the rescreening, and the potential polymorphism examined in exon 5 was not investigated further. The primers from Dr E W Jabs (the EWJ primers) labelled exons 6, 7 and 8 were used to amplify product from the affected (A) patient samples 3353, 3256, 3212 and 3214, and the unaffected (U) patient sample 3254, a sample from a random blood donor BB821, and 6 CEPH parents. The products were screened by SSCA on 10% and 37.5% MDE gels.

An extra band in exon 6 was detected in samples 3256(A), 3212(A), 3214(A),3254(U), BB821(U) and CEPH parent 2102 (Fig. 5.5). Mendelian inheritance of this polymorphism was demonstrated in CEPH family 21 (Fig. 5.6).

Fig. 5.5: SSCA of EWJ exon 6. Lanes, 1: 3256(A), 2: 3254(U), 3: BB821, 4: 3353(A), 5: 3212(A), 6: 3214 (A), 7: 1201, 8: 1202, 9: 1701, 10: 1702, 11:2101, 12: 2102. + indicates presence of the extra band, arrow indicates position of extra band.

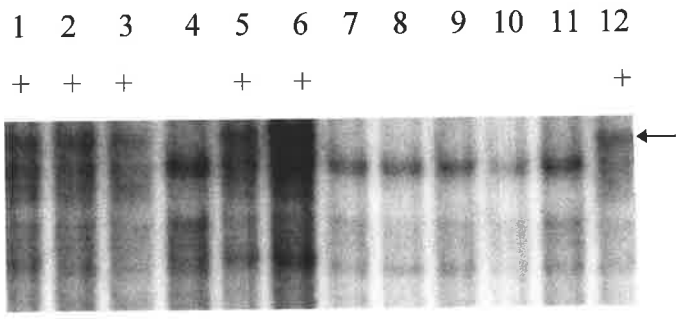
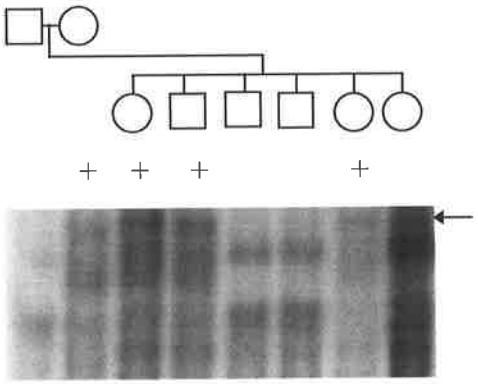


Fig. 5.6: SSCA of EWJ exon 6 in CEPH family 21. + indicates presence of, and arrow indicates position of, the polymorphic band.



No evidence of variation was detected using the EWJ exon 7 primers on 10% or 37.5% MDE gels.

The random blood donor sample BB821, and CEPH parents 1201, 1701 and 2102, were found to have an extra band in the region covered by the EWJ exon 8 primers, on 10% SSCA and 37.5% MDE gels (Fig 5.7). Mendelian inheritance of this polymorphism was demonstrated in CEPH families 12 and 21 (Fig. 5.8).

Fig 5.7: SSCA screen of EWJ exon 8. Lanes, 1: 3256(A), 2: 3254(U), 3: BB821, 4: 3353 (A), 5: 3212(A), 6: 3214(A), 7: 1201, 8: 1202, 9: 1701, 10: 1702, 11: 2101, 12: 2102. Arrows indicate polymorphic bands.

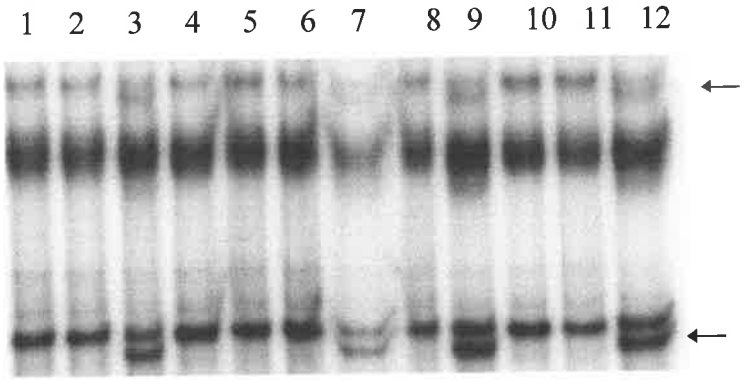


Fig 5.8 A: EWJ exon 8 SSCA polymorphism in CEPH family 12. Arrow indicates polymorphic band.

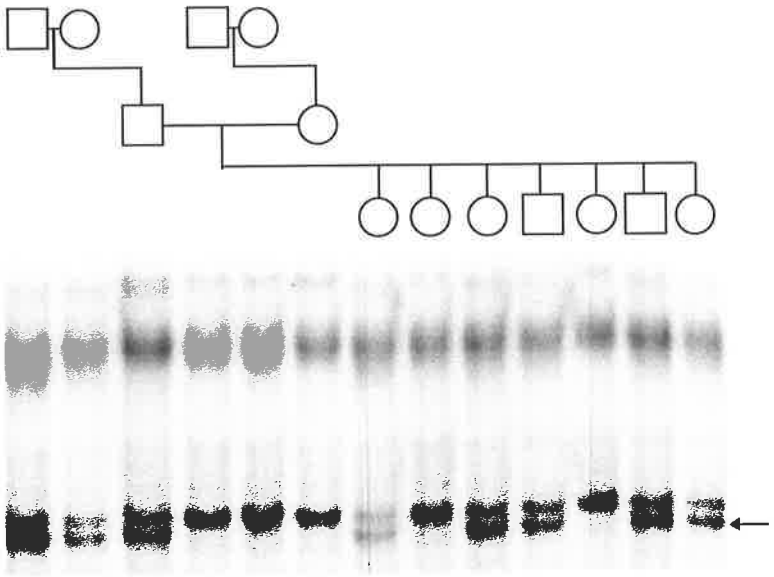
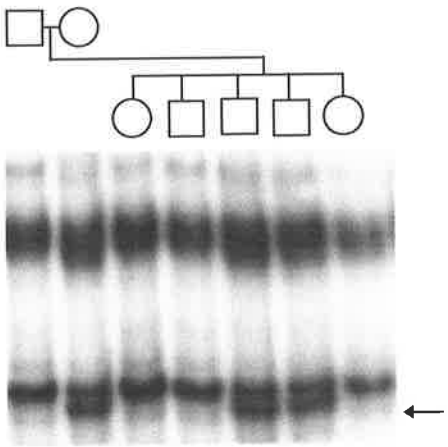


Fig 5.8B: EWJ exon 8 SSCA polymorphism in CEPH family 21. Arrow indicates polymorphic band.



The primers used to amplify exon 7 for the detection of the mutation (primers from Prof M Muenke, and published by Bellus *et al.* (1996) - the MM primers) were also used to screen this same group of affected and unaffected patient samples, and a random blood bank sample and 6 CEPH parent samples. Two bandshifts were evident (Fig. 5.9). The four affected individuals had an extra band which was not present in any of the unaffected samples, and presumably this is due to the C749G mutation. The other extra band was present in 3256(A), 3212(A), 3214(A), 3254(U), BB821 and CEPH parent 2102. Mendelian inheritance was demonstrated in CEPH family 21 (Fig. 5.10).

Fig. 5.9 SSCA screen of MM exon 7. Lanes, 1: 3353(A), 2: 3256(A), 3: 3212(A), 4: 3214(A), 5: 3254(U), 6: BB821, 7: 1201, 8: 1202. Arrows indicate polymorphic bands. Double stranded DNA is shown under single stranded DNA.

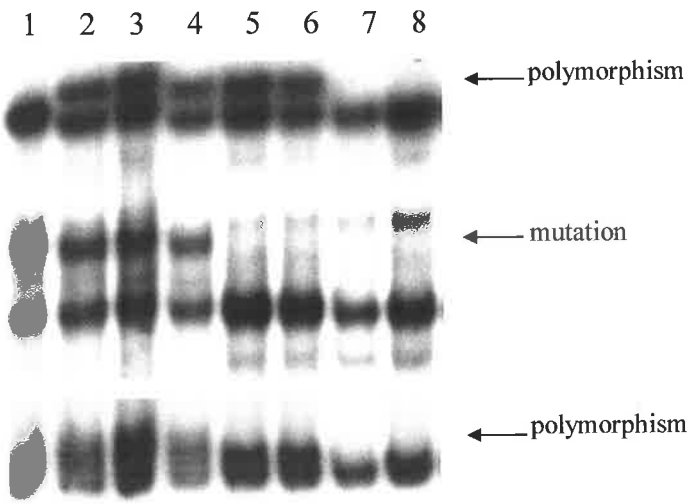
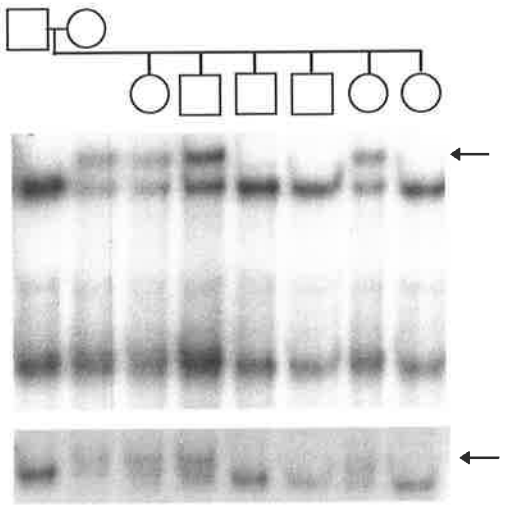
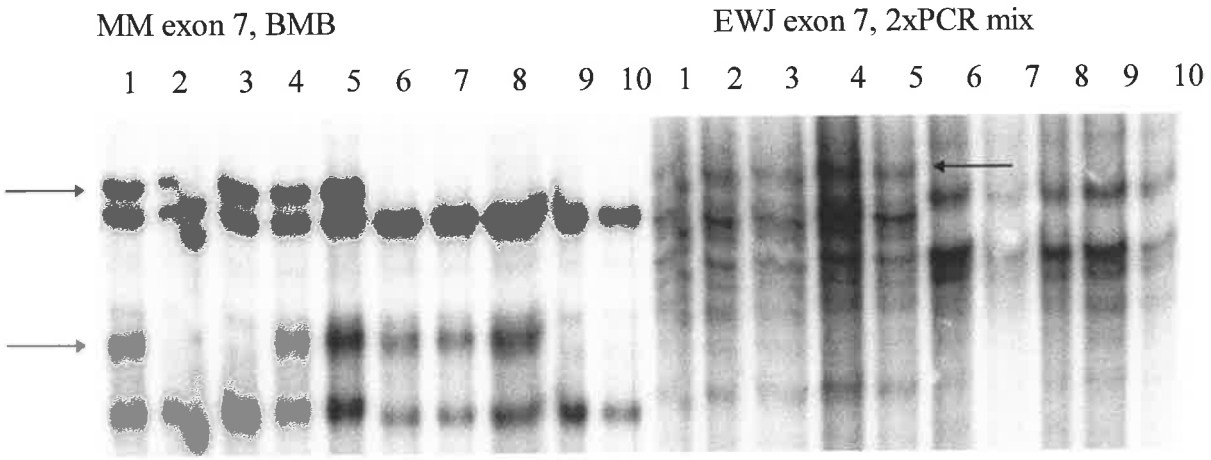


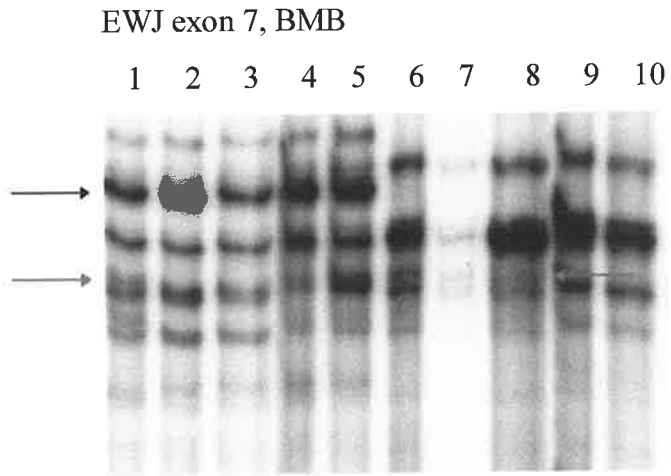
Fig. 5.10: MM exon 7 SSCA polymorphism on CEPH family 21. Arrows indicate polymorphic bands. Double stranded DNA is shown under single stranded DNA.



Direct comparison of the EWJ exon 6 and the MM exon 7 primers shows that on MDE gel the polymorphism can be seen in both cases, but the band present in the affected samples is only detectable in the samples amplified with the MM exon 7 primers (Fig. 5.11). There may be some difference in the double stranded DNA of the affected and unaffected samples with the EWJ exon 6 primers, however this is not clear. When these samples were electrophoresed on 10% SSCA gel, the polymorphic band can be seen with both primer sets, however the affected samples are not distinguishable with either primer set (results not shown). One possible cause of the difference on MDE gel may be the fact that the EWJ exon 6 primers were used with 2xPCR mix, and the MM exon 7 primers were used with the exact conditions recommended by Muenke *et al.* (Boehringer Mannheim buffer). Assuming that there was no mix up with the labelling of the EWJ exon 6 primers, then the different amplification conditions could be the cause of the SSCA result differences. The comparison was repeated with exactly the same PCR conditions used for the two sets of primers (those recommended by Prof M Muenke), and the samples were electrophoresed on the same gel. From this gel it appears that the affected samples can be distinguished from the unaffected samples using the EWJ exon 6 primers, however this is more obvious when the MM primers are used.

Fig. 5.11: SSCA screening of exon 7 using MM and EWJ primers. Lanes, 1: 3256(A), 2: 3254(U), 3: BB821, 4: 3212(A), 5: 3214(A), 6: 3360(A), 7: 3159(A), 8: 3232(A), 9: 3233(U), 10: 3358(U). BMB-Boehringer Mannheim buffer. Black arrows indicate polymorphic bands, red arrows indicate mutation bands.





(The MM exon 7 products were also included on this gel, they appeared as above, and are not included here)

5.5 DISCUSSION

The mutation found to be causing the craniosynostosis syndrome in the affected members of the Craniosynostosis Adelaide Type pedigree is C749G of the *FGFR3* gene. The mutation was found in all pedigree members which were coded as affected, and in none that were coded as unaffected which agrees entirely with the clinical description of this

family (Ades *et al.*, 1994). This confirmed the importance of radiological examination as carried out by Ades *et al.* (1994). This mutation causes a Pro250Arg substitution in the linker region between the second and third Ig-like domains of the extracellular portion of the protein. The C749G change of *FGFR3* was not detected in over 120 normal chromosomes (Bellus *et al.*, 1996).

Mutations at the corresponding point have also been found in FGFR1 (Pro252Arg) in Pfeiffer syndrome patients (Muenke *et al.*, 1994; Meyers *et al.*, 1996 and Schell *et al*, 1995) and in FGFR2 (Pro253Arg) in Apert syndrome patients (Wilkie *et al.*, 1995b; Park *et al.*, 1995b). This suggests a common pathogenesis (since they are identical mutations affecting the homologous codons in separate genes). Possible modes of action and consequences of the Pro250Arg FGFR3 mutation are discussed in Chapter 6.

The individuals found to have the polymorphic extra band with the MM exon 7 primers were the same as those who had a polymorphic extra band with the EWJ exon 6 primers. When the primer sequences were compared to each other and to the (now available) genomic sequence, it appears likely that the region covered by the EWJ exon 6 primers is the same as that covered by the MM exon 7 primers. The product size given for the EWJ exon 6 primer pair was 215bp, however when these are tested beside the MM exon 7 primers (341bp), they appear very similar in size. The reverse primer is identical to that published by Bellus *et al.* (1996), there is one mismatch between the forward primers, and the EWJ forward primer has an extra G at the 3'end (Fig. 5.12). Both the MM and EWJ forward primers differ from the published sequence (Perez-Castro *et al.*, 1997), they have an A where the published sequence has a G. Thus the region amplified by the EWJ exon 6 primers should correspond to the region amplified by the MM exon 7 primers, that is, both should amplify exon 7 (191bp) and 150bp of flanking sequence. Initially, when these two primer sets were directly compared it appeared that the mutation could be detected with the MM primers only. However when they were compared using the same amplification conditions, the affected samples can be distinguished from the unaffected samples with the EWJ exon 6 primers, however the products of the two primer sets do not appear identical, and the mutation is more easily detected with the MM primer set. It is not clear that the mismatch and extra base in the forward primer would cause the differences in the observed results, it presumably would

not be enough to cause the primer to fail to bind at the expected position. For the EWJ exon 6 primers the difference between the results of the two different amplification conditions is apparent, and demonstrates the importance of choice of PCR amplification conditions in mutation detection work using SSCA.

Fig 5.12: Comparison of FGFR3 exon 7 primer sequences. Regions of difference are boxed.

MM F: CGG CAG TGA CGG TGG TGG TGA

EWJ exon 6F: CGA CAG TGA CGG TGG TGG TGA G

PC: CGG CAG TGG CGG TGG TGG TGA G

MM R: CCA AAT CCT CAC GCA ACC C

EWJ exon 6R: CCA AAT CCT CAC GCA ACC C

PC: CCA AAT CCT CAC GCA ACC C

(MM - primers from Prof M Muenke, and published by Bellus et al., 1996; EWJ - primers from Dr E W Jabs, PC - sequence from Perez-Castro *et al.*, 1997)

Given that the EWJ exon 6 primers most likely amplify exon7, the polymorphisms detected with the EWJ exon 6 and exon 8 primers presumably represent polymorphisms in exons 7 and 9.

Sample 3353 appeared different by SSCA to all other samples tested for exons 9, 16 and 17/18. Also samples 3353 and BB821 appeared different to the other samples for the exon 5 region. For the exon 16 and 17/18 regions, the different patterns present in 3353 could have been due to partial digestion of the PCR products prior to SSCA, or might have represented a rare polymorphism. The fact that sample 3353 appeared different for a number of different exon regions indicates that there may be something unusual about the DNA preparation for this sample.

When conducting the mutation detection work in this family, it was the only published craniosynostosis pedigree mapping to chromosome 4p16, that the candidate was aware of. *MSX1* and *FGFR3* were initially both considered as candidate genes. Following the linkage mapping of Craniosynostosis Adelaide Type, *FGFR* genes were implicated in a

number of craniosynostosis syndromes, and thus *FGFR3* became an even stronger candidate for being the gene mutated in the individuals affected by Craniosynostosis Adelaide Type. The genomic sequence had just been obtained from Dr A Winterpacht, and the primers that the candidate had subsequently designed were abandoned after contact was made by Prof M Muenke *et al.* who informed the candidate that a mutation in FGFR3 had been identified. The *FGFR3* genomic organisation (splice donor and acceptor sequences), 5' flanking sequence and suggested primer sequences for mutation detection have now been published (Perez-Castro *et al.*, 1997; Wuchner *et al.*, 1997), thus greatly facilitating any future mutation detection work in the *FGFR3* gene.

In addition to the Pro250Arg craniosynostosis mutation, various mutations of *FGFR3* have been shown to be responsible for a number of disorders in recent years. Achondroplasia (ACH) is the most common type of genetic dwarfism, and is inherited as an autosomal partially dominant condition. The heterozygous form is characterised by short-limbed dwarfism (rhizomelic form) and macrocephaly, the homozygous condition is more severe and usually leads to death in the neonatal period. Following the localisation of the gene causing ACH to the telomeric region of 4p, (Velinov *et al.*, 1994; Le Merrer *et al.*, 1994; and Francomano *et al.*, 1994) ACH was shown to be caused by mutations in the transmembrane domain of the *FGFR3* gene (Shiang *et al.*, 1994; Rousseau *et al.*, 1994). Shiang *et al.* (1994) found G1138A on 15 of the 16 ACH affected chromosomes that they tested, the other had G1138C. Both of these mutations result in Gly380Arg in the transmembrane domain of FGFR3. Rousseau *et al.* (1994) found the same Gly380Arg change in 17 sporadic and 6 familial cases of ACH, 22 were caused by G1138A, and one was caused by G1138C. The *FGFR3* gene has thus been shown to have two highly mutable sites, one leading to dwarfism (at 1138) and one leading to craniosynostosis (at 749).

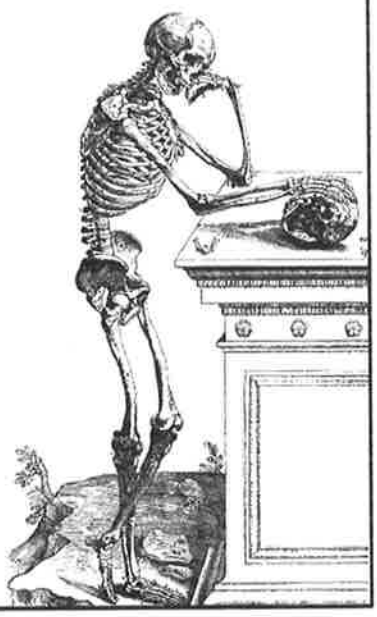
Following the discovery of the ACH mutations, *FGFR3* mutations were found in two other forms of dwarfism. Thanatophoric dysplasia (TD) is a more severe form of dwarfism, resembling homozygous ACH. The TD skeletal dysplasia is lethal in the neonatal period, and its features include micromelic shortening of the limbs, relative macrocephaly and reduced thoracic cavity. TD has been divided into subtypes, TDI patients have curved, short femurs with or without clover leaf skull, while TDII patients

have straight, relatively long femurs and severe clover leaf skull deformity. Tavormina *et al.* (1995a) found a recurrent A1948G (Lys650Glu) mutation in *FGFR3* of all TDII patients tested. Amongst the TDI patients tested, they found C742T (Arg248Cys), and A1111T (Ser371Cys), and in some cases no mutation was found. The TDII mutation affects the tyrosine kinase domain, while the two TDI mutations affect the extracellular region. An additional mutation, C746G (Ser249Cys) was later found in some of the TDI cases in which mutations had not previously been found (Tavormina *et al.*, 1995b). Rousseau *et al.* (1995), also found mutations in *FGFR3* in TDI patients, namely T2458G, T2458A and A2460T which all disrupt the stop codon at 807.

Hypochondroplasia is a milder form of dwarfism, with short stature (radiologically resembling ACH) but relatively few clinical symptoms. This disorder was mapped to 4p16.3 at the same time as ACH (Le Merrer *et al.*, 1994). Mutations causing Asn540Lys of the tyrosine kinase domain of FGFR3 have been found in hypochondroplasia patients (Prinos *et al.*, 1995; Bellus *et al.*, 1995). Furthermore, an FGFR3 transmembrane mutation (Ala391Glu) has been found in at least six unrelated cases of Crouzon syndrome with acanthosis nigricans (Meyers *et al.*, 1995; Wilkes *et al.*, 1996).

The sequence of events leading to the discovery of the Pro250Arg mutation of FGFR3 was initiated by the mapping of Craniosynostosis Adelaide Type to 4p16, and at that time it was the only craniosynostosis syndrome mapping to 4p16 that the candidate was aware of. Bellus *et al.* (1996) identified an *FGFR3* mutation in some craniosynostosis patients, and this led to the identification of the gene defect causing Craniosynostosis Adelaide Type. The discovery of the Pro250Arg mutation in numerous cases of craniosynostosis (additional cases have been identified in Chapter 9) has identified *FGFR3* as a gene crucial to the normal development of the skull as well as the normal development of the long bones.

CHAPTER 6: EXPLORATION OF THE FUNCTIONAL IMPLICATIONS OF THE PRO250ARG MUTATION OF FGFR3 BY HIGH SENSITIVITY IMMUNOFLUORESENCE FLOW CYTOMETRY



6.1 SUMMARY

Determination of the mutation responsible for Craniosynostosis Adelaide Type led to experiments to investigate the effect of the Pro250Arg mutation on the FGFR3 protein. The effect(s) of the Pro250Arg mutation may relate to the ligand binding, receptor dimerization or subsequent signalling aspects of the functioning of the FGFR3 protein. Antiserum to part of the extracellular region of the FGFR3 protein was used in flow cytometry experiments on skin fibroblast cells from an affected member of the Craniosynostosis Adelaide Type family and from normal controls. Flow cytometry on untransformed fibroblasts was the experimental approach taken, to reflect the *in vivo* conditions. The most likely consequence of the mutation was considered to be alteration of the structure of the extracellular domain, causing differing responses to ligands or ligand-independent activity. The results of these experiments were inconclusive because the expression of FGFR3 appeared to be below the level detectable by the high sensitivity immunofluoresence flow cytometry technology which was used.

6.2 INTRODUCTION

FGFR3 was the third member of the FGFR family of tyrosine kinase receptors to be isolated (Keegan *et al.*, 1991a). Following screening of a cDNA library with the chicken v-*sea* gene (a receptor-like tyrosine kinase) under low stringency conditions, sequencing of a positive cDNA revealed domains which are present in other FGFRs. The distinguishing feature of this class of growth factor receptors is that their transmembrane region is longer than that of other tyrosine kinases.

Keegan *et al.* (1991a) demonstrated that their clone encoded a 125kDa transmembrane glycoprotein, by assaying transient expression in COS cells. They expressed the protein in *Xenopus* oocytes and demonstrated that the FGFR3 protein is capable of being activated by aFGF(FGF1) and bFGF(FGF2), but they did not determine which ligands it interacts with *in vivo*. On finding this third fibroblast growth factor receptor, they suggested that receptor previously known as FGFR/*flg* be called FGFR1 and the *bek* encoded protein be called FGFR2, since they seem to belong to a distinctive family of receptors, the fibroblast growth factor receptors.

The FGFR3 protein was then further characterised. Keegan *et al.* (1991b) made two polyclonal antisera in rabbits against specific regions of the FGFR3 protein. SB141 antiserum recognised amino acids 94-255 of the extracellular domain, and SB102 antiserum recognised amino acids 578-806 of the kinase domain. They demonstrated recognition of FGFR3 by the antisera, then investigated cross reactivity to other FGFRs, which was important given the sequence homology between these receptors. They used immunoprecipitation and immunoblotting on protein from overexpressing cells (FGFR1 and FGFR2) or derived from in vitro transcription and translation (FGFR1). Chicken FGFR1 was weakly recognised by SB102, but not by SB141, human FGFR1 was recognised by SB102, but not by SB141 and human FGFR2 was not recognised by SB102 or SB141.

They expressed FGFR3 in mammalian cells and found proteins with molecular weights of approximately 97kDa, 125kDa and 135kDa. The 125kDa and 135kDa proteins were modified forms of the 97kDa soluble, non-glycosylated protein. The 125kDa protein was shown to be an immature, high-mannose, membrane associated form of FGFR3, while the 135kDa protein was shown to have acquired complex carbohydrates during passage through the Golgi apparatus and so was most likely the cell-surface form of FGFR3 (Keegan *et al.*, 1991b).

Alternative splicing has been demonstrated in the Ig-like III domain of *FGFR3* (diagram of FGFR shown in Fig. 7.1). Chellaiah *et al.* (1994) found, in addition to the form of FGFR3 that corresponds to the IIIc splice variants of FGFRs 1 and 2, another form corresponding to the IIIb splice variants of FGFRs 1 and 2, that is they found evidence of alternative splicing in FGFR3. The third Ig-like domain of membrane bound FGFR3 is coded for by two exons, the 5' half is coded for by iiia, and the 3' half is coded for by either iiib or iiic, and this leads to the two FGFR3 isoforms FGFR3IIIb and FGFR3IIIc. This is also the situation for FGFRs 1 and 2. Chellaiah *et al.* (1994) investigated the expression domains of these two FGFR3 isoforms, and found FGFR3IIIb expressed in skin, and at a low level in kidney, liver and lung, but no expression in brain, whereas the highest level of FGFR3IIIc expression was in the brain. This suggests possible different biological functions for these FGFR3 isoforms. Expression in bone was not tested.

Chellaiah *et al.* (1994) also tested the ligand binding abilities of the isoforms with respect to aFGF (FGF1), bFGF (FGF2) and kFGF (FGF7). The only difference detected in the soluble receptor binding assay was a small difference in FGF2 binding: it bound more to FGFR3IIIc than to IIIb. The ligand binding was further investigated by overexpressing the receptors in BaF3 cells (growth factor-dependent) and assaying the mitogenic response of the resultant cell lines, using ligands FGF-1, -2, -4, -5, -6 and -7. BaF3 cells are a pro B cell line, they are growth factor dependent, and don't express FGFRs (Ornitz *et al.*, 1992). When BaF3 cells are transfected with an FGFR cDNA, they show a dose-dependent mitogenic (proliferative) response to FGF (Ornitz and Leder, 1992). Cells expressing FGFRIIIc responded well to FGFs -1, -2 and -4, and poorly to FGFs -5 and -6, while cells expressing FGFR3 IIIb responded only to FGF-1. Neither receptor isoform caused a response to FGF-7. From experiments with chimeric FGFR2/FGFR3 receptors, they concluded that the information necessary to determine the ligand binding of FGFRs 2 and 3 with respect to at least two ligands, is encoded by a single alternatively spliced domain. Because of the very restricted ligand binding of FGFR3 IIIb they speculated that there may exist a novel FGF that can activate this receptor, and that such an FGF would be likely to be expressed in epithelial tissues.

A number of dwarfism disorders, including Achondroplasia (ACH), Hypochondroplasia (HD) and Thanatophoric Dysplasia (TD), have been shown to be caused by various mutations of FGFR3 (Chapter 5). These mutations occur in the transmembrane domain (ACH), the tyrosine kinase domain (TD II and HD) and the extracellular region (TD I) of FGFR3. The mutations in TD type I occur in the extracellular domain, at C742T (Arg248Cys) in some patients (Tavormina *et al.*, 1995a), and at C746G (Ser249Cys) in some patients (Tavormina *et al.*, 1995b). Hence these extracellular mutations are extremely close to the Craniosynostosis Adelaide Type mutation at codon 250, but with vastly different phenotypic effects.

The Neu receptor is a member of the Epidermal Growth Factor Receptor (EGFR) family, and these receptors are characterised by an extracellular ligand binding domain composed of two cysteine-rich subdomains (Heldin, 1995; Webster and Donoghue, 1996). Webster and Donoghue (1996) substituted the transmembrane domain of the Neu receptor with the transmembrane domains of mutant (ACH) and wildtype FGFR3,

then assayed the effect on signalling through the Neu tyrosine kinase. They concluded that the ACH mutation (Gly380Arg) results in ligand-independent activation of the tyrosine kinase activity of FGFR3 (which they predicted was caused by constitutive dimer stabilization), and allows constitutive signalling through a chimeric Neu:FGFR3 receptor. They suggested that the unregulated signalling through FGFR3 with the ACH mutation (Gly380Arg) might cause abnormal maturation at long bone growth plates by either inhibition of the normal proliferation of chondrocytes or by their premature differentiation. A Val664Glu mutation of Neu causes ligand independent activation of the Neu tyrosine kinase, and Webster and Donoghue (1996) also tested various amino acids at positions 664 of Neu and 380 of FGFR3 and showed that for these proteins to dimerise constitutively, the essential feature is an amino acid (in the appropriate position of the transmembrane domain) with a side chain that is capable of hydrogen bonding. There are also steric factors involved. Residues capable of hydrogen bond formation, through either N-H or O-H groups in their side chain include Arg, Glu, Asp and to a lesser degree Gln, His and Lys. The Pro250Arg mutation responsible for Craniosynostosis Adelaide Type is in the extracellular domain, thus dimerisation in the absence of the usual ligand (inappropriate dimerisation) due to hydrogen bonding (because of the Arg) is a possible result of this mutation.

Naski *et al.* (1996) made chimeric receptors: mutant (ACH and TD) and wildtype FGFR3 attached to the intracellular portion of FGFR1 (to amplify the sensitivity of the assay). Activity was assayed in BaF3 cells, which don't express FGFRs, but when transfected with an FGFR cDNA, the BaF3 cells exhibit a dose dependent proliferative response to FGF. They found ligand independent activity. The ACH mutation was found to be partially activating while the Arg248Cys TD type I mutation fully activated the receptor. Autophosphorylation of a set of tyrosine residues in the intracellular domain of the FGFR (one of the hallmarks of FGFR activation) was found to be constitutive for the TD mutation (Arg248Cys), but this was not detected for the chimera with the ACH mutation, and was found only after FGF1 treatment for the wildtype receptor.

There are a number of possible ways the Pro250Arg mutation may affect the FGFR3 protein. Of the ways that may lead to haploinsufficiency, the mutation may affect expression such that no protein is produced from the mutated copy of the gene.

Alternatively, the protein may be produced, but during processing the Pro250Arg receptors may be recognised as abnormal and be retained in the endoplasmic reticulum. In some diseases mutant proteins are recognised and despite apparently normal levels of mRNA and protein synthesis, there are reduced intracellular levels of protein, leading to disease (Brooks, 1997). The active retention and degradation of abnormal proteins is presumably to prevent functionally altered proteins from entering the cellular machinery. In Mucopolysaccharidosis type VI, catalytically active protein is synthesised, but it is conformationally abnormal, and is retained and degraded in the endoplasmic reticulum, directly contributing to the disease pathology (Brooks, 1997). Both possibities would imply that the mutant phenotype is caused by haploinsufficiency for the FGFR3 product. Wolf-Hirschhorn syndrome (abnormal development of a variety of organs) is caused by deletion of part of chromosome 4p. In some patients the deletion includes the *FGFR3* locus, hence they only have one copy of the *FGFR3* gene (Tommerup *et al.*, 1993). Since these patients do not develop craniosynostosis, it is unlikely that haploinsufficiency for FGFR3 could cause Craniosynostosis Adelaide Type. Also, Deng *et al.* (1996) derived mice with a targeted disruption (null mutation) of the *Fgfr3* gene, and mice heterozygous for the null mutation were phenotypically normal. Colvin *et al.* (1996) also generated mice with a targeted disruption of *Fgfr3*, and the heterozygous mice appeared normal.

The mutant gene may be expressed such that the temporal and spatial pattern is different from that of the normal allele, that is, it is not expressed in some cells where it should be expressed. This possibility would still amount to haploinsufficiency, in certain cell types.

The mutation may affect the protein in other ways, which do not involve haploinsufficiency. The mutated receptor may be expressed temporally and spatially as normal, but the receptors may not be targeted to the cell surface correctly, or the receptors may stay there a longer or shorter time than normal. Another possibility is that the receptors are expressed and targeted correctly, but they are functionally changed, for instance they may react to a ligand that they wouldn't normally react to.

Another alternative is that the mutation may cause the receptor to act in a ligand-independent way, that is, cause activation of downstream intracellular pathways

constitutively. Different mutations may cause different degrees of ligand independent activation, (or affect different pathways). In this way the Pro250Arg mutation could give a different phenotype to that of the ACH mutation. Naski *et al.* (1996) found ACH (Gly380Arg) mutation weakly activating, while the TD I mutation (Arg248Cys) was fully activating, and proposed that the ACH mutation may alter the equilibrium for receptor dimerization, such that a small but transient fraction of the receptor molecules dimerise in the absence of ligand, causing partial activation of signalling cascades.

Bellus *et al.* (1996) suggested that the Pro250Arg mutation of FGFR3 may affect the interactions between the mutant FGFR3 receptor and the FGFR2 receptors, possibly causing more heterodimers and causing craniosynostosis by affecting FGFR2. Bellus *et al.* (1996) suggested that the FGFR1 Pro252Arg (Pfeiffer) and the FGFR3 Pro250Arg mutation (Craniosynostosis Adelaide Type) may cause craniosynostosis by affecting FGFR2 signalling, that is, they may interact in some way such that mutations in any of the three, at this position, can cause a similar phenotypic effect.

Another possibility is that the phenotype is caused by overexpression of FGFR3; however it is difficult to imagine how an extracellular mutation of a receptor could cause it to be overexpressed.

The most likely hypothesis is that the extracellular portion of the receptor is changed in conformation as a consequence of the Pro250Arg mutation. This conformational change may affect the interactions of the receptor with ligands, such that the mutated receptor can be activated by a ligand that the wildtype cannot, or no longer responds to a ligand that activates the wildtype receptor. This hypothesis is favoured since it can explain the fact that this Pro250Arg mutation affects the development of the skull, with no effect on the long bones, whereas the Arg248Cys mutation (in the adjacent codon) severely affects long bone development (TD I) and has been shown to fully activate the receptor (Naski *et al.*, 1996). If the Pro250Arg molecules were to react differently to a ligand and such a ligand may have a tissue specific expression pattern that includes the cranial sutures but not the growth plates of the long bones, then the phenotype could be accounted for.

To investigate the C749G mutation of *FGFR3*, one possibility was to use the Neu receptor and make chimeric receptors, that is, replace the extracellular region of Neu with the mutated (C749G) and wildtype sequences coding for the extracellular regions from FGFR3. The chimeric receptors would then be transfected into growth factor dependent cells (such as BaF3) and the activation of the receptors determined, by assessing the activation of the Neu tyrosine kinase domain. This is the approach taken by other studies of FGFR3 mutations, because the activation of the Neu kinase domain can be readily assessed using focus formation assays (formation of foci of transformed cells - indicative of oncogenic transformation), whereas, for instance, assays for the activation of the FGFR2 kinase domain are less well characterised (Galvin *et al.*, 1996).

One disadvantage of using chimaeric receptors is that it involves transfection leading to an artificially high number of receptors expressed in cells, and thus the results may not accurately reflect the *in vivo* situation. A chimeric gene may not produce the same splice variants as are produced from *FGFR3*. The BaF3 cells would not produce any FGFRs from their own genome, thus there would not be any heterodimers formed between the FGFR3 chimeric molecules and any other FGFRs. Since one of the possible explanations for the action of the mutation places much importance on the formation of heterodimers (Bellus *et al.*, 1996), it is reasonable to consider the lack of the other FGFR molecules as a drawback. Even if the other FGFR molecules were present, heterodimers may not form between the chimeric recptors and the other FGFR1, 2 and 3 proteins in the cells because of the Neu content of the chimera, also the presence of such a high number of chimeric receptors would alter the likelihood of heterodimers forming (more likely to get homodimers). Thus the results obtained from a chimera experiment may be misleading, because any changes observed may be due, at least in part, to the overexpression of the receptor (and absence of other FGFRs from the cell), and not due to the mutation.

Analysis of cells from an untransformed skin cell line or fresh tissue, would more closely reflect the *in vivo* situation. Analysis of such cells may give a more accurate indication of what actually occurs *in vivo* when FGF receptors are mutated, rather than what happens when mutated receptors are expressed at artificially high numbers on cultured cells. Flow cytometry technology was chosen for these experiments since it provides

objective, cell by cell analysis. Thus it was decided to apply these techniques to study the Pro250Ag mutation of FGFR3.

Using cells from an untransformed fibroblast cell line has a number of other advantages. An untransformed (and non-transfected) cell line with one mutated copy of the *FGFR3* gene will presumably produce any splice variants that this mutated gene produces *in vivo*. An untransformed fibroblast cell line from a Craniosynostosis Adelalaide Type patient will presumably still produce receptor proteins from other *FGFR* genes, and various downstream signalling molecules, in normal amounts. Detection of receptors on untransfected cells can be difficult, which is a disadvantage. Normal levels of expression of these fibroblast growth factor receptors in cells are low, and most studies involve overexpression of the mutant receptor since the levels of receptor expression are not sufficient to facilitate easy detection (personal communication - Prof M Hayman, Stony Brook, New York). Thus to study untransformed cells requires a technique to detect receptors present in low numbers.

Zola (1995) described a technique for the detection of cytokine receptors, which are "often present and active at concentrations that are below the limit of sensitivity of conventional immunofluoresence flow cytometry". Concentrations of 100-500 molecules per cell are below the level of conventional immunofluoresence, whereas Zola *et al.* (1990a) found high sensitivity immunofluoresence could consistently detect fewer than 100 molecules of antibody bound per cell. Zola *et al.* (1990b) used the high sensitivity immunofluorescence technique to detect p70, which is expressed at low levels estimated to be approximately 540 molecules per cell (Ben Aribia *et al.*, 1989), on the majority of lymphocytes from normal blood samples, without *in vitro* stimulation.

The SB141 antiserum (Keegan *et al.* 1991b) recognises amino acids 94-255 of the extracellular domain of FGFR3, and this includes the codon containing the Craniosynostosis Adelaide Type mutation, codon 250. This made SB141 a suitable antibody to investigate whether the Pro250Arg mutation causes a conformation change of the extracellular (ligand binding) region. A conformation change may cause altered binding (increased or decreased) of the antiserum to the receptors, or a change in dimerization. If this could be demonstrated, it was planned to test the receptors with

various ligands, to determine whether the mutated receptors react differently to a particular ligand.

6.3 MATERIALS AND METHODS

Materials

Fibroblasts (cell line T96/25) were grown from a skin sample taken from 3161 (IV-5), an affected member of the Craniosynostosis Adelaide type pedigree (Fig. 3.1), and also from normal controls T97/30 (post mortem sample from accidental death, young female with no known abnormalities), T77/60 (normal but carries a balanced translocation), and SF4130 normal control skin fibroblast cell line from Department of Chemical Pathology (WCH). No growth factor receptor or other growth related genes were known to be affected in the controls. The fibroblast cell lines were grown at 37°C in OPTI-MEM I (GIBCO BRL, Life Technologies) supplemented with 8% fetal calf serum (CSL) and were maintained by Cathy Derwas (Cytogenetics and Molecular Genetics Department, WCH).

The SB141 antiserum (rabbit anti human) was kindly supplied by Prof M Hayman (Stony Brook Health Sciences Centre, New York). The control antiserum (rabbit anti human Ig), biotinylated goat anti rabbit serum, normal (pre immune) rabbit serum and normal sheep serum were kindly supplied by Prof H Zola (The Child Health Research Institute (CHRI), WCH).

Solutions

PBS: purchased as powder to be diluted with water, from MultiCel, Trace, or 0.8% w/v NaCl, 0.2% w/v KCl, 0.115% w/v Na_2HPO_4, 0.02% w/v KH_2PO_4

PBS-azide: PBS with 0.1% azide

trypsin/EDTA: 1:250 ratio of trypsin to EDTA, pH 7.0 MultiCel, Trace

EDTA: 0.02% w/v solution in PBS

FACS fixative: 2%w/v D-glucose, 1% v/v formaldehyde, 4.8mM Na N_3 in PBS, pH 7.3.

trypan blue: 0.08% solution in PBS

Methods

Cell Harvesting (Cathy Derwas)

To prepare for cell counting the fibroblast cells were washed twice with PBS, then detached from the tissue culture flask surface ($75cm^2$) by adding 2ml of trypsin/EDTA followed by gentle tapping to dislodge the cells. The cells were then poured into a 10ml centrifuge tube and pelleted by centrifugation at 200g for five mins in a Heraeus Sepatech Megafuge 1.0. The cell pellet was then washed twice with PBS.

Alternative Cell Harvesting Techniques (Cathy Derwas and Georgina Hollway)

Alternative cell harvesting techniques attempted were the use of sterile cell scrapers, EDTA and trypsin with rapid addition of medium. The washing of the cells before and after their detachment from the flask surface was the same as for the standard cell harvesting technique, in each case. The cell scrapers were used to physically detach the cells from the flask surface, while they were covered with 5ml of PBS. The cell suspension was then poured into a 10ml tube. Another alternative attempted was the addition of 2ml of a 0.02% w/v solution of EDTA (Martin, 1994) to the flask, and tapping the flask. Once the cells appeared detached (viewed using an inverted microscope) the cell suspension was poured into a 10ml tube and treated as described above. Another cell harvesting technique attempted was the use of trypsin/EDTA, very briefly (1-2mins), followed by the immediate addition of 8ml of OPTI-MEM I, as soon as the cells appeared to be detatched, to prevent any further trypsin activity. The cells were then poured into a 10ml tube and treated as before.

Cell Counting (Cathy Derwas and Georgina Hollway)

Cell numbers were estimated using a Neubaur Haemocytometer. Cells were fully suspended, then ~9ul was pipetted under a coverslip placed on the Haemocytometer. Cells were counted in specific squares of the grids marked on the Haemocytometer, using an inverted microscope. The cell number was estimated by averaging the counts from 4 squares, and multiplying by 10^4, to give the number of cells in one ml. On one

occasion the number of viable cells was assayed (in addition to total cell count) by using trypan blue. This stains nonviable cells only, since it can only penetrate cells which have a damaged cell membrane. This demonstrated that there were very few non viable cells and was not repeated.

Cell Preparation for Flow Cytometry (Georgina Hollway)

The cells were prepared for flow cytometry using a method described by Zola (1995). Cells were diluted to a concentration of ~10^5-10^6 per 50ul, and the concentrations were made uniform between the different cell samples within each experiment. The 50ul cell aliquots were mixed with 50ul of antiserum (at a specified dilution, 1:10, 1:100, 1:1,000 and 1:10,000) in a labelled 3ml polystyrene tube, mixed by tapping the tube, then placed on ice for 30 minutes, with tapping after 15 mins to resuspend settling cells. The cells were then washed with three ml of ice-cold PBS-azide, and centrifuged at 1,500rpm (200g) in an IEC Centra-8R centrifuge (International Equipment Company) at 4°C for five minutes. The wash was poured off while the tubes were held in a rack (for uniform supernatant removal from each tube). This wash was then repeated. The cells were then blocked with 50ul normal serum, and due to the unavailability of goat serum, sheep serum was used, the tubes were flick mixed, and incubated on ice for 10 mins. The second antibody was then added, in these experiments 50ul of biotinylated goat anti rabbit serum (Vector Laboratories) (1:100) was used. Following flick mixing, the tubes were incubated on ice for 30 mins, and settling cells were resuspended after 15 mins. The cells were then washed twice in PBS-azide as before. For detection of the biotinylated antibodies 50ul of phycoerythrin conjugated to streptavidin (PE-SA) (Sigma) was then added to each tube and following flick mixing, they were incubated on ice for 30 mins, with resuspension after 15 mins. The cells were then washed once with PBS-azide and stored at 4°C, wrapped in foil until they were analysed later that day. If sample reading was not to occur until the next day, then the cells were fixed by the addition of 50ul of FACS fixative (Lanier and Warner, 1981).

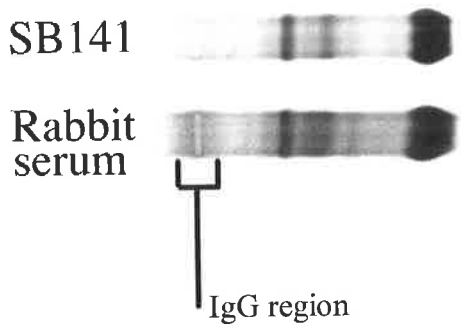
Flow Cytometry (Silvia Nobbs)

Five thousand cells per sample were read on a Coulter Epics Elite ESP Flow Cytometer. Forward and side scatter was used to gate to the cell population of interest, and all cell samples were analysed at a wavelength of 488nm using an argon laser.

6.4 RESULTS

It was necessary to repeat these experiments several times due to a number of problems. Normal rabbit serum (preimmune serum) was used as a negative control, since the SB141 antiserum was produced in a rabbit. Any fluoresence in the SB141-treated samples that was above the level of fluorescence produced by the corresponding normal rabbit serum control, could be considered to be due to the SB141, FGFR3-specific antibodies. The result from the normal rabbit serum was generally equal to the result from the corresponding SB141 sample. This may indicate that the FGFR3 antibodies were not active, however analysis of total protein content: spectrophotometry at OD280 and a total IgG gel, conducted by A. Nikolotsopoulos, Flinders Medical Centre, Adelaide, (Fig. 6.1) demonstrated that the SB141 antiserum was less concentrated than the normal rabbit serum (as indicated by the paler smear around the well the SB141 was loaded into, compared to the rabbit serum well). Thus the samples being compared were not of equal titres. This was rectified in subsequent experiments, by using a range of dilutions of normal rabbit serum.

Fig 6.1: Comparison of Ig content of SB141 and normal rabbit serum



It was noted when culturing the cells that the patient cell line consistently became confluent earlier than the negative control being used, T97/30. It was speculated that this may be as a result of the *FGFR3* mutation in the T96/25 patient cell line and so doubling time experiments were conducted by Bree Davis (Department of Cytogenetics and Molecular Genetics, WCH) on these cell lines and three additional control fibroblast cell lines, to quantitate the growth rate difference. Contrary to expectations the T96/25 cell line produced a relatively normal growth curve, whereas the control cell line, T97/30, did not grow in a normal manner and its growth curve did not follow the normal three-phase pattern (results not shown). Thus it seemed the cell line being used as a

normal control was abnormal, for reasons unknown. Results obtained to this point were therefore disregarded and a different normal fibroblast cell line (SF4130) was used as a normal control in subsequent experiments.

A number of different cell harvest techniques were tried, including the use of trypsin/EDTA, cell scraping in PBS, the use of EDTA and a modified trypsin method. The trypsin method used initially may have been damaging the extracellular portion of the FGFR3 receptors, since trypsin is a protease. Because of this a cell scraper was then used to harvest the cells while they were in PBS. However when a scraper was used the cells formed clumps which were not suitable for flow cytometry, and passaging of the solution through a 10 ml pipette could not alleviate this. When the cells were treated with a PBS/0.02% w/v EDTA solution, they lifted from the flask surface in sheets, again, not suitable for use in flow cytometry. The trypsin method was modified to brief exposure to trypsin followed by immediate addition of media to flask, to minimise the time the cells were exposed to trypsin, and hence hopefully minimise potential damage to the cell surface receptors by trypsin.

Table 6.1 shows the results obtained when flow cytometry was performed on cells harvested using the modified trypsin method. Samples 1-3 and 16-18 were negative controls (no first antibody), which were included to determine whether the detection reagents alone were capable of producing positive signal. Very low levels of signal (background levels) resulted from these controls. At ratios of 1:10 and 1:100 the normal rabbit serum did react more than the background level. This was probably due to the high level of antibodies in this serum, and cross reactivity between rabbit and human. There was no clear difference between the reaction of the SF4130 (control) cells and the T96/25 (affected patient) cells, to the SB141 antiserum.

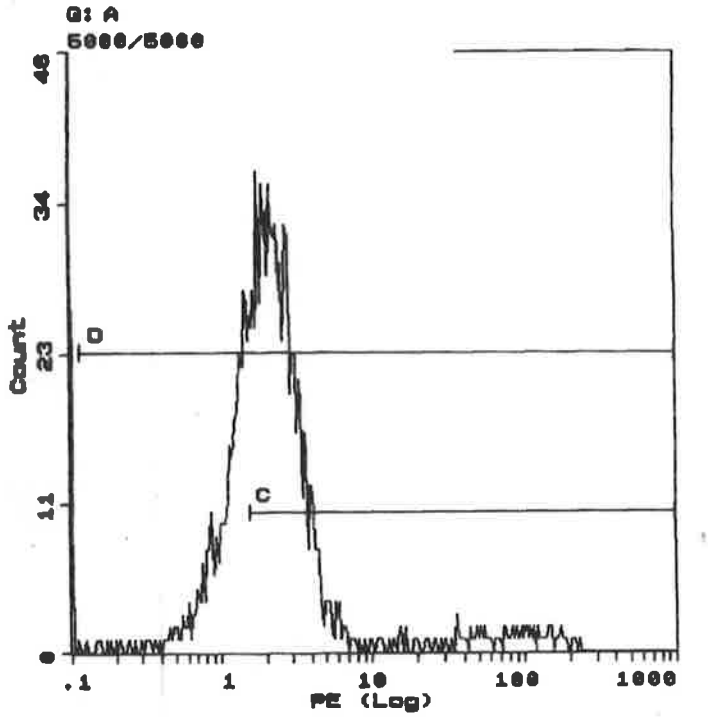
Table 6.1: Mean Fluoresence Values for Flow Cytometry of Craniosynostosis Adelaide Type and Control Cells treated with Normal Rabbit Serum (nrs), FGFR3-specific Antiserum (SB141) or Rabbit anti Human Ig Antiserum (RαHIg). BiGαR - biotinylated goat anti rabbit antiserum.

No.	Cells	1st Antibody	sheep serum	2nd Antibody (BiGαR)	PE-SA	Mean Fluoresence (D)
1	SF4130	-	-	-	+	0.208
2	SF4130	-	-	+	+	0.208
3	SF4130	-	+	+	+	0.253
4	SF4130	nrs 1:10	+	+	+	3.31
5	SF4130	nrs 1:100	+	+	+	0.756
6	SF4130	nrs 1:1,000	+	+	+	0.298
7	SF4130	nrs 1:10,000	+	+	+	0.235
8	SF4130	SB141 1:10	+	+	+	1.29
9	SF4130	SB141 1:100	+	+	+	0.459
10	SF4130	SB141 1:1,000	+	+	+	0.264
11	SF4130	SB141 1:10,000	+	+	+	0.198
12	SF4130	RαHIg 1:10	+	+	+	4.40
13	SF4130	RαHIg 1:100	+	+	+	3.97
14	SF4130	RαHIg 1:1,000	+	+	+	1.05
15	SF4130	RαHIg 1:10,000	+	+	+	0.296
16	T96/25	-	-	-	+	0.232
17	T96/25	-	-	+	+	0.227
18	T96/25	-	+	+	+	0.234
19	T96/25	nrs 1:10	+	+	+	2.56
20	T96/25	nrs 1:100	+	+	+	0.723
21	T96/25	nrs 1:1,000	+	+	+	0.319
22	T96/25	nrs 1:10,000	+	+	+	0.274
23	T96/25	SB141 1:10	+	+	+	1.47
24	T96/25	SB141 1:100	+	+	+	0.422
25	T96/25	SB141 1:1,000	+	+	+	0.261
26	T96/25	SB141 1:10,000	+	+	+	0.253
27	T96/25	RαHIg 1:10	+	+	+	6.22
28	T96/25	RαHIg 1:100	+	+	+	4.40
29	T96/25	RαHIg 1:1,000	+	+	+	1.14
30	T96/25	RαHIg 1:1,0000	+	+	+	0.352

+ added, - not added

Fig. 6.2: Graphs of flow cytometry results (cell count versus fluorescence level - PE), for samples19, 23 and 27.

6.2A: Sample 19 - T96/25 cells with normal rabbit serum



6.2B: Sample 23 - T96/25 cells with SB141 antiserum

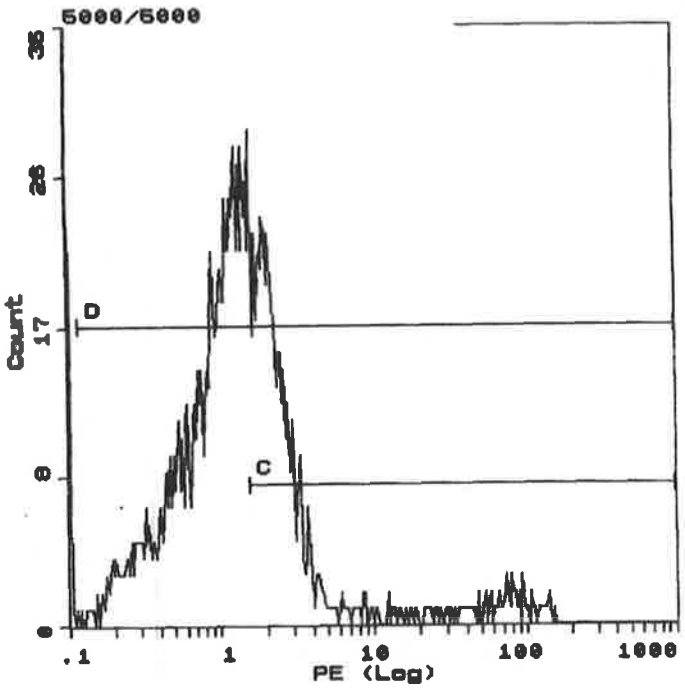
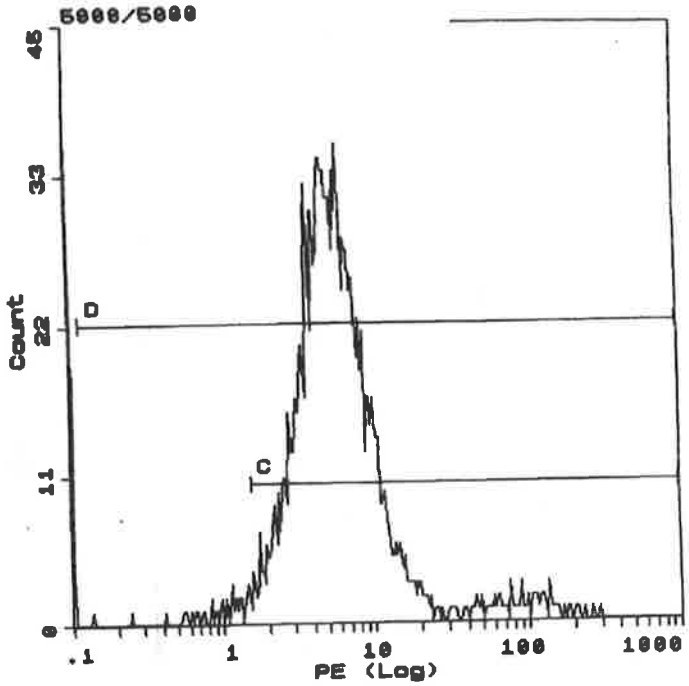


Fig. 6.2 continued

6.2C: Sample 27 - T96/25 cells with rabbit anti human Ig antiserum



6.5 DISCUSSION

The negative control antiserum used, (Rabbit anti Human Ig antiserum) reacted positively to both the control and patient skin fibroblast cells. This was unexpected and presumably indicates some cross reactivity between the anti-Ig antiserum and skin fibroblast cells. BaF3 cells were initially considered as a possible negative control for the flow cytometry experiments, however they were not used since they are a pro B cell line and thus represent a different cell type to the skin fibroblasts, and thus valid comparisons could not be made.

The levels of fluorescence obtained from the SB141 antiserum bound to normal skin fibroblasts was not enough to clearly demonstrate specific binding. Thus, because specific binding to the normal, unmutated cells could not be demonstrated and there was no difference when using skin fibroblasts from a Craniosynostosis Adelaide Type patient, the results of these experiments are inconclusive.

There are a number of possible reasons for the low fluoresence levels. The high sensitivity technique for flow cytometry Zola (1995), has been used successfully to

detect less than 100 molecules per cell, levels below the detection of conventional immunofluorescence (Zola *et al.*, 1990a). One possibility is that despite using high sensitivity flow cytometry, the numbers of FGFR3 receptors at the cell surface are still below the level of detection. Since this technique is capable of detecting less than 100 molecules per cell if a high affinity antibody is used, this may indicate that the number of FGFR3 molecules present at the cell surface is in fact very low. The technique has so far been used with monoclonal antibodies, which tend to give much clearer results due to a better ratio of specific signal : background binding, than polyclonal antisera, also the commercially available detection reagents for monoclonal antibodies are more effective than those available for the detection of polyclonal antibodies. Use of a monoclonal antibody would have been preferable in these experiments rather than the polyclonal SB141. Lin *et al.* (1996) made an anti-FGFR3 monoclonal antibody, 8.34, against amino acids 94-255 of the extracellular region of FGFR3. This would have been extremely useful for these experiments; however, it was not available.

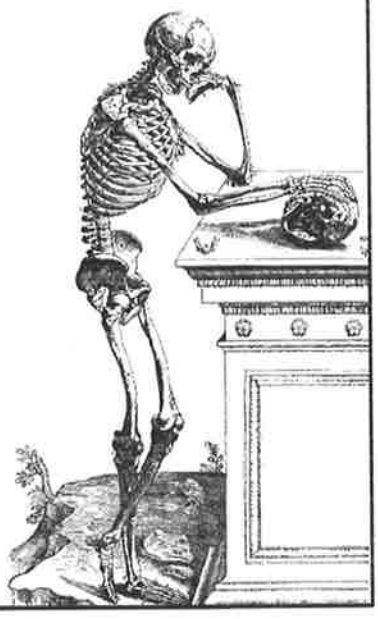
Another possibility is that the SB141 antiserum was not completely active when it arrived, and hence the chances of producing detectable fluorescence were reduced. This possible cause of low fluoresence levels could be resolved by testing the antiserum on a cell line transfected with an FGFR3-expressing plasmid. If this was negative, then it would be most likely that the antiserum was not in an active state. A useful positive control for these experiments would have been a cell line which overexpresses FGFR3, but the candidate was unable to obtain such a cell line in the remaining time available.

If a known active FGFR3 antiserum (that did not have to be transported to Adelaide) failed to detect the receptors on untransfected fibroblast cells, using this experimental design, then it could be concluded that the receptors probably cannot be detected by current flow cytometry techniques. If this were the case, then development of a technique to detect receptors present at the cell surface in very low numbers remains an objective, whether this is by flow cytometry or some other technology. One possibility may be the addition of extra rounds of amplification, for example, SB141, goat anti rabbit, mouse anti goat, horse anti mouse, biotinylated rat anti horse, PE-SA, to further amplify the signal. If functional antisera still failed to detect the FGFR3 receptors, then it may be that they are present in even lower numbers than was previously suspected.

One possibility of confirming whether or not the SB141 antisera was received in an active state would be to use it as a "probe" against a Western blot. However there is no guarantee that an antibody that works on a Western blot, will be active in flow cytometry experiments and vice versa. This is because the proteins on Western blots are usually denatured, whereas they are native on the cells used in flow cytometry experiments. Therefore these two techniques present different targets to antibodies and hence can give different results.

The aim of these flow cytometry experiments was to investigate the implications of the Pro250Arg mutation on the FGFR3 receptor. The aim was to detect the receptors on untransformed and untransfected cells, to give a more accurate reflection of the *in vivo* situation, rather than the investigation of cells overexpressing mutant receptors. However if the detection of receptors on native cells does not prove possible, as suggested by these experiments, then experiments such as those undertaken to investigate the ACH and TD mutations will be necessary to gain a better understanding of the molecular effects of the Pro250Arg mutation of FGFR3.

CHAPTER 7: MUTATION DETECTION IN *FGFR2* AND IDENTIFICATION OF THREE NOVEL MUTATIONS



7.1 SUMMARY

Patients diagnosed with a craniosynostosis syndrome, or with an imprecise diagnosis including craniosynostosis, were tested for mutations in exons U and B of *Fibroblast Growth Factor Receptor 2* (*FGFR2*). *FGFR2* mutations were found in all 12 unrelated Apert syndrome patients (C770G and C767G), eight unrelated Crouzon syndrome patients (T1030C, C1052G, G1044A, G1037A, T1036C, C1064G, G1021C, T797C), three unrelated Pfeiffer syndrome patients (T1030C, A(-2)G and G(-1)C relative to the start of exon B) and six unrelated patients of mixed diagnosis (C1038G, T1036C, T875A, C1073G, C767G, G(+1)T). Mutations were found in 47%, 60% and 35% of the unrelated Crouzon, Pfeiffer and mixed diagnosis patients tested, respectively. Three of the mutations found were novel; G(-1)C relative to the start of exon B, T875A and T797C. The mutations were confirmed by direct enzyme digest where base changes created restriction enzymes sites, or by the design and application of novel mismatch primers to introduce artificial enzyme sites to either the wildtype or mutant DNA. The mutations found are likely to cause phenotypic abnormalities by affecting the structure of the extracellular portion of the FGFR2 protein. One of the mutations, C1038G, was found in two related patients, one with clinical features of Pfeiffer syndrome and the other having mild Crouzon syndrome. This demonstrates the phenotypic variability associated with FGFR mutations can occur within as well as between families. The first ten of these mutations found have been published (Appendix 7.1 - Hollway *et al.*, 1997).

7.2 INTRODUCTION

Progress in the elucidation of the genetic bases of human disorders of morphology demonstrated that the fibroblast growth factor receptors (FGFRs) play a significant role in limb and craniofacial development. Mutations in *FGFR3* are responsible for achondroplasia (Rousseau *et al.*, 1994; Shiang *et al.*, 1994), thanatophoric dysplasia (Rousseau *et al.*, 1995; Tavormina *et al.*, 1995), hypochondroplasia (Bellus *et al.*, 1995) and Crouzon syndrome with acanthosis nigricans (Meyers *et al.*, 1995). A mutation in *FGFR1* can cause Pfeiffer syndrome and mutations in *FGFR2* cause Apert, Crouzon, Pfeiffer or Jackson Weiss syndromes, all of which have craniosynostosis with varying degrees of digital involvement (Jabs *et al.*,

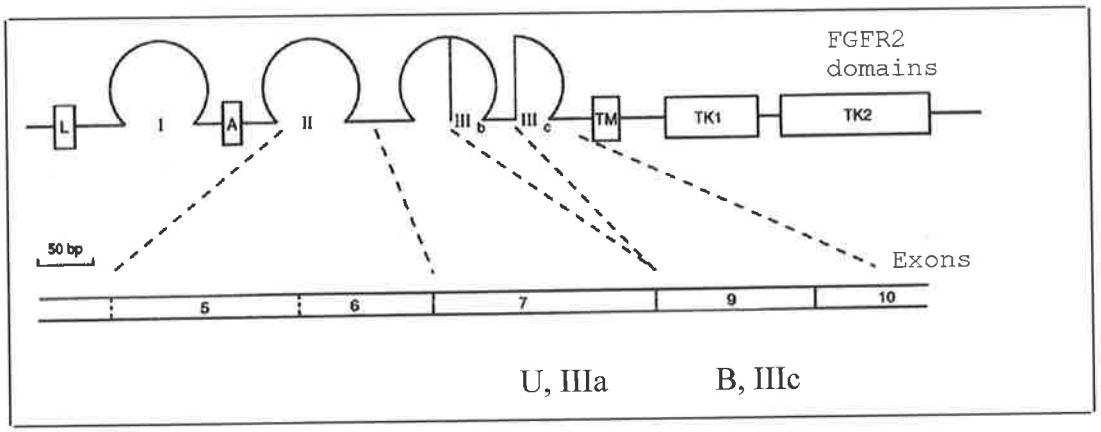
1994; Muenke *et al.,* 1994; Reardon *et al.*, 1994; Gorry *et al.*, 1995; Lajeunie *et al.*, 1995; Oldridge *et al.*, 1995; Park *et al.,* 1995a; Park *et al.,* 1995b; Rutland *et al.,* 1995; Schell *et al.*, 1995; Wilkie *et al.*, 1995b; Meyers *et al.*, 1996; Steinberger *et al.*, 1996a, Steinberger *et al.*, 1996b, Pulleyn *et al.*, 1996, Passos-Bueno *et al.*, 1997. Also, *FGFR2* mutations have been shown to cause Beare-Stevenson cutis gyrata syndrome (Przylepa *et al.*, 1996). Some of the *FGFR2* mutations have been shown to be capable of causing more than one of the syndromes, with the phenotypic variablility occurring both within and between families (Meyers *et al.*, 1996; Appendix 7.1 - Hollway *et al.*, 1997).

Apert syndrome has been shown to be caused by either of two specific mutations in exon U of *FGFR2* in 99% of cases (Park *et al.*, 1995b, Wilkie *et al.*, 1995b), whereas a variety of *FGFR2* mutations have been implicated in cases of Crouzon, Pfeiffer and Jackson Weiss syndromes. Even though a variety of mutations have been detected, they are generally restricted to certain parts of the *FGFR2* gene such that most new cases tested will now have a mutation previously described in the literature. Current screening procedures detect mutations in 50% of Crouzon cases and 80% of Pfeiffer cases (Meyers *et al.*, 1996). The location(s) of the remaining mutations is not known. *FGFR2* mutations have been found in all of the five cases (one familial and four sporadic) of Jackson Weiss syndrome so far screened for mutations (Jabs *et al.*, 1994; Park *et al.*, 1995a; Meyers *et al.*, 1996 and Tartaglia *et al.*, 1997).

The exons of *FGFR2* are referred to by various alternative nomenclatures, exon B is also known as IIIc or 9, and exon U is also known as IIIa or 7. Wilkie *et al.* (1995b) stated that the complete genomic structure of *FGFR2* was not known and that exon assignments should be regarded as provisional. The IIIa and IIIc numbering system was used by Johnson *et al.* (1991), the 7 and 9 numbering was used by Givol and Yayon (1992), and Miki *et al.* (1992) used the exon B and U nomenclature. Different mutation reports use different nomenclature systems, in this study the exons are referred to as exon B and U.

The cDNA sequence has been reported by Houssaint *et al.* (1990) and Dionne *et al.* (1990), and while the amino acid numbering is the same, these publications differ in their nucleotide numbering. The numbering in Houssaint *et al.* (1990) differs to that in Dionne *et al.* (1990) due to shorter 5' and 3' untranslated regions and there is a 9 nucleotide duplication in Houssaint *et al.* (1990), which was not confirmed by further studies. The numbering used by Reardon *et al.* (1994) was that of Houssaint *et al.* (1990) with the 9 duplicated bases omitted, and this is the numbering system used in this chapter. Other mutation reports, such as Wilkie *et al.* (1995b), use the numbering system in Dionne *et al.* (1990). For exons B and U, the numbering used here can be converted to the numbering used in Wilkie *et al.* (1995b) by adding 167. In some places in this chapter, the alternative numbering is shown in parentheses.

Fig 7.1 Diagram of FGFR2, modified from Wilkie *et al.* (1995b). L-hydrophobic leader sequence, A-acidic domain, TM-transmembrane region, TK-intracellular tyrosine kinase domain and I, II and III-immunoglobulin-like domains I, II and III. The alternative second half of the third immunoglobulin domain is coded for by exon 8.



7.3 MATERIALS AND METHODS

Patients studied

Patients were ascertained through the South Australian Clinical Genetics Service and the Australian Cranio-Facial Unit at the Women's and Children's Hospital, Adelaide. The clinical diagnosis of the patients in whom mutation detection work was conducted, are shown in Tables 7.2-7.5. Blood samples were taken from the

patients, and where possible blood was also taken from the patient's parents. Genomic DNA was extracted from the blood samples as previously described (Chapter 2).

Mutation Detection

Single strand conformation analysis (SSCA) and dye terminator sequencing were conducted on *FGFR2* exon U of the first Apert patient (5502) to be screened. Subsequent patients with a clinical diagnosis of Apert syndrome were screened by *Bst*UI, *Bgl*I, *Hae*III and *Mbo*I digestion of exon IIIa PCR products. Products were electrophoresed on 2% agarose gels. The C767G mutation creates a *Hae*III restriction enzyme site and destroys an *Mbo*I site, the C770G mutation creates a *Bst*UI site and destroys a *Bgl*I site (Wilkie *et al.*, 1995b).

Initial mutation screening of patients diagnosed with Crouzon, Pfeiffer or Jackson-Weiss syndrome, or unclassified craniosynostosis consisted of single stranded conformation analysis (SSCA), and in some cases also heteroduplex analysis (HA) (patients 6062, 6075, 6100, 6126, 6327, 6398, 6875 and 6962) of *FGFR2* exons B or U. *FGFR2* exon B was amplified by PCR from genomic DNA using primers published by Reardon *et al.* (1994) and exon U was amplified using the primers published by Park *et al.* (1995a)(Table 7.1).

Table 7.1: Standard *FGFR2* primers

Primer Sequence (5'-3')	Reference
Exon B f:ATC ATT CCT GTG TCG TCT AAC	Reardon *et al.* (1994)
r:AAA AAA CCC AGA GAG AAA GAA CAG TAT A	
Exon U f:TGA CAG CCT CTG ACA ACA CAA C	Park *et al.* (1995a)
r:GGA AAT CAA AGA ACC TGT GGC	

The radioactive PCRs were conducted according to the method in Chapter 2, using Boehringer Mannheim buffer and file 21. Early in the project, the exon U and B products were electrophoresed on 4.5%, 6% and 10% polyacrylamide gels, and if SSCA revealed no variation, these products were also electrophoresed on 37.5% MDE gels (HydroLink). Later in the project, all samples were electrophoresed on 10% SSCA and 37.5% MDE gels, when it was noticed that the 4.5% and 6% gels

generally did not detect any changes not seen on the 10% and/or MDE gels. SSCA and HA gels were prepared, loaded and electrophoresed as described in Chapter 2.

For the majority of samples analysed during this project, DNA samples giving an aberrant band by SSCA or HA were sequenced on an ABI 373A DNA sequencer by Dye Terminator or Dye Primer sequencing (Chapter 2). Sequence changes found were confirmed (or further consistent evidence obtained) by enzyme digests if they created (or destroyed) a restriction enzyme recognition site. The digest products were electrophoresed on 2% agarose gels. Initially PCR products to be digested were purified using the Qiaquick kit; however, the candidate and other members of the laboratory noted this sometimes caused the restriction enzymes to fail to cut the positive controls. Thus, digests were conducted on unpurified PCR products. In cases where direct restriction enzyme confirmation was not possible, mismatch primers were designed (Table 7.6) using *FGFR2* sequence data in Houssaint *et al.* (1990) to create enzyme sites in the normal or mutated allele. When designing a mismatch primer, the relevant exon was checked for additional restriction sites for the enzyme to be used, since an additional site near the artificial site would make the normal and mutated alleles difficult to distinguish by agarose gel electrophoresis of the digest products. Products were amplified using one mismatch primer and one standard primer, in 100ul reactions with Boehringer Mannheim buffer. A standard PCR file was used (94°Cx(1min), annealing temperaturex(1min), 72°Cx(1min))x35 cycles, with different annealing temperatures used, primer 2836: 55°C primer 3147: 55°C, primer 3449: 52°C, primer 3798: 45°C and primer 4192: 52°C.

In the later stages of the project, a number of patient samples, of known mutations (determined earlier in the project) were included on the SSCA gels, with the samples of unknown mutation. It was anticipated that by comparison of different SSCA banding patterns, in some cases it would be possible to bypass the sequencing step, and proceed straight to the relevant mutation confirmation (enzyme digest, or use of mismatch primer followed by enzyme digest). Samples that gave patterns that did not match any of those previously found, were sequenced as before.

Patient samples were tested for *FGFR1* mutations by SSCA on 10% and MDE gels, using the primers published by Muenke *et al.* (1994), F: GGA ATT CCA TCT TCC ACA GAG CGG, R: GGA ATT CCT CAA GAT CTG GAC ATA AGG CAG.

7.4 RESULTS

The patients tested are summarised in tables 7.2-7.5, according to the clinical diagnosis. Mutations that were found are indicated. FGFR2 exons B and U were screened from 100 normal chromosomes, and no SSCA changes were seen. Any SSCA variants found in patients were therefore known not to be common polymorphisms present in the normal population. Mutations were confirmed by enzyme digest or the use of mismatch primers followed by enzyme digest. In those cases where an enzyme site is created, the exact sequence change is confirmed. However, in those cases where an enzyme site is destroyed, the exact nature of the base change is not confirmed, but the presence of a mutation within the recognition sequence of the enzyme is demonstrated, consistent with the sequencing results.

Table 7.2 : Summary of Apert syndrome patients tested and mutations that were found.

Patient DNA Number	Mutation*
5502 @	C770G (C937G) Pro253Arg
6403 @	C767G (C934G) Ser252Trp
6326 @	C767G
6273 @	C767G
6274/6275 (unaffected parents of 6273)	C767G absent
6841	C770G
7222/7223 (unaffected parents of 6841)	C770G absent
7284	C767G
7317	C767G
7515	C770G
7646	C767G
8144	C767G
8152	C770G
8190	C767G

@ included in Appendix 7.1 - Hollway *et al.* (1997)
*Follows nomenclature of Houssaint *et al.* (1990), (Dionne *et al.* (1990) in parentheses).

Table 7.3 : Summary of Crouzon syndrome patients tested and mutations that were found

Patient DNA Number	Mutation	Confirmation (if site created) Consistent with (if site destroyed)	Reference (if previously reported)
5135/5134/5140/5141/ 5136 @	T1030C Tyr340His		Jabs *et al.*, 1994 Reardon *et al.*, 1994
5137/5138/5139 (unaffected relatives of 5135)	T1030C absent		
5788 @	C1052G Ser347Cys	mismatch primer	Jabs *et al.*, 1994
5786/5787 (unaffected parents of 5788)	C1052G absent		
6360/6409	G1044A creation of donor splice site at 344	mismatch primer	Reardon *et al.*, 1994
6075	no mutation found		
6126	no mutation found		
6127/6250 (unaffected parents of 6126)			
6962	no mutation found		
7368	G1037A Cys342Tyr	creates *Rsa*I site	Reardon *et al.*, 1994
7387	no mutation found		
7514	no mutation found		
7873	T1036C $ Cys342Arg	creates *Cfo*I site	
7816	C1064G Ser351Cys	destroys *Eco*RV site	Pulleyn *et al.*, 1996
7935/7936	G1021C exon B Ala337Pro	creates *Bst*NI site	Passos-Bueno *et al.*, 1997
8101/8102	T797C exon U Leu262Pro	creates *Aci*I site	this study
8103	no mutation found		
8147	no mutation found		
7611, 7612, 7613	see Chapter 9		
7910, 7911, 7912	see Chapter 9		

$ mutation confirmed without sequencing
@ included in Hollway *et al.* (1997)
bold type - novel mutations found in this study

Fig. 7.2: Pedigree of Crouzon family, (+) indicates presence of T1030C mutation, (-) indicates its absence.

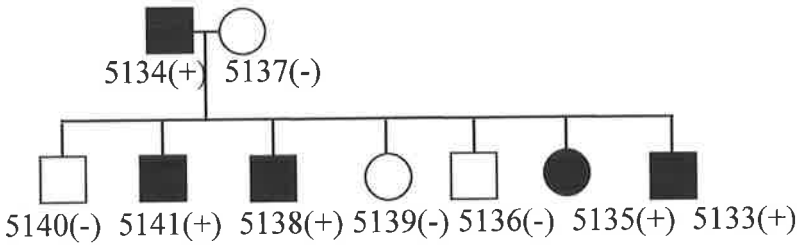


Table 7.4 : Summary of Pfeiffer syndrome patients tested and mutations that were found

Patient DNA Number	Mutation	Confirmation (if site created) Consistent with (if site destroyed)	Reference (if previously reported)
1647 @	T1036C	creates *Cfo*I site	Pfeiffer and Crouzon(Rutland *et al.*, 1995: Schell *et al.*, 1995)
2201 (=7151)	A(-2)G (exon B)	destroys *Bfa*I site	Lajeunie *et al.*, 1995
6062	no mutation found		
6177 @	G(-1)C (exon B)	destroys *Hae*III site	this study
7460	no mutation found		

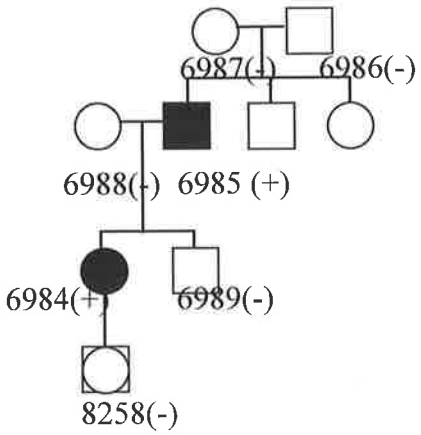
@ included in Appendix 7.1 - Hollway *et al.* (1997)
bold type - novel mutation found in this study

Table 7.5: Summary of clinically unclassified craniosynostosis patients tested and the mutations that were found.

Patient DNA Number	Clinical Diagnosis	Mutation	Confirmation (if site created) Consistent with (if site destroyed)	Reference (if previously reported)
6012 @	Pfeiffer	C1038G Cys342Trp	mismatch primer	
6011	unaffected half sister of 6012	C1038G absent		
6018 @	Crouzon	C1038G Cys342Trp	mismatch primer	Park *et al.*, 1995a (Crouzon)
6327	Misc. Craniosynostosis	no mutation found		
6875	Craniosynostosis	no mutation found		
6984/6985	Crouzon or JWS	T1036C Cys342Arg	creates *Cfo*I site	Reardon *et al.*, 1994 (Crouzon) Park *et al.*, 1995a (JWS)
6986/69876 988/6989	unaffected relatives of 6984 & 6985	T1036C absent		
8258	fetus of 6984	T1036C absent		
7152	Crouzon or JWS	T875A exonU Ile288Asn	mismatch primer	this study
7323	Pfeiffer or Apert	no mutation found		
7269	FGFR?, Crouzon, Pfeiffer or JWS	C1073G Ser354Cys	mismatch primer	Reardon *et al.*, 1994 (Crouzon)
7409	Craniosynostosis	no mutation found		
7416	Craniosynostosis, developmental delay, epilepsy	no mutation found		
7801	Craniosynostosis	no mutation found		
7804	Craniosynostosis	C767G *FGFR2*exU Ser252Trp	as for the Apert mutation	
7822	*FGFR2* Craniosynostosis	no mutation found		
7824	Craniosynostosis	no mutation found		
6398	Crouzon/Pfeiffer	see Chapter 9		
7922	Crouzon/Peiffer/ JWS	no mutation found		
7999	Apert/Crouzon	G(+1)T *FGFR2*exB Ala314Ser	destroys *Hae*III site	Schell *et al.*, 1995
8233	*FGFR1* or *FGFR2* mutation?	no mutation found		

Note: 6018 is mother of 6011 and 6012
6984 is mother of 8258
@ included in Appendix 7.1 - Hollway *et al.* (1997)
bold type - novel mutations found in this study

Fig.7.3: Pedigree of Crouzon/JWS family, (+) indicates presence of T1036C, (-) indicates its absence.



Samples which did not appear to have mutations in *FGFR2* exons B or U, were then subjected to mutation detection in *FGFR1* exon 5, *FGFR3* (Chapter 9), and some were also screened for *FGF8* mutations (Chapter 8). The *FGFR1* mutation screen did not detect any aberrant bands.

A chorionic villus sample (CVS) (8258) was prenatally taken from the fetus of 6984. DNA was extracted from the sample by K. Friend, who also excluded the possibility of maternal contamination of the sample, by the use of AC repeat marker analysis. The candidate then tested the sample for the presence of the T1036C mutation of *FGFR2*, present in the mother. Both SSCA and *Cfo*I digest showed no evidence of the T1036C mutation.

To confirm mutations which were found by sequencing, or suspected on the basis of comparison of bandshifts, restriction enzyme digests were used. In five cases mismatch primers were designed to artificially create recognition sequences in either the normal or mutated sequence (Fig. 7.4). This was necessary since there were no natural recognition sequences created or destroyed by the mutation. The primer sequences are shown in Table 7.6.

Fig. 7.4 Mismatch primer design for the C1038G, C1052G, C1073G, T875A and G1044A mutations, mismatched bases shown in red type and mutated bases shown in blue type.

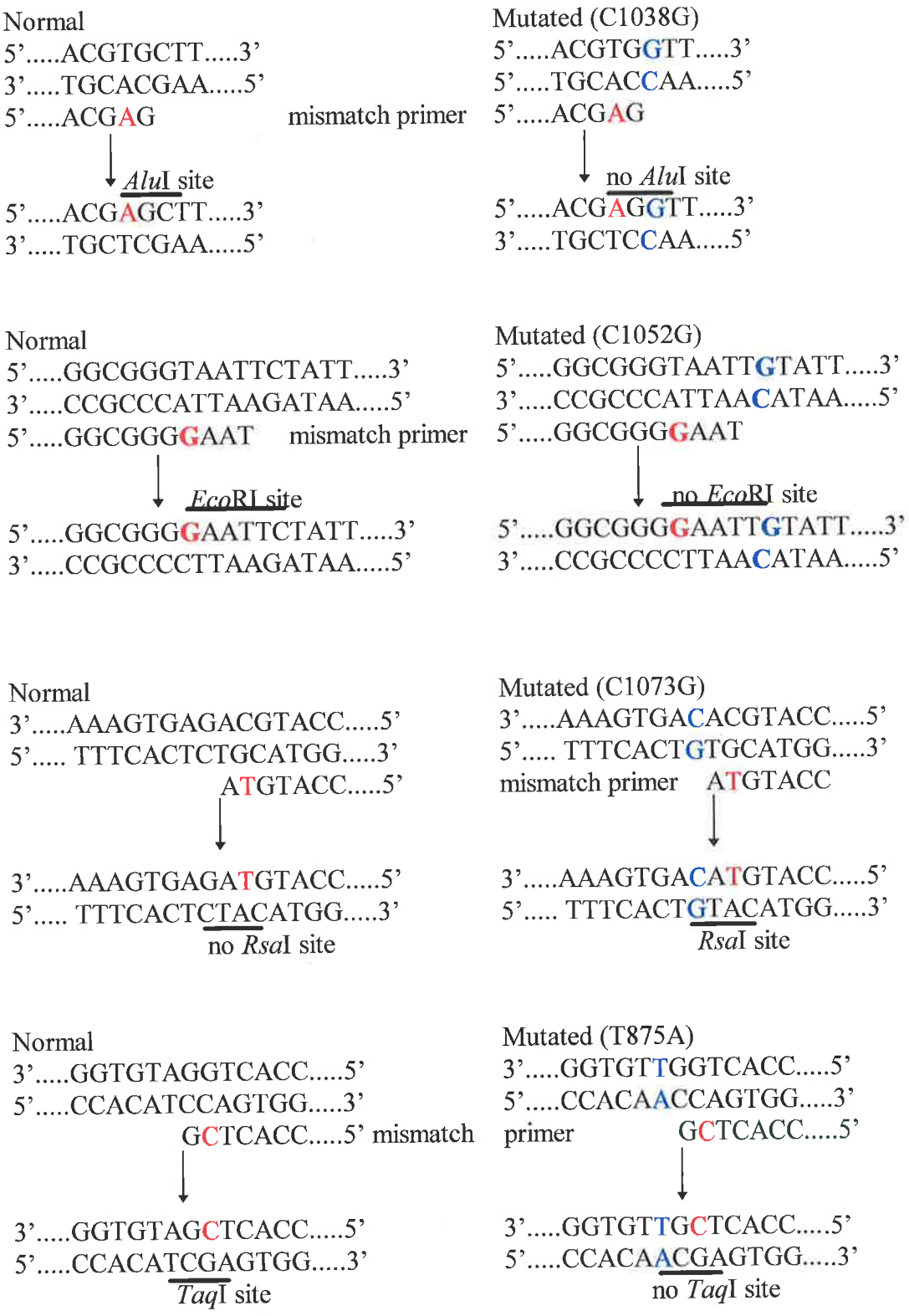


Fig. 7.4 continued

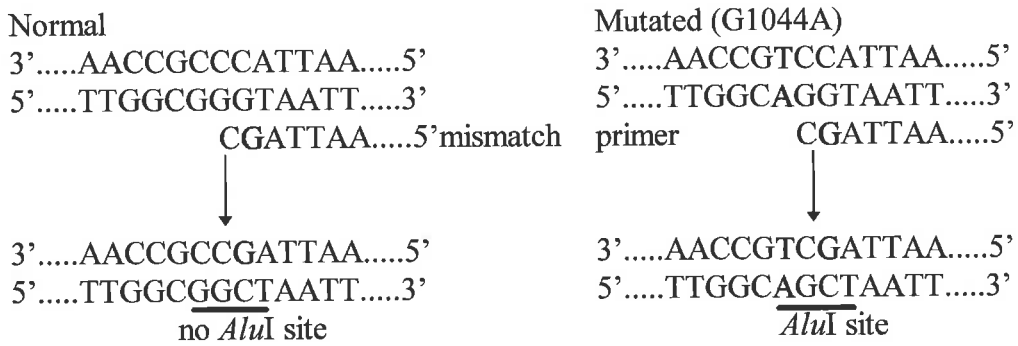


Table 7.6 : Mismatch Primers

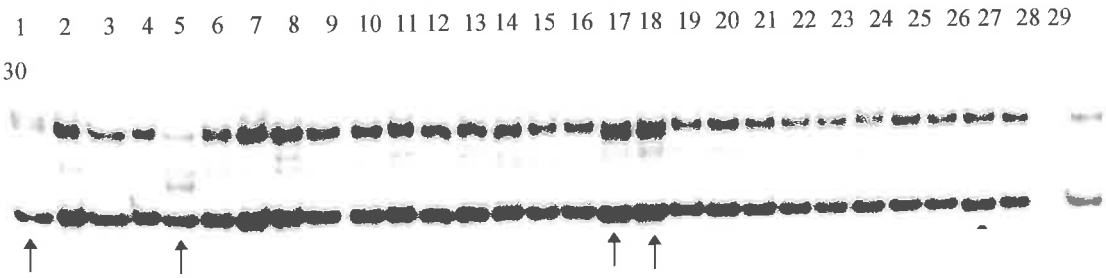
mutation	Sequence of Primers (a)	Restriction Enzyme Site	product size (bp)
exon B C1038G (C1205G)	f 5' GAC GCT GGG GAA TAT ACG **A**G-3'(2836) r: standard ex B reverse primer (2056)	*Alu*I site created in normal DNA sequence	110→ 90+20
exon B C1052G (C1219G)	f: 5'GAA TAT ACG TGC TTG GCG GG**G** AAT-3' (3147) r: standard ex B reverse primer (2056)	*Eco*RI site created in normal DNA sequence	101→ 78+23
exon B G1044A (G1211A)	f: standard exon B forward primer (2055) r: 5'-GGA TAT CCC AAT AGA ATT A**G**C-3'(3449)	*Alu*I site created in mutated DNA sequence	163→ 20+143
exon U T875A (T1042A)	f: standard exon U forward primer (2760) r: 5'-TCC ACG TGC TTG ATC CAC T**C**G-3'(3798)	*Taq*I site created in normal DNA sequence	217→ 20+197
exon B C1073G (C1240G)	f: standard exon B forward primer (2055) r: 5'-CTG GCA GAA CTG TCA ACC ATG **T**A-3' (4192)	*Rsa*I site created in mutated DNA sequence	196→ 174+22

a : Mismatch base in **bold** type

In an attempt to reduce the number of times an exon had to be sequenced from a patient sample to determine the sequence change causing a bandshift, several patient summary gels were conducted (examples shown in Fig.7.5). Patient sample 7873 (lane 27, Fig. 7.5C) appeared on 10% gel to have the same bandshift as patient sample 1647 (lane7, Fig 7.5C). Exon B from patient sample 7873 was not sequenced, the T1036C (Cys342Arg) mutation was confirmed directly by *Cfo*I digest (mutation creates a *Cfo*I site).

Fig 7.5: Samples on Patient Summary Gel, 1-30: exon B, 31-39: exon U. 1. 7151 [A(-2)G], 2. 7269 [C1073G], 3. 7409, 4. 7368 [G1037A], 5. 6177 [G(-1)C], 6.6018 [C1038G], 7. 1647 [T1036C], 8. 6360 [G1044A], 9. 6409 [G1044A], 10. 5788 [C1052G], 11. 5135 [T1030C], 12. 7709, 13. 7707, 14. 7514, 15. 7557, 16. 7611, 17. 6984 [T1030C], 18. 6985 [T1030C], 19. 6986, 20. 6987, 21. 6988, 22. 6989, 23. 6011, 24. 6012, 25. 7726, 26. 7727, 27. 7873, 28. BB888, 29. no DNA, 30. 7152, 31. 7873, 32. 7409, 33. 7152 T875A, 34. 7707, 35. 7709, 36. 7726, 37. 7727, 38. BB888, 39. no DNA.
Arrows denote samples with a SSC/heteroduplex change.

7.5A: Exon B on MDE gel, single stranded DNA.



7.5B: Exon U on MDE gel, single stranded DNA.

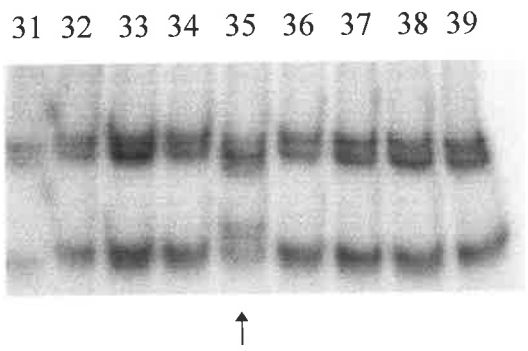
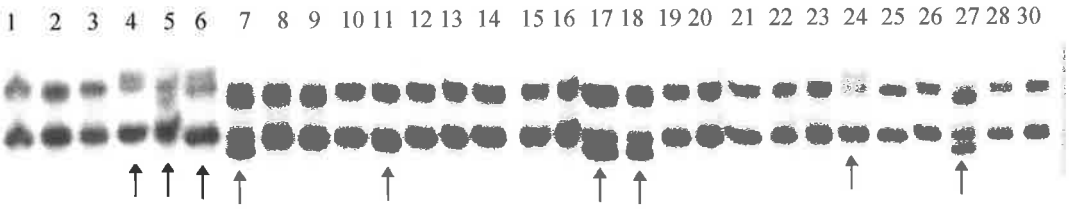
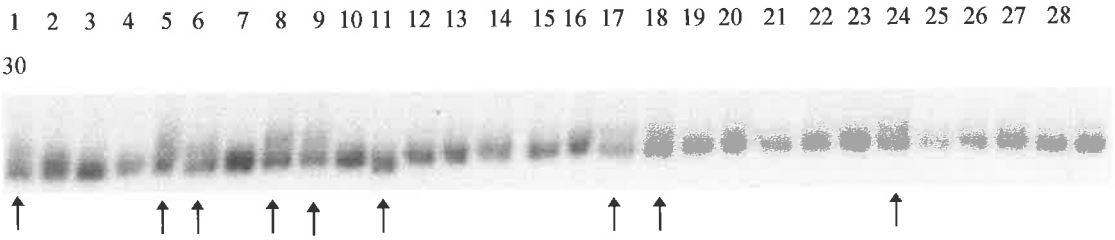


Fig. 7.5 continued

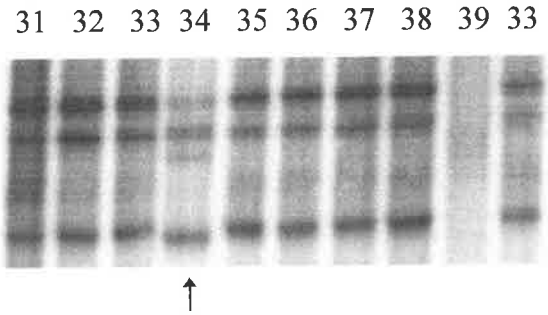
7.5C: Exon B on 10% SSCA gel, single stranded DNA.



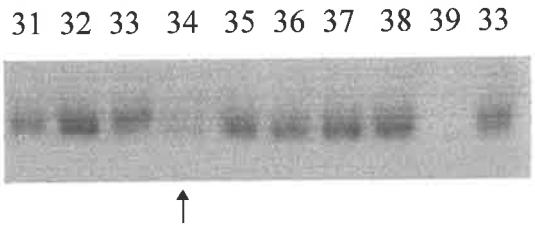
7.5D: Exon B on 10% SSCA gel, double stranded DNA.



7.5E Exon U on 10% SSCA gel, single stranded DNA.



7.5F Exon U on 10% SSCA gel, double stranded DNA.

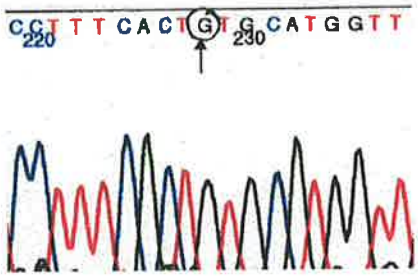


Examples of mutation confirmation in some patient samples are shown in Fig.s 7.6 - 7.8.

Fig 7.6: Elucidation and confirmation of the sequence basis of the mutation present in patient sample 7269.

7.6A: Dye Primer forward sequence

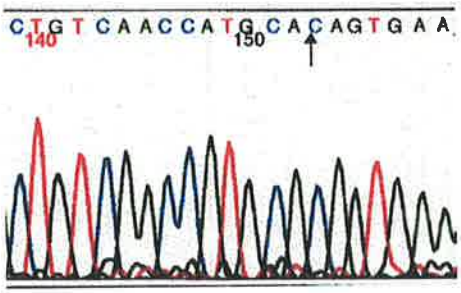
CCTTTCACTCTGCATGGTT normal seq



mutant seq

7.6B: Dye Primer reverse sequence

CTGTCAACCATGCAGAGTGAA



7.6C: Digestion of mismatch primed PCR product to confirm the C1073G (Ser354Cys) mutation in 7269. Lanes, 1: Puc/HpaII, 2: control/*Rsa*I, 3: 7269/*Rsa*I, 4: undigested 7269.

1 2 3 4

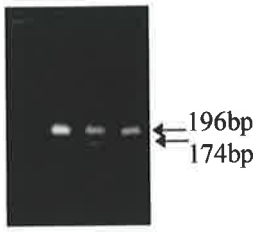
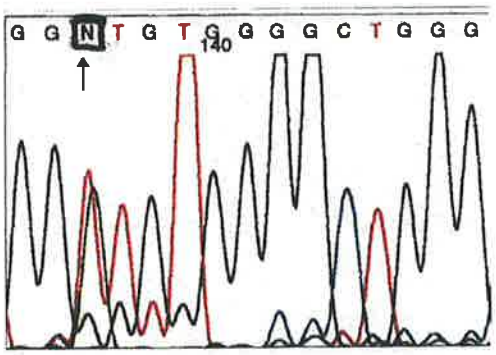


Fig7.7: Elucidation and confirmation of the sequence basis of the mutation in *FGFR2* exon U of patient sample 7152.

7.7A Dye Terminator sequencing with primer 2761.

G G A T G T G G G G C T G G G - normal sequence



- mutant sequence

7.7B: Digestion of mismatch primed PCR product to confirm the T875A (Ile288Asn) mutation in patient sample 7152. Lanes, 1: control/*Taq*I, 2: 7152/*Taq*I, 3: undigested control, 4: puc/*Hpa*II

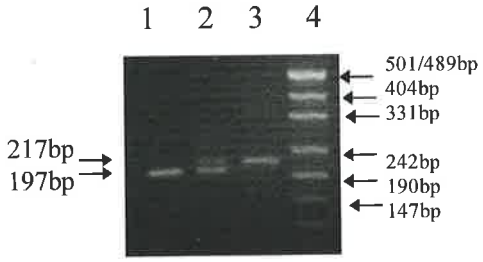
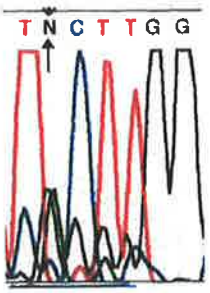


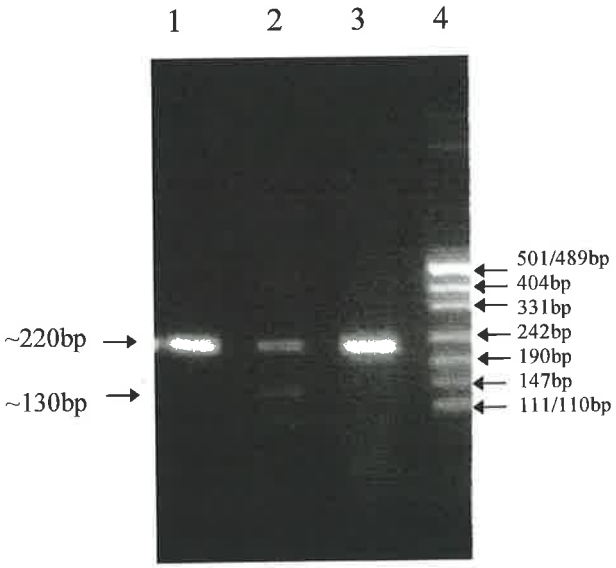
Fig. 7.8: Elucidation and confirmation of the sequence basis of the mutation present in patient sample 7368.

7.8A: Dye Terminator sequencing with primer 2055.

T G C T T G G



7.8B: Digestion confirmation of G1037A (Cys342Tyr) in patient sample 7368. Lanes, 1: control/*Rsa*I, 2: 7368/*Rsa*I, 3: undigested control, 4: Puc/*Hpa*II



7.5 DISCUSSION

Apert syndrome is characterised by craniosynostosis and severe symmetrical syndactyly of the hands and feet. There are also other abnormalities which occur with lower frequencies, including variable mental impairment, various internal organ abnormalities (including heart defects) and skeletal alterations (including progressive shoulder mobility limitation, short humeri, limited elbow extension and abnormalities of the hip joint) (Blank, 1960; Cohen and Kreiborg, 1993). It is

autosomal dominant and has a birth prevalence of approximately 15.5 per 1,000,000 births (Cohen *et al.*, 1992), and this was found to be fairly uniform over a number of different populations and the mutation rate was calculated at 7.8×10^{-6} per gene per generation.

Two specific mutations, in adjacent amino acids of *FGFR2* have been shown to cause Apert syndrome, C767G (Ser252Trp) and C770G (Pro253Arg) (Wilkie *et al.*, 1995b and Park *et al.*, 1995b). These mutations occur in the linker between the second and third immunoglobulin-like domains of *FGFR2* (Fig. 7.1*)*. There has been some debate in the literature as to whether there are any phenotypic differences between those Apert patients with the C767G mutation, and those with the C770G mutation. When initially reporting the *FGFR2* mutations in Apert syndrome patients (and its allelism to Crouzon syndrome), Wilkie *et al.* (1995b) noted that the mean syndactyly severity score for the feet, and for hands and feet combined, was significantly higher for the C767G mutation patients, in the 40 unrelated cases of Apert syndrome that they studied. Park *et al.* (1995b) found the same mutations in all but one of the 36 Apert syndrome patients that they studied, but they found no statistically significant differences for 29 clinical features they examined. Wilkie's group then studied a further 30 patients and found either of the two mutations in all of them. They also found that the two groups of patients differed significantly with respect to frequency of posterior cleft palate, congenital heart disease and for three measures of syndactyly severity (Slaney *et al.*, 1996). According to Slaney *et al.* (1996) the significance criteria used by Park *et al.* (1995b) were probably excessively cautious, also there could have been differences in the classification of syndactyly severity.

Apart from the differences which may or may not exist between the two mutation groups, the phenotypes caused by the two mutations are very similar, which would imply that these mutations have similar effects on the function of the FGFR2 protein. It has been suggested that the mutations could alter the relative orientation of two of the immunoglobulin-like domains (II and III) or cause some alteration to the conformation of the ligand binding site, or both and so mimic or enhance binding of FGFs (Wilkie *et al.*, 1995a). Apert syndrome is a disorder caused largely

by two alternative mutations, and the relative frequency of these mutations has been consistent across a number of studies. Combining the relative frequencies from studies by Moloney *et al.* (1996) (including those patients in Wilkie *et al.*, 1995b and Slaney *et al.*, 1996)-118 unrelated patients, Park *et al.* (1995b) - 35 unrelated patients including one in whom no mutation was found, and Meyers *et al.* (1996) 13 patients, leads to the combined numbers of C767G-108 and C770G- 57. This gives 65% C767G and 34% C770G. The results of this study are 8 patients with C767G (66.6%) and 4 patients with C770G (33.3%) which are consistent with the results found elsewhere. Oldridge *et al.* (1997) reported an Apert syndrome patient who did not have either of the common mutations, but was found to have a 366bp insertion of Alu sequence, 19bp upstream of exon U. This was not present in either parent and was concluded to be the cause of the Apert syndrome. The patient had characteristic features of Apert syndrome, plus some additional atypical findings. Uncommon mutations which give the same phenotype as the common mutations may lead to a better understanding of how the Apert syndrome phenotype is actually caused.

Moloney *et al.* (1996) combined their data with those of Park *et al.* (1995b) and Meyers *et al.* (1996), and used the birth prevalence figure of 1 in 65,000 (Cohen *et al.* 1992) and determined that the germline mutation rates appear to be $5x10^{-6}$ for C767G and $2.7x10^{-6}$ for C770G. Moloney *et al.* (1996) noted this made them the most frequent germline transversions known in the human genome, at that time.

Blank (1960) reported a parental age effect among sporadic cases of Apert syndrome most likely due to increased age of father, that is, the prevalence of sporadic cases was found to be greater, the older the father. Erickson and Cohen (1974) confirmed the parental age effect and also concluded it is probably due entirely to increased paternal age. Moloney *et al.* (1996) determined the parent of origin of the mutation in 57 cases, and in every case it was paternal. They concluded therefore that most, if not all cases of Apert syndrome are due to a mutation of paternal origin, and that the parental age effect is due to paternal age.
In contrast to Apert syndrome, where two mutations are responsible for the vast majority of cases, many different mutations have been implicated in cases of

Crouzon, Pfeiffer and Jackson-Weiss syndromes. All the mutations detected in this study are thought to affect the structure of the extracellular domain of FGFR2 protein (Fig. 7.1). There was one sporadic case (5788), where the parents could be tested, and the mutation was found to be *de novo*. No SSCA changes were found in the 100 normal chromosomes tested. The mutations found in the three familial cases tested, were found to segregate with the affected family members. For these reasons the mutations are likely to be causative. Also, the mutations found in this study have been previously implicated in FGFR2 craniosynostosis syndromes, except the novel mutations found in 6177, 7152 and 8101/8102.

In this study, *FGFR2* mutations were detected in 8 of 17 unrelated Crouzon cases examined, which is consistent with the 50% mutation detection rate for Crouzon patients published by Meyers *et al.* (1996). Of the 5 Pfeiffer cases tested here, *FGFR2* mutations were found in 3, Meyers *et al.* (1996) reported a mutation detection rate in Pfeiffer cases of 67%, which was increased to 80% when the *FGFR1* gene was also considered. The other group of patients considered in this study were those which had a mixed diagnosis, either the patient had some features of, for example, Crouzon and Jackson-Weiss syndromes, or the clinicians didn't specify, and stated craniosynostosis, or requested testing for FGFR mutations. Of the 17 unrelated cases in this category, *FGFR2* mutations were found in 6 cases. One possibility is that some of the patients, in whom no mutations were found, have mutations in other regions of the *FGFR2* gene, that weren't screened in this study. Another possibility is that there are other genes involved, and one candidate is the *FGF8* gene (discussed further in Chapter 8).

There is considerable clinical overlap between the various craniosynostosis syndromes. In Crouzon syndrome patients, the craniosynostosis causes skull abnormalities and characteristic facial features which includes ocular proptosis (prominent eyes). The development of the limbs is generally normal, however there have been reports of limb abnormalities in Crouzon patients. Murdoch-Kinch and Ward (1997) reported a study which confirmed that there is a radiographically detectable abnormality of the hands of Crouzon syndrome patients. This and other studies have been eroding the previously held clinical distinctions between the

different syndromes, leading to an alternative concept of FGFR2 craniosynostoses, that is, the different syndromes are phenotypic extremes of one syndrome. In Jackson Weiss syndrome patients there are skull abnormalities plus clinical and or radiographic abnormalities of the feet. Pfeiffer syndrome patients have, in addition to skull abnormalities broad thumbs, variable cutaneous syndactyly and broad great toes, severe ocular proptosis and elbow ankylosis may or may not be present (Cohen, 1993b). The phenotype may even vary between affected individuals within a single family (Appendix 7.1 - Hollway *et al.* 1997). There is considerable variation in phenotype within each syndrome and the overlapping phenotypes complicate diagnosis, especially for isolated cases.

An intriguing finding to come out of the mutation detection work conducted in this study and similar studies conducted elsewhere, is that different syndromes present in different people, can be caused by the same mutation. There are now several reported instances of a particular mutation being implicated in more that one syndrome. Reardon *et al.* (1994) reported the *FGFR2* mutations Cys342Arg (T1036C) and Cys342Try (G1037A) in Crouzon patients and these mutations were later also found in cases of Pfeiffer syndrome (Rutland *et al.*, 1995). The Cys342Arg mutation was also observed in a Jackson-Weiss syndrome patient (Park *et al*, 1995a) and a Crouzon/JWS patient in this study. Another mutation of this codon, Cys342Ser (T1036A) has been found in cases of Crouzon (Reardon *et al.*, 1994) and Pfeiffer syndrome (Meyers *et al*, 1996) as has the mutation Cys278Phe (G1027T) (Pfeiffer - Meyers *et al*, 1996; Crouzon-Oldridge *et al.*, 1995). Two mutations have been observed in both Crouzon and JWS patients, Ala344Gly (C1043G) (Crouzon-Gorry *et al.*, 1995, JWS; Jabs *et al.*, 1994) and Gln289Pro (A878C) (Crouzon-Oldridge *et al.*, 1995; JWS-Meyers *et al.*, 1996). In addition, Tartaglia *et al.* (1997) reported Cys342Ser (T1036A and G1037C) in Jackson Weiss syndrome patients.

In addition to identical mutations giving different phenotypes in unrelated individuals, there have also been cases where different phenotypes occur within the same family (in people who have the same mutation). One such case occurred in this study, where molecular studies were conducted in a family with clinical

heterogeneity, a mother (6018) had features of mild Crouzon syndrome and her daughter (6012) had features of Pfeiffer syndrome (Appendix 7.1). Both had the *FGFR2* mutation Cys342Trp (C1205G), (confirmed using a mismatch primer), which has previously been found in Crouzon patients only (Park *et al*, 1995a). Other mutations within the same codon (342) are known to have variable phenotypic effects. The T1203C mutation in codon 342 is responsible for Pfeiffer, Crouzon or Jackson Weiss syndromes and the T1204A mutation in codon 342 is responsible for Pfeiffer or Crouzon syndromes (Park *et al.*, 1995a).

Meyers *et al* (1996) also identified a family where different phenotypes were present in family members with the same mutation. Two members of this family had features of Crouzon syndrome, while another had features of Pfeiffer syndrome, including broad thumbs and great toes. All had the mutation Val359Phe (G1254T). Previously some individuals among the many affected family members from within the large, original Jackson Weiss syndrome family had features suggestive of Crouzon syndrome while others were suggestive of Pfeiffer syndrome (Jackson *et al.*, 1976). If examined in isolation, some of these individuals may have been diagnosed as having Pfeiffer or Crouzon syndromes but in the context of the whole family they were diagnosed as having Jackson-Weiss syndrome. The small family in the present study showed similar clinical variability, as did members of the family reported by Meyers *et al.* (1996).

According to Wolf (1997), this is not the only example of such phenotypic heterogeneity, that is, identical mutations giving different phenotypes in unrelated individuals, and sometimes even in different members of a family. For example two distinct diseases, fatal familial insomnia (FFI) and a subtype of Creutzfeldt-Jakob disease (CJD) are both linked to the same mutation (codon 178) in the prion protein gene (*PRNP*). There is a polymorphism, common in the normal population, at codon 129 of the *PRNP* gene. In those with the mutation at codon 178, the codon 129 polymorphism on the mutant allele appears to determine the phenotype (Goldfarb *et al.*, 1992). There are a number of possible causes of identical mutations giving variable phenotypes. These include additional sequence variations within the mutated genes (as in the *PRNP* example) and general genetic

background variation, in particular modifier genes and epigenetic effects modulating the expression of the mutated gene. Stochastic effects during morphogenesis, and environmental influences may also be involved (Wolf, 1997).

Rutland *et al.* (1995) and Gorry *et al.* (1995) both suggested that there may be sequence polymorphisms in *FGFR2* affecting the phenotypic expression of the mutant allele. Gorry *et al.* (1995) suggested that these may lie outside the two well characterised exons. Another possibility is that variations at other loci, the products of which interact with the FGFR2 product, may cause variation. Possibilities include the FGFR2 product from the other allele present, other FGFR protein products and the FGF ligands. According to Gorry *et al.* (1995) another possibility is clinical difficulty in distinguishing Crouzon and JW syndromes, but presumably the phenotypic variation is real, irrespective of the diagnostic label assigned to the patient.

Meyers *et al.* (1996) suggested changing the clinical concepts of these syndromes. These disorders could be regarded as the same condition with phenotypic variability, since the same gene (*FGFR2*) is involved. There are now examples of major phenotypic variability occurring within families, especially large families. Families examined by clinicians generally have a low number of affected members, often only one, so the phenotype variability seen within each family can be low. When rare large families are examined, then intrafamilial phenotypic variability may seem normal rather than the exception (and overall it may seem like one disorder). The phenotype is generally consistent within small families and this may be due to similar genetic backgrounds, specific modifying genes or environmental factors. There are examples of affected kindreds that vary from the standard characteristics of a particular syndrome. For example Kerr *et al.* (1996) reported a Pfeiffer syndrome patients with normal thumbs (that is, not characteristic Pfeiffer thumbs), no *FGFR1* nor *FGFR2* mutations were detected in this patient. Steinberger *et al.* (1997) reported members of a family with signs of Crouzon syndrome and plagiocephaly (not usually associated with Crouzon syndrome). They considered this as evidence that the syndromes named are really just phenotypic extremes of

the *FGFR* mutation spectrum. They found the novel mutation Lys292Glu (A886G) in the affected members of this family.

Whether these disorders should be considered as one condition or several conditions associated with the one gene is merely a question of nomenclature. These syndromes (including Crouzon, Pfeiffer and Jackson Weiss syndrome) could be considered as the one craniosynostotic syndrome (an *FGFR2* craniosynostotic syndrome), as was suggested by Meyers *et al.*, (1996). The only complication with this approach is that Pfeiffer syndrome can then be either an FGFR1 or FGFR2 craniosynostosis syndrome, caused by mutations in two different genes with identical phenotypic effects. Passos-Bueno *et al.* (1997) reported an Apert patient with a Pfeiffer mutation. Thus Apert syndrome may also be part of the FGFR2 craniosynostosis syndrome, and may not be completely mutationally distinct.

Steinberger *et al.* (1996b) reported a high level of phenotypic variability in a family segregating the G1044A mutation of exon B. The range of phenotypes meant they could not clinically assign the patients one of the known craniosynostosis syndromes, further demonstrating that FGFR2 craniosynostosis syndrome may be the more appropriate terminology.

Genotype-phenotype correlations for pathogenic mutations within *FGFR2* might be difficult to establish if the phenotype is modified by naturally occurring genetic variants elsewhere within *FGFR2*, other *FGFR*s or other genes which affect the same developmental pathways. The challenge for future studies is to identify these modifiers. At the present time it is not possible to infer the mutation from the phenotype or the phenotype from the mutation. It is necessary to further explore the genotype-phenotype correlations in *FGFR2* craniosynostosis syndromes as knowledge of the genotype-phenotype relations could have prognostic implications in genetic counselling. The ability to predict the most likely phenotypic outcome of a particular mutation might be useful to enable craniofacial surgeons to make more informed decisions, earlier, about how best to proceed. Candidate modifiers include the polymorphisms found and described in chapters 4, 5 and 8.

As well as the phenotypic heterogeneity that occurs in the craniosynostosis disorder, there is also genetic heterogeneity, that is, different mutations capable of causing the same phenotype, which further complicates genotype-phenotype correlations. There are many mutations of *FGFR2* capable of causing Crouzon syndrome, and Pfeiffer syndrome can be cause by a mutation in FGFR1 (Muenke *et al.*, 1994) or various *FGFR2* mutations.

Currently it is not known precisely how these various mutations affect the *FGFR2* protein to cause craniosynostosis. Many of the mutations found in this study involve cysteine residues, seven involved the creation of an additional cysteine residue or the replacement of a cysteine by another amino acid (patients 5788, 7269, 7368, 7873, 1647, 6012/6018 and 6984/6985 - the mutation in the last five all involved the replacement of Cys 342 by another residue). This is consistent with the result of other studies. According to Wilkie *et al.* (1995a) cysteine 342 and cysteine 278 are predicted to be connected by a disulphide bond in normal individuals. It is thought that these residues are important for structural stability since they are conserved in all FGFRs. If one of these cysteines is replaced by another amino acid, there may be unfolding of the IgIII domain in the vicinity, also the other, unmutated cysteine of the pair is left free and unpaired. There is speculation that the unpaired cysteine could cause intermolecular disulphide bonding between receptor molecules, that is, dimerization without ligand. This could lead to activation of receptors and subsequent signalling, without ligand, which could account for the dominant nature of the mutations. Mutations that create an additional cysteine could act by a similar mechanism since they directly create an additional unbound cysteine. In this study cysteine-creating mutations occurred at codons 347 and 354.

Another group of mutations found in this study involved disruption of splice sites, or activation of cryptic splice sites. The G1044A (A344A) mutation was found in two related patients 6360 and 6409. This mutation was previously investigated by Del Gatto and Breathnach (1995). They found that the mutation makes a rarely used 5' splice site (at that point) more closely resemble the 5' splice site consensus sequence and this then becomes the predominantly used 5' splice site. This leads to

receptor molecules which are lacking 17 amino acids of the third Ig domain of the extracellular region. This would presumably affect the ligand binding capabilities of these molecules.

Three patients were found to have mutations in the 3' (acceptor) splice site of *FGFR2* exon B. The mutations found were A(-2)G in 7151, G(-1)C in 6177 and G(+1)T in 7999 all relative to the start of exon B. Two of these mutations have also been found in other studies (Schell *et al.*, 1995), while the G(-1)C mutation in Pfeiffer syndrome patient 6177 was novel. This G (-1)C mutation in the 3' splice site of *FGFR2* exon IIIc could reasonably be considered pathogenic given the highly conserved nature of the -1 position of the splice acceptor site. Shapiro and Senapathy (1987) examined the sequences of splice acceptor sites in many organisms, and at the -1 (relative to the start of the exon) position they found 100% conservation of the base G, in primates, rodents and other mammals. Su *et al.* (1990) found a G to C base change at -1 with respect to the start of exon 16 of the argininosuccinate synthetase gene in a fibroblast cell line from a citrullinemia patient. They directly demonstrated the disease causing nature of that mutation through the detection of three transcripts resulting from abnormal mRNA splicing. It was not possible to get a further sample from patient 6177, who had a splice site mutation. While Schell *et al.* (1995) did not directly show (by cDNA analysis) that the splice site mutations they found, cause splicing abnormalities, they concluded this would most likely be the case considering the positions of the mutations, the low occurrence of other nucleotides at these positions of the acceptor (3') splice site consensus sequence and by comparison with splice site mutations found in other disorders. These splice site mutations may cause exon B to be skipped, which would result in a frameshift and the creation of a stop codon 48bp downstream from the 5' end of the next exon (Miki *et al.*, 1992), or cryptic splice sites within exon B may be activated.

Krawczak *et al.* (1992) estimated that as many as 15% of the point mutations that cause human genetic disease result in defective mRNA splicing. The mutations and deletions of pre-existing splice sites so far described (Park *et al.*, 1995a; De Moerlooze and Dickson, 1997) appear to be more prevalent among Pfeiffer

patients than among Crouzon patients; however, only a relatively small number of mutations have so far been characterised.

Another novel mutation found in this study was T1042A (Ile288Asn) in a Crouzon/JW syndrome patient, 7152. This is likely to be pathogenic because the same codon was previously implicated in a patient with Crouzon syndrome who had a nine base pair deletion (1038-1046 or 871-879) (Oldridge *et al.*, 1995).

A novel mutation in Crouzon patient samples 8101 and 8102 was also identified in this study, namely T797C (Leu262Pro). No mutations or deletions of this codon have previously been reported, the closest reported mutation is Ser267Pro (Oldridge *et al.*, 1995).

In cases where the mutation did not create or destroy an enzyme site, mismatch primers were designed using *FGFR2* sequence data in Houssaint *et al.*, (1990) to create enzyme sites in PCR products from normal individuals that are absent from the patients, or present on the mutated DNA, but absent from normal. It is worth reiterating that in cases where a mutation destroys a restriction enzyme site, then failure to digest with the particular enzyme doesn't confirm the exact mutation. However, it does confirm that a mutation has occurred within the recognition site of the enzyme, and this in combination with interpretation of the sequencing trace in that region can be considered as confirmation of the mutation. Creation of a restriction enzyme site confirms the exact sequence change that has occurred.

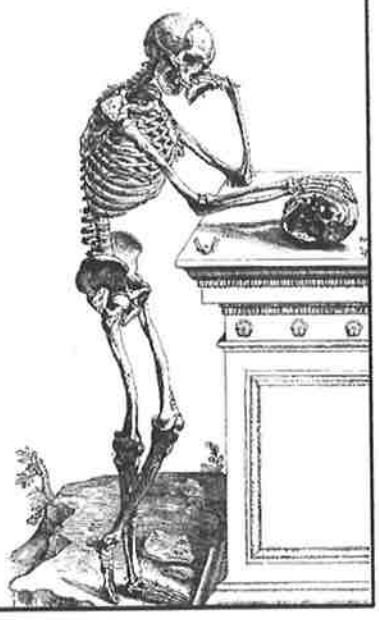
When designing mismatch primers, there is sometimes no choice as to whether an enzyme site is created on the normal or mutated allele. If there is a choice, then the creation of a site in normal DNA means there is an internal positive control in patient samples. These are dominant conditions and affected individuals have both a mutated and a normal chromosome. However, creation of an enzyme site on the mutated allele confirms the exact nature of the sequence change.

The mutation in patient 7873 was not sequenced. The mutation was predicted by comparison to known SSCA patterns, and then the relevant confirmation technique

was applied. Given that mutations are only 100% confirmed by digest in cases where a site is created, perhaps all other samples should still be sequenced. In this study only one sample was not sequenced. It is thought that the frequency of this may increase as a larger bank of samples of known mutation are accrued.

As mutations are found in more patients, genotype-phenotype relations may be uncovered. Further research is required to determine how these mutations act, and what factors modify/influence the mutations to give various phenotypic effects.

CHAPTER 8: MUTATION SCREENING IN *FGF8*



8.1 SUMMARY

The candidate gene *FGF8* was screened for mutations in six craniosynostosis syndrome patients in whom mutations were not found in *FGFR2*. The hypothesis was that *FGF8* might be involved, based on the facts that

1) the gene had been shown to map to the same chromosomal location as *FGFR2*,

2) it had been demonstrated that some FGF8 isoforms were capable of interacting with FGFR2, and

3) the expression pattern of FGF8 was consistent with a role in craniofacial development.

Because of the potential of an interaction between the FGF8 and FGFR2 gene products it was hypothesised that a mutation in either could cause a similar phenotype. Polymorphisms were detected in exons 1C, 2 and 3 (regions 3, 5 and 6), but no mutations of the *FGF8* gene were found in the six craniosynostosis patients with clinical features of Crouzon, Pfeiffer or unclassified craniosynostosis syndromes, who had previously tested negative for *FGFR2* mutations. The nature of the undetected mutations in the patients examined remains unknown.

8.2 INTRODUCTION

In both the *FGFR2* mutation detection studies previously described (Appendix 7.1 and Chapter 7), and in similar studies conducted elsewhere (Meyers *et al.*, 1996), the mutation detection rate in Crouzon patients is about 50%. Schell *et al.* (1995a) linked three Pfeiffer families to 10q, but did not find *FGFR2* (exon B) mutations in two of the families in their study. Reardon *et al.* (1994) found *FGFR2* mutations in only ~50% of the Crouzon patients they looked at and noted that there was no evidence of genetic heterogeneity in the linkage to chromosome 10q25-q26 reported by Preston *et al.* (1994). These results suggest either:

1) that there are mutations in regions of *FGFR2* that weren't screened (promoter regions, some coding regions and intronic sequences of the gene were not screened),

2) mutations may have been missed by SSCA screening, or

3) that there is another gene which maps to the same region that is also involved, and mutations in this second gene are responsible for craniosynostosis (Crouzon and Pfeiffer syndromes) in some patients. *FGF8* was a prime candidate based on map location and functional relationship with *FGFR2*.

Mouse Fgf8 was initially isolated by Tanaka *et al.*(1992). They stimulated a mouse carcinoma cell line with testosterone and purified the androgen-induced growth factor (AIGF) from the conditioned medium of the stimulated cells. They then derived partial amino acid sequence of this product and via the use of degenerate oligonucleotide primers, isolated some clones from a cDNA library. They found two alternatively spliced transcripts, which they suggested corresponded to different protein isoforms. By analysing the amino acid sequence of AIGF, they found significant homology with the FGF family, and the *AIGF* gene is now known as *FGF8*.

In addition to the two alternate cDNAs (representing two protein isoforms) that Tanaka *et al.* (1992) found, Crossley and Martin (1995) reported a further five RNAs of *Fgf8*, encoding different protein isoforms (produced by alternative splicing). Crossley and Martin (1995) detected *Fgf8* expression in many tissues of the developing mouse embryo, including the developing head and limbs and also in the inner ear. Many of the sites where *Fgf8* expression was found are known to be involved in directing outgrowth and patterning. These sites included the apical ectodermal ridge of the limb bud, the primitive streak and tail bud, the surface ectoderm overlying the facial primordia and the mid-brain-hindbrain junction (Crossley and Martin, 1995).

Tanaka *et al.* (1995) isolated the human copy of the *AIGF/FGF8* gene (from a placental genomic library) and showed that the amino acid sequence which it encodes is identical to that of the mouse AIGF/Fgf8 protein.

MacArthur *et al.* (1995) investigated the spatial and temporal localisations of the FGF8 isoforms, and tested their interactions with various FGFRs. They analysed the expression of several FGF8 isoforms by *in situ* hybridisation, immunohistochemistry and by RNase protection methods, but they did not find any major differences in the spatial and temporal localisations. They then tested the ligand/receptor interactions of three recombinant (r) FGF8 isoforms, and found none of these activated FGFR1b, FGFR2b or FGFR3b. The rFGF8b isoform demonstrated a very weak interaction with FGFR1c, a reasonably strong interaction with FGFR2c and a strong interaction with FGFR3a. The rFGF8c isoform was shown to interact weakly with FGFR2c, strongly with FGFR3c, but not at all with FGFR1c. The rFGF8a isoform did not activate any

of the FGFRs tested. Thus both rfGF8b and rFGF8c were found to activate FGFR2c (to varying degrees) demonstrating the potential for an *in vivo* interaction between these ligands and receptors, assuming some overlap in their spatial and temporal expression patterns.

White *et al.* (1995) mapped the human *FGF8* gene to chromosome 10q25-q26, near *FGFR2*, and in July 1996 the genomic structure and sequence of the gene was published by Gemel *et al.* (1996). This provided the basis for screening genomic DNA for *FGF8* mutations.

Thus, there were a number of factors which indicated that mutations in *FGF8* may be responsible for those craniosynostosis cases which map to 10q25-q26, but in whom no mutations of *FGFR2* have been found. Namely, the gene had been shown to map to the same chromosomal location as *FGFR2*, it had been demonstrated that some FGF8 isoforms were capable of interacting with FGFR2, and the expression pattern of *FGF8* (including the developing head and limbs) was consistent with a role in craniofacial development. Because of the potential of an interaction between the *FGF8* and *FGFR2* gene products it was hypothesised that a mutation in either gene could cause a similar phenotype. The aim of the work presented in this chapter was to test the hypothesis that *FGF8* might be involved in craniosynostosis in those patients without *FGFR2* mutations.

8.3 MATERIALS AND METHODS

Patient Samples

DNA from six patients with clinical features including craniosynostosis (Table 8.1), in which abnormalities of the *FGFR2* gene had not been detected were selected for mutation screening of *FGF8*. These patients had been tested for *FGFR2* mutations by SSCA (10% and MDE gels) and in some cases HA (Chapter 7), and had been tested for the Pro250Arg mutation of *FGFR3* (Chapter 9). Clinical diagnosis is known to be difficult due to overlapping phenotypes, thus the mutation detection screen of *FGF8* was not restricted to those patients with a diagnosis of Crouzon or Pfeiffer syndrome (Table 8.1).

Table 8.1: Diagnoses of Patients included in *FGF8* Mutation Screen

Patient Number	Original Diagnosis
6062	Pfeiffer syndrome
6075	sagittal and lambdoidal craniosynostosis, query Crouzon syndrome
6126	Crouzon Syndrome
6327	short stature, craniosynostosis, midface hypoplasia and other dysmorphic features
6875	multiple craniosynostoses
6962	Crouzon Syndrome

SSCA

Seven pairs of primers were designed to amplify the coding sequence of *FGF8*, all inton/exon boundaries were included (Appendix 8.1). The exons screened, primers used and product sizes are shown in Table 8.2. All products were amplified using file 21 and PCR conditions described in Chapter 2. The regions covered by these primers were amplified from each patient and SSCA was conducted on 10% SSCA and 37.5% MDE gels.

Table 8.2: Primers used to screen *FGF8*

Primer Region and Primer Sequences	Exons Screened	Product Size
1. FGF8-11(f): GAG CAC GAC GTT CCA CGG GA FGF8-12(r): CAG CAA GTG CAA CAG CCT GT	1A and part of 1B	300bp
2. FGF8-1BF(f): TCT CTC TCC CGC CCG CTT TT FGF8-1BR(r): AGT GCT GGG CTC CGA GAC CTT	remainder of 1B	308bp
3. FGF8-1CF(f): AGG GCT GCC TCC CTA CTT AA FGF8-1CR(r): CAG CCC AGG ATG AAC GAG	1C	166bp
4. FGF8-1DF(f): GGG CGG AGT AGC ATT ATA ATG FGF8-13(r): AGT GCA GTT GGG ACT GGT GGT	1D	295bp
5. FGF8-14(f): GGT CAG GGA TCT GCC AAT AG FGF8-15(r): ACT GTC TTG GAG GAG TCC AG	2	226bp
6. FGF8-16(f): TGG GAA GGA CAG GAA GTT GC FGF8-17(r): ATG TAC CAG CCC TCG TAC TTG	part of 3	290bp
7. FGF8-3F(f): CAG AGC AAC GGC AAA GGC AA FGF8-3R(r): TCT CTG CGG TCT GGC ATT GTG	remainder of 3	331bp

8.4 RESULTS

In the initial screen the six patient and six control samples were analysed by SSCA (10% SSCA and 37.5% MDE) for regions 1-7. Since a number of different patterns were noted for regions 2, 3, 5 and 6 in this initial screen (Table 8.3), the mutation screen was expanded to include a variable number of additional DNA samples from random blood donors, plus those samples which had shown possible different patterns in the initial screen.

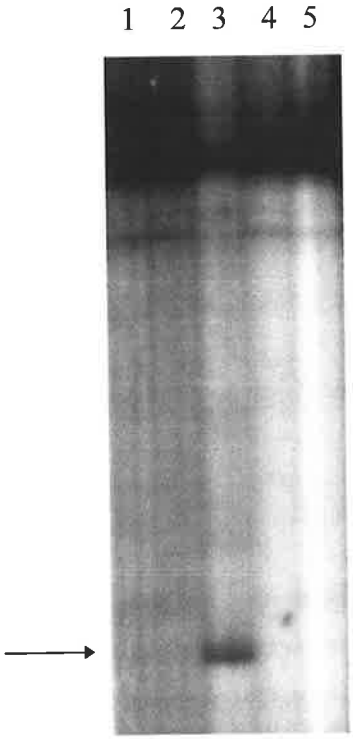
Table 8.3: Results of Initial Mutation Screen of *FGF8* in six patient and six control samples

Primer Region	Changes seen in Initial Screen
2	possible heteroduplex changes on 10% and MDE gels in patient and control samples
3	patient sample 7460 and a control sample - band shift on 10% SSCA gel
5	patient sample 7460-altered on MDE and 10% gels, and a control sample-altered on MDE and 10% gels, but different to 7460
6	patient sample 7416-band shift on 10%SSCA gel

Results of Expanded Mutation Screen

For region 2, patient samples 6075, 7557, 7416 and 7460 plus 20 control samples were tested on 10% and MDE gels. On both 10% and MDE gels, sample 7460 had an extra band some distance from the other single stranded bands, no other changes were evident (Fig. 8.1).

Fig. 8.1: Region 2 samples on 10% SSCA gel. Lanes 1: 7757, 2: 7416, 3: 7460, 4: BB953, 5:BB952. BB samples from random blood donors. Extra band in patient sample 7460 indicated by an arrow.



For region 3, patient samples 7557, 7416 and 7460, plus 39 control samples, were tested on 10% and MDE gels. Three control samples (BB895, BB893 and BB914) had an altered pattern on both 10% and MDE gels, none of the patient samples had any observable changes (Fig. 8.2). Seventy three CEPH parent DNA samples were then screened for the polymorphism; however, none of these were found to have the bandshift, whereas it was consistently detected in BB895, BB893 and BB914.

Fig. 8.2: Example of SSCA change in region 3 seen on 10% gel. Lanes, 1: BB914, 2: BB728, 3: 7475. Single and double stranded extra bands indicated by arrows.

For region 5 patient sample 7460 and 46 control samples were tested on MDE gel. Different bandshifts were observed in control sample BB896 and patient sample 7460 (Fig. 8.3). The extra band present in BB896 was also present in two of the 42 CEPH parent DNA samples tested, samples 2101 and 2102. Transmission of this polymorphism in family 21 was consistent with Mendelian inheritance (Fig. 8.4).

Fig. 8.3: SSCA of region 5 of patient and control samples on MDE gel. Lanes, 1: 7460, 2: BB896, 3: BB921, 4: BB955 and 5: BB915. Arrows indicate extra bands seen.

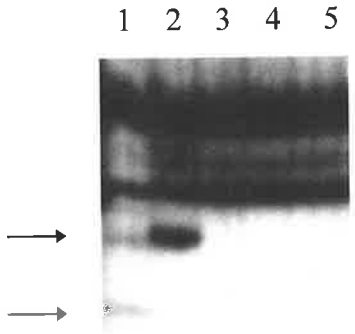
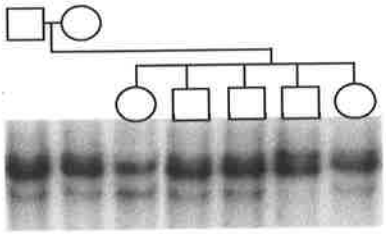


Fig. 8.4: Mendelian inheritance of region 5 polymorphism in CEPH family 21.



For region 6, patient samples 7557, 7416 and 7460 and 44 control samples were screened on MDE and 10% gels. On MDE gel, sample 7416 had an altered pattern which was also present in six control samples. Another pattern was present in two control samples, and a different pattern again was present in a single control sample, indicating a high degree of polymorphism in this region (Fig. 8.5). On 10% gel an altered pattern was seen in one control sample, while 7460 had an altered pattern also seen in a control individual (Fig. 8.6). The most frequent polymorphism, seen in samples 7416, BB914, BB893 and BB895, was also found in 4 of the 40 CEPH parent DNA samples tested. Three were heterozygous for the bandshift, and one was

homozygous (Fig. 8.7). Mendelian inheritance of the most frequent polymorphism was demonstrated in CEPH families 12 and 1408 (Fig. 8.8). The other bandshifts detected in this region were not examined any further.

Fig. 8.5: Region 6 SSCA samples on MDE gel. Lanes, 1: BB895, 2: BB894, 3: BB893, 4: BB922, 5: BB921, 6: BB920, 7: BB918, 8: no DNA control, 9: BB916, 10: BB915, 11: BB914, 12: BB911, 13: BB910, 14: BB885, 15: BB905, 16: BB909, 17: BB907, 18: BB903, 19: BB902, 20: 7557, 21: 7416, 22: BB811.

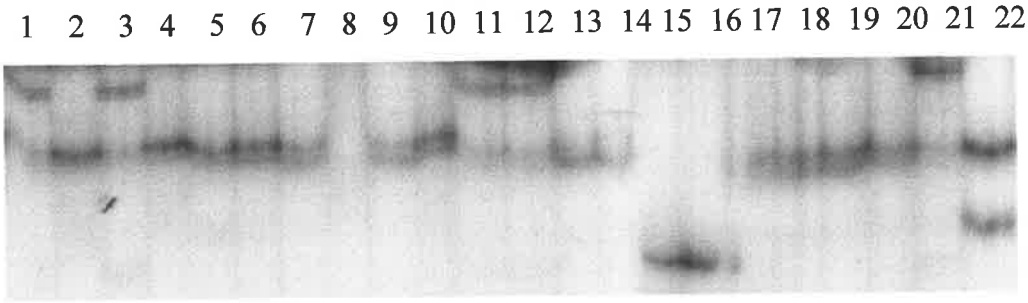


Fig 8.6: Region 6 samples on 10% SSCA gel. Lanes, 1: BB949, 2: 7460, 3: BB925.

Fig. 8.7: SSCA of region 6 from CEPH parent samples on MDE gel. Lanes, 1: 141302, 2: 133401, 3: 133402, 4: 134001, 5: 134002, 6: 140801, 7: 140802. CEPH parent 141302 was heterozygous for the bandshift, and CEPH parent 140802 was homozygous for the bandshift.

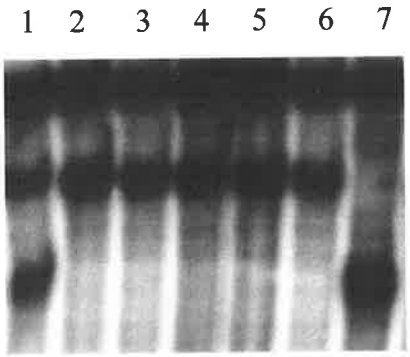
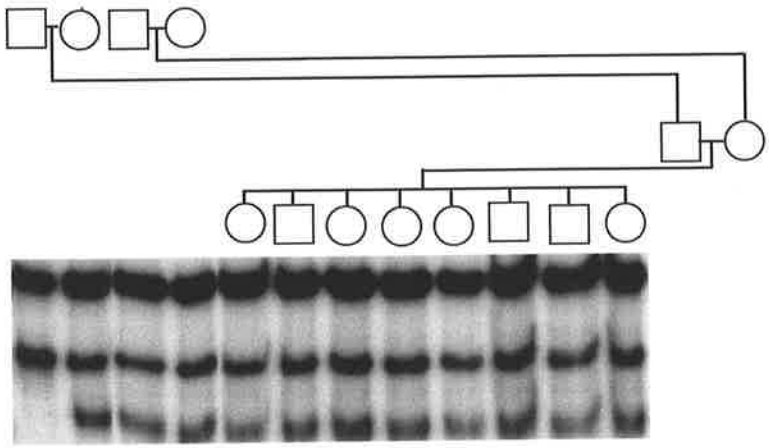


Fig. 8.8: Region 6 polymorphism in CEPH family 1408. Parents (140801 and 140802 are shown in Fig. 8.7). All children of 140801 and 140802 were heterozygous for the polymorphism.



Patient sample 7460 gave a unique pattern for regions 2, 3 and 5. Investigation revealed that this sample had been a very small blood sample and that DNA was extracted from it using the Isoquick kit (according to manufacturers instructions by Jean Spence). To investigate whether the preparation of the sample was in some way contributing to the results observed, another larger sample (7475) was obtained from the same patient, and DNA was extracted using the normal method. SSCA was

conducted on regions 2 and 5 from both 7460 and 7475, and altered patterns were seen in 7460 only. This created serious doubts about sample 7460, and the altered patterns it had produced. Sample 7460 was not considered further.

8.5 DISCUSSION

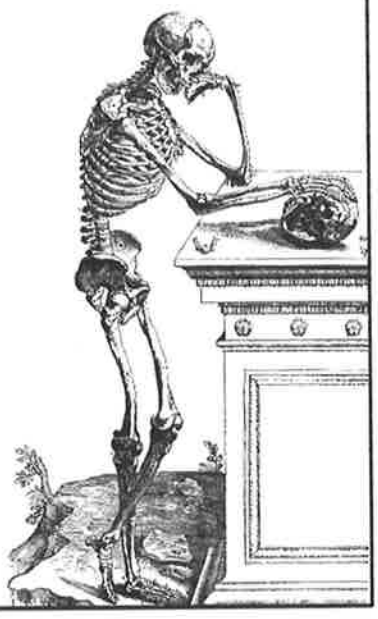
Patient sample 7416 was the only patient sample confirmed as having a band shift in an *FGF8* exon, this was in region 6 (exon 3), and the same pattern was also seen in control samples. Polymorphisms were also found in regions 3 (exon 1C) and 5 (exon 2). Mendelian inheritance of the polymorphisms was demonstrated using DNA from CEPH families. This was not possible for the polymorphism found in region 3 since the bandshift was not found in any of the 73 CEPH parents tested. This was unexpected since the bandshift was found in three of the 39 control DNA samples tested. The control DNA samples were from random blood donors in Adelaide, Australia. The CEPH parent samples are from French, North American (Utah) and Venezualan families. The difference in population origin could explain the failure to detect the polymorphism in the CEPH parent DNA samples, alternatively it may have been due to chance alone. Another occurrence, presumably attributable to chance, is the fact that when region 5 was screened in 42 CEPH parents the only two samples found to have the bandshift were a married couple 2101 and 2102.

The exons were not sequenced to discover the molecular basis of the polymorphisms found, since they were clearly of no major pathological significance (being present in random blood donors and CEPH family members, in some cases). These polymorphisms are potentially useful to exclude the *FGF8* gene, by the examination of recombination events, if it becomes a candidate in family studies for any other disorder. It is also possible that while these polymorphisms appear neutral in members of the normal population, in combination with a mutation (for instance, in this gene, other *FGF* genes or *FGFR* genes) they may act as modifiers of the phenotype (analogous to the fatal familial insomnia (FFI) and Creutzfeldt-Jakob disease (CJD) situation, see Chapter 7). In this study three polymorphisms but no mutations were found, however the number of patient samples tested was low.

As the present study was in progress, Yoshiura *et al.* (1996; 1997) independently reported screening *FGF8* in two Pfeiffer syndrome kindreds, a craniosynostosis type Philadelphia family (Robin *et al.*, 1996) and 65 DNA samples from patients with sporadic Pfeiffer syndrome or other craniosynostosis syndromes. They identified six variants by SSCA and sequenced them. Five of these were base substitutions in introns, and did not appear to affect splicing. The other mutation was an 18bp (6 amino acid) inframe duplication in exon 1C. It is possible that the exon 1C (region 3) polymorphism detected by the candidate in this study is the same as this duplication (since the large size of the bandshift is consistent with a duplication). Yoshiura *et al.* (1997) reported a frequency of 0.0025 of the duplication in a Caucasian population. In this study polymorphisms were detected in the PCR products covering exons 1C, 2 and 3, however it is possible that the variation actually occurred in the flanking intronic sequence included in the amplicons, and that the exon 2 and 3 polymorphisms also correspond to polymorphisms found in the Yoshiura *et al.* (1997) study.

Yoshiura *et al.* (1997) found no evidence for involvement of *FGF8* in craniosynostosis, in agreement with this study, and they concluded that mutations of the coding region of *FGF8* are unlikely to be a common cause of craniosynostosis. In the present study, it is possible that some mutations of *FGF8* were missed by the screening technique used, SSCA. It is also possible that mutations in the promoter or intron regions of *FGF8* (not screened in this study) cause craniosynostosis. Promoter mutations could disrupt the expression of the gene, and intronic mutations could be pathogenic if they disrupt an existing splice site, or create a new splice site. The mutations in those patients in whom *FGFR2* mutations were not detected, could lie in the coding regions of *FGFR2* which were not screened, or in the promoter or intron regions. Another possibility is that there is another unknown gene in the region of *FGF8* and *FGFR2*, mutations of which cause craniosynostosis. Subsequent additional craniosynostosis syndrome patients in whom no *FGFR2* mutations were found, were not tested for *FGF8* mutations on the basis of the negative results already described in this chapter, and described by Yoshiura *et al.* (1997).

CHAPTER 9: DETECTION OF THE PRO250ARG MUTATION OF FGFR3 IN PATIENTS WITH CRANIOSYNOSTOSIS AND DEAFNESS



9.1 SUMMARY

Craniosynostosis patients in whom no *FGFR2* mutations were found (Chapter 7), were tested for the C749G (Pro250Arg) mutation of *FGFR3*. The mutation was found in six unrelated patients from a total of 27 unrelated patient samples which were tested. The three familial and three sporadic positive cases were originally diagnosed with Crouzon syndrome, Pfeiffer syndrome, Saethre Chotzen syndrome or Craniosynostosis Adelaide Type and some were diagnosed with uncharacterised craniosynostosis. One of the familial cases had four members with craniosynostosis (one of whom was deaf). A further 13 family members were deaf, with no evidence of craniosynostosis. The presence of the Pro250Arg mutation in the members of this family with craniosynostosis and/or deafness raises the possibility that some families with the Pro250Arg mutation of FGFR3 could present with deafness alone (Appendix 9.1 - Hollway *et al.*, 1998).

9.2 INTRODUCTION

A group of eight patients, diagnosed with a craniosynostosis syndrome or uncharacterised craniosynostosis, were tested for the Pro250Arg mutation of FGFR3. The patients tested were those that were negative for the FGFR2 mutation screen (Chapter 7) or diagnosed with Craniosynostosis Adelaide Type. The Craniosynostosis Adelaide Type family members with the Pro250Arg mutation displayed a wide range of phenotypes (Chapter 3) and so no discrimination on the basis of original diagnosis was made to determine who should be tested for the FGFR3 mutation. Following this initial screen, patients in whom no FGFR2 mutations were found, were routinely screened for the Pro250Arg mutation of FGFR3. Several samples were tested for the Pro250Arg FGFR3 mutation, without prior FGFR2 testing, because the clinical features were reminiscent of those of the Craniosynostosis Adelaide Type family (namely deafness was present), or because the test was specifically requested by a clinician after this syndrome was defined (Chapter 5, Appendix 5.1).

9.3 MATERIALS AND METHODS

The screening for the Pro250Arg FGFR3 mutation was conducted by *Nci* I digests as described in Chapter 5.

Linkage analysis in the craniosynostosis-deafness pedigree (Fig. 9.3) was conducted as described in Chapter 3 with the Pro250Arg mutation tested against the craniosynostosis-deafness phenotype. Persons marrying into the family were coded as unaffected, and a gene frequency of 0.0001 was used for the disease allele. The phenotype was analysed as a dominant disorder with 90% penetrance, and no phenocopy rate was used. Two patients were coded as affection status unknown, 8196 because her deafness may have had a different cause, and 7727 because she was too young for her hearing to be adequately tested.

9.4 RESULTS

Following the finding of the Pro250Arg mutation in the Craniosynostosis Adelaide Type family, an initial subset of eight patients were tested for the C749G mutation of *FGFR3* (Fig 9.1). These eight patients (6062, 6075, 6100, 6126, 6327, 6398, 6875 and 6962) had been negative for *FGFR*2 mutation screening. The mutation was found in 6100 and 6398. Thus, samples that were negative to *FGFR*2 mutation detection (Chapter 7), were then routinely checked for the *FGFR3* mutation, before proceeding to *FGF8* mutation screening (Chapter 8). Also, as awareness of this mutation increased, three patient samples (8143, 8142 and 8172) arrived with a specific request to test for the C749G (Pro250Arg) mutation. The mutation was found in one (8143) of these patients.

The Pro250Arg mutation was found in a total of six unrelated patient samples (three sporadic and three familial cases) of craniosynostosis. A total of 27 unrelated patient samples were tested for the mutation. The patients who tested positive had a wide range of original clinical diagnoses (Table 9.1) and had differing phenotypes.

Fig 9.1: *Nci* I digests of patient samples. Lanes, P:puc/*Hpa*II, 1: 6062, 2: 6075, 3: 6100, 4: 6126, 5: 6327, 6: 6398, 7: 6875, 8: 6962, 9: 3211, 10: 3210, 11: undigested control sample. 3211: positive control from Craniosynostosis Adelaide Type family, 3210: negative control from Craniosynostosis Adelaide Type family. + indicates presence of the mutation.

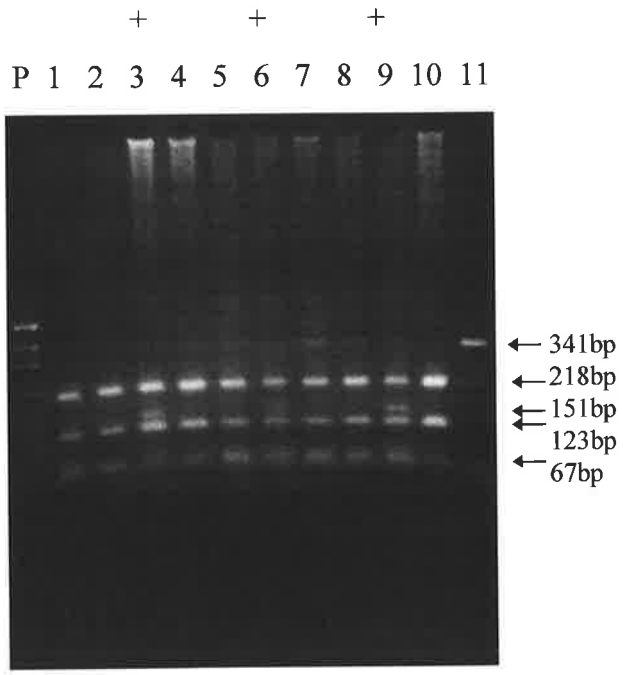


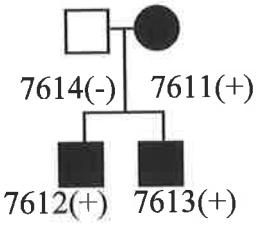
Table 9.1: Original Diagnosis of Patients with the C749 (Pro250Arg) mutation of *FGFR3*

DNA Number	Original Diagnosis
6100	Saethre Chotzen syndrome
7611, 7612 and 7613	Crouzon syndrome
6398	Crouzon/Pfeiffer syndrome
7910, 7911 and 7912	query Crouzon syndrome
8143	Craniosynostosis Adelaide Type
7709, 7726 and 7727*	Craniosynostosis, deafness

*- 15 additional family members were later tested (8196, 8197, 8198, 8199, 8200, 8201, 8202, 8203, 8204, 8205, 8206, 8207, 8208, 8289 and 8293)

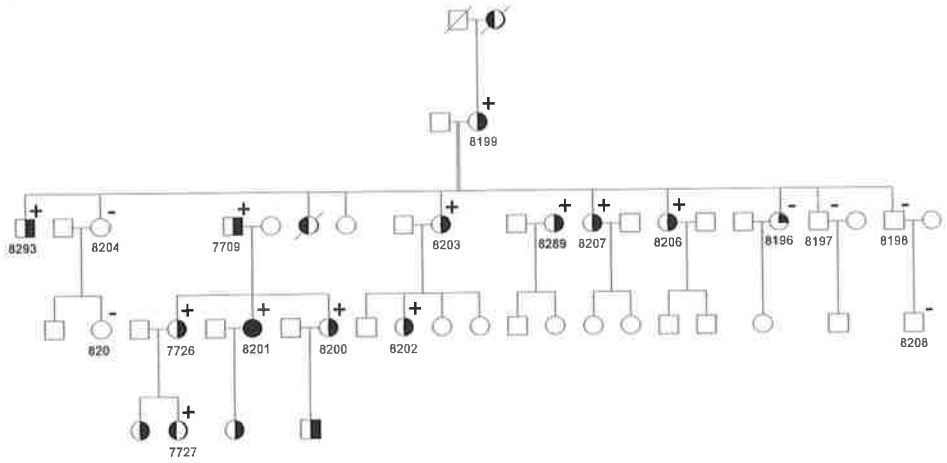
The Pro250Arg mutation was found to segregate in the familial cases tested (Fig. 9.2).

Fig. 9.2: Crouzon pedigree (+mutation present, -mutation absent)



Samples arrived from three patients (7709, 7726 and 7727) with uncharacterised craniosynostosis and due to the presence of some other clinical features reminiscent of Craniosynostosis Adelaide Type (including deafness), these samples were tested for the Pro250Arg FGFR3 mutation without prior FGFR2 mutation screening. These three samples were positive for the Pro250Arg mutation. Patients 7709 and 7726 had bilateral deafness in addition to craniosynostosis (7727 was too young for hearing to be tested). Other members of the family were known to have bilateral deafness without any clinical signs of craniosynostosis (clinical data are presented in Appendix 9.1). Samples were collected from a further fifteen members of the family, and tested for the Pro250Arg mutation of FGFR3. The mutation was found to cosegregate with craniosynostosis or deafness in the family (Fig. 9.3) with one exception. One woman, 8196, had unilateral deafness (in contrast to the other affected family members), no evident craniosynostosis and did not have the mutation. Her child was reported to be normal. If it was assumed that 8196 had a coincidental cause for her deafness and hence she was coded as being of unknown phenotype, the mutation showed no recombination with the craniosynostosis-deafness phenotype, with a maximum lod score of 4.19 at theta=0.

Fig. 9.3: Craniosynostosis/deafness pedigree, showing segegation of the Pro250Arg mutation with the craniosynostosis/deafness phenotype. Symbols, + Pro250Arg mutation present, - Pro250Arg mutation absent,  - bilateral deafness,  - unilateral deafness,  - craniosynostosis.



9.5 DISCUSSION

The C749G (Pro250Arg) mutation of *FGFR*3 has now been detected in people with a wide range of phenotypes. Reardon *et al.* (1997) found the mutation in four familial and five sporadic cases of craniosynostosis, in 165 craniosynostosis patients tested. The extent of craniosynostosis in those with the mutation ranged from unilateral or bilateral coronal craniosynostosis (in most cases), to craniosynostosis involving all sutures except the lambdoidal in one sporadic case. Other features present in some patients included broad thumbs and halluces, broad first and second toes, facial asymmetry, flat forehead and prominent ear crura. Some of the patients in which they found the mutation were originally thought to have Saethre Chotzen syndrome (SCS). Golla *et al.* (1997) found the Pro250Arg mutation in a family which also had features suggestive of SCS. The affected pedigree members had uni or bilateral coronal craniosynostosis and low frontal hair line and other features present in some family members included hypertelorism, facial asymmetry, strabismus, ptosis, partial cutaneous syndactyly and brachydactyly. One member had "clover leaf skull" deformity.

Moloney *et al.* (1997) also noted the variable expressivity of the mutation. In the patients they found to be positive for the mutation, skull symptoms ranged from normal, to uni or bilateral coronal craniosynostosis. Macrocephaly and brachydactyly were also observed in some patients. The range of phenotypes that Bellus *et al.* (1996) described (when originally reporting the mutation) was, similar: uni or bilateral coronal craniosynostosis and macrocephaly. Bellus *et al.* (1996) reported most hands and feet were clinically normal, but radiologically they observed some abnormalities. The range of phenotypes has also been documented by Muenke *et al.* (1997) (Table 1 of Appendix 5.1, which includes the Craniosynostosis Adelaide Type family).

The range of phenotypes, of the patients who tested positive for the mutation, in this present study, is indicated by the range of original clinical diagnoses (Table 9.1).

From these studies (including Appendix 5.1 and Appendix 9.1) it is clear that there is a wide range of phenotypes that can result from the Pro250Arg FGFR3 mutation. There are some features which are frequently noted in these studies, such as uni or bilateral coronal craniosynostosis. The many different features are by no means present in all cases, and are not characteristic of this mutation. A number of cases found to have Pro250Arg were originally diagnosed as having SCS (for instance Reardon *et al.*, 1997; Golla *et al.*, 1997; this study), and Reardon *et al.* (1997) noted the significant phenotypic overlap between SCS and Pro250Arg craniosynostosis.

Because of the wide variation in phenotype, it is not possible to determine from clinical examination alone who will have the Pro250Arg mutation. However, there are a number of more common features that suggest that the presence of this mutation should be considered (as part of the differential diagnosis), such as coronal craniosynostosis. The overall clinical picture associated with this mutation may be different from that emerging from "craniosynostosis-orientated" studies, since the studies so far have been biased towards the severe end of the phenotypic spectrum, as Moloney *et al.* (1997) noted with regards to their study.

This notion is certainly supported by the finding of the Pro250Arg mutation in this study in a family predominantly affected by deafness (Appendix 9.1). The initial three samples

to arrive from this family were tested for Pro250Arg (C749G) of *FGFR3* (before *FGFR2* testing), since they were said to have craniosynostosis and sensorineural deafness which was a feature of the Craniosynostosis Adelaide Type family (Chapter 3) and since Muenke *et al.* (1997) (Appendix 5.1) reported that approximately one third of people with the Pro250Arg *FGFR3* mutation have mild to moderate sensorineural hearing loss. A further 15 samples arrived from this family and were tested. Overall four family members had craniosynostosis as determined by symptoms, that is, required surgery (8201 and 7727) or by review of clinical photographs. One of these was deaf. A further thirteen had no evidence of craniosynostosis but were either symptomatically deaf (bilaterally) or required bilateral hearing aids; in two individuals (8293 and 7726) audiometry revealed a uniform 20-30 decibel bilateral sensorineural hearing loss. The low penetrance for symptomatic craniosynostosis in this family raises the possibility that some families with the Pro250Arg mutation could present with deafness alone.

Sites of *Fgfr3* expression in the mouse include the developing nervous system, cartilage rudiments of developing bone, the lens of the eye, and the developing cochlea of the inner ear. In particular, high expression has been found in the differentiating hair cells and the underlying support cells of the developing cochlear duct (Peters *et al.*, 1993). Mice homozygous for a targeted disruption of *Fgfr3* have skeletal abnormalities and inner ear defects (Colvin *et al.*, 1996). The ears of these mice have no recognisable inner and outer pillar cells and no tunnel within the organ of Corti. This strongly supports the idea that an *FGFR3* mutation could cause deafness.

In addition there have been thirteen localisations for autosomal dominant non-syndromal deafness (Van Camp *et al.*, 1997). One locus, DFNA6, has been mapped to 4p16.3 (Lesperance *et al.*, 1995), which led to speculation that the Pro250Arg mutation of *FGFR3* might be the defect responsible for DFNA6 (Appendix 9.1).

The widely variable phenotype associated with the Pro250Arg mutation raises some important points relating to genetic counselling. The parents of an apparently sporadic case (with the mutation) should be tested for the mutation because they may appear clinically normal (possibly they have mild radiological abnormalities) but still have the mutation, thus increasing the recurrence risk of another affected child. The wide

variation in phenotype represents a complication for prenatal diagnosis: it would be very difficult to predict how severe the phenotype may be in a fetus which tests positive to this mutation.

It is important to now determine what factors influence the phenotype to cause such variability. One possibility is polymorphic variation (as discussed in Chapter 7). There may be polymorphic loci, which on their own don't have phenotypic effects, but in combination with the Pro250Arg mutation act as modifiers to the phenotype. Such polymorphisms may occur in the *FGFR*3 gene or in any of the genes coding for proteins which interact with the FGFR3 protein. Candidate polymorphisms within the *FGFR3* gene include the T1150C (Phe384Leu) change which was found in some affected and unaffected members (and unrelated controls) of a Pro250Arg kindred (Golla *et al.*, 1997) and also the *FGFR*3 and *FGF*8 polymorphisms found in this study (Chapters 5 and 9).

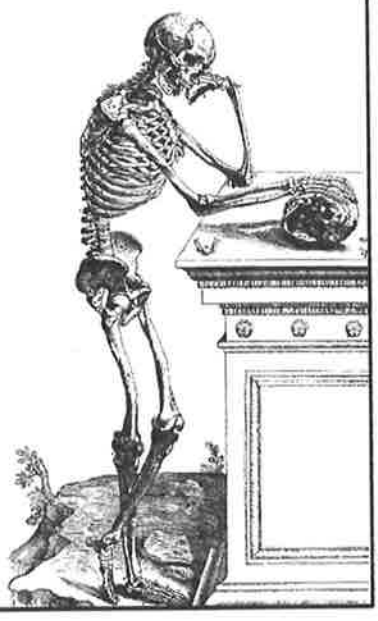
Based on the results of their study and a frequency of coronal craniosynostosis of 0.8-1.0 in 10,000 with 61% sporadic cases, Moloney *et al.* (1997) calculated a mutation rate at nucleotide 749 of about $8x10^{-6}$ per haploid genome. According to Moloney *et al.* (1997) this would represent the highest nucleotide transversion rate currently known in the human genome. The frequency is comparable to that of G1138A of *FGFR*3 (ACH) and C767G of *FGFR*2 (Apert). Moloney *et al.* (1996) reported the germ line mutation rates of the Apert mutations C767G and C770G as $5x10^{-6}$ and $2.7x10^{-6}$ respectively. Based on an ACH prevalence of between 1/15,000 and 1/77,000, Bellus et al. (1995) estimated the mutation rate at the 1138 nucleotide of *FGFR3* to be between $5.5x10^{-6}$ and $2.8x10^{-5}$ per gamete per generation.

Koeberl *et al.* (1990) investigated regions of the factor IX gene from 60 unrelated haemophiliacs and made some mutation rate estimations based on their results. They calculated the mutation rate per base per generation for transitions was $27x10^{-10}$, and $4.1x10^{-10}$ for transversions and $0.9x10^{-10}$ for deletions. Their total mutation rate estimate was $3.2x10^{-9}$. They found that the proportion of transitions at non CpG nucleotides was seven times more than if all base substitutions were of equal likelihood, and at CpG dinucleotides they were 24 times more than if all substitutions were of equal likelihood.

The mutation rates at positions 749 and 1138 of *FGFR3* and 767 (934) and 770 (937) of *FGFR2* are significantly higher than those reported for the factor IX gene. Both Apert mutations are C to G transversions, but the C767G mutation occurs in a CpG dinucleotide, whereas the C770G mutation does not. The nucleotide most commmonly mutated in ACH, 1138 of *FGFR3*, is also a CpG dinucleotide. Thus the greater frequency of the C767G mutation may be due to the CpG dinucleotide. Cooper and Youssoufian (1988) examined reports of single base pair mutations and found that 35% of those they looked at occurred within CpG dinucleotides. Their findings were consistent with methylation inducing deamination of 5-methyl cytosine and they suggest that methylation of DNA within coding regions may make a significant contribution to the incidence of genetic disease in humans.

Some people with the Pro250Arg mutation of FGFR3 have minimal abnormalities, and in some cases only minor radiological abnormalities (Ades *et al.*,1994). Thus, there may be many people with the mutation who will not be tested for craniosynostosis associated mutations. Also, there may be families with the Pro250Arg mutation who present with deafness alone. There are many causes of deafness. Deafness may be congenital or develop postnatally. Estimates of the prevalence of congenital deafness vary, 0.45-1 per 1,000 (Baraitser and Winter, 1984). Approximately half the cases of congenital deafness are genetic in cause, the others are caused by environmental or unknown factors. Non-congenital deafness may also have a genetic cause. Isolated hearing loss (non-syndromic) may be inherited as an autosomal dominant, autosomal recessive or X-linked recessive trait. Deafness can also be syndromal, and occurs with a variety of abnormalities (including eye, musculo-skeletal, renal and nervous abnormalities) (Garver, 1987). There may be people with minor radiological abnormalities, or deafness, due to the Pro250Arg mutation of FGFR3 who would not be tested for this mutation (which is currently considered as a craniosynostosis causing mutation). Thus it is possible that the frequency of the Pro250Arg FGFR3 mutation is even higher than current estimates, due to under ascertainment. It is intriguing that there are a group of *FGFR* mutations that appear to occur at much higher frequencies than other mutations previously described.

CHAPTER 10: CONCLUSION



Rapid advances have been made since 1994 in the understanding of the genetic basis of craniosynostosis disorders. Prior to 1994 only three craniosynostosis syndromes had been assigned chromosomal locations; Greig cephalopolysyndactyly to chromosome 7p, Saethre Chotzen syndrome to chromosome 7p21 and Craniosynostosis Boston type to chromosome 5q. For two of these three syndromes the causal gene had been identified: *GLI3* was implicated in Greig cephalopolysyndactyly and a mutation of *MSX2* was shown to cause Craniosynostosis Boston type.

Since 1994 the causal genes have been identified for many other craniosynostosis syndromes. The rapid progress that has recently occurred in the field of craniosynostosis genetics demonstrates the success of the positional candidate approach to identifying genes, which when mutated, give rise to certain syndromes. Mutations of *FGFR2* have been shown to cause Apert, Crouzon, Jackson Weiss, Pfeiffer and Beare-Stevenson cutis gyrata syndromes. Mutations of *FGFR1* were also shown to cause Pfeiffer syndrome, and mutations of *FGFR3* were shown to cause Craniosynostosis Adelaide Type (also known as FGFR3 craniosynostosis) and Crouzon syndrome with acanthosis nigricans. In each case the causal genes were localised by linkage mapping in affected families, and mutation screening in positional candidate genes led to the identification of the genes involved. This approach also led to the identification of the *TWIST* gene as causal in Saethre Chotzen syndrome.

The mapping of Craniosynostosis Adelaide Type to chromosome 4p16 (Chapter 3) delineated a new syndrome based on gene localisation. Examination of candidate genes from the region then implicated *FGFR3* as having a key role in the normal development of the skull. An international effort led to the identification of an *FGFR3* point mutation (C749G, Pro250Arg) as causal in a subset of craniosynostosis patients, including the Craniosynostosis Adelaide Type pedigree (Chapter 5). Members of the *FGFR* gene family have been shown to be crucial to the normal development of the skull and face. The *TWIST* gene product is also thought to act in the same pathway and the FGFRs may be downstream targets of TWIST. The importance of the *FGFR3* gene to development is further demonstrated by the fact that different mutations of *FGFR3* can cause a range of dwarfism disorders including achondroplasia, thanatophoric dysplasia and hypochondroplasia, which are disorders of the long bones.

During this study the Pro250Arg mutation of FGFR3 proved to be a relatively common mutation among craniosynostosis syndrome patients (Chapter 9). It has been found in a wide range of phenotypes and represents a highly mutable site within FGFR3. The ACH and Apert mutations of *FGFR3* and *FGFR2* respectively, also appear to occur at high frequencies (greater than or equal to $5x10^{-6}$). The finding of some patients with the Pro250Arg FGFR3 mutation, with deafness but no evidence of craniosynostosis has led to the hypothesis that some people with this mutation may present with deafness alone, that is with no obvious signs of craniosynostosis (Chapter 9). If the Pro250Arg FGFR3 mutation is found to cause a proportion of autosomal dominant deafness cases, then the mutation frequency at codon 250 of FGFR3, could be even higher than current estimates (greater than or equal to $8x10^{-6}$). In FGFR1 and FGFR2, mutations have been found at the codons corresponding to codon 250 of FGFR3. This may indicate that they all cause the craniosynostosis phenotype by a similar mechanism (since the same codon is affected in each case).

The degree of allelic heterogeneity is different for the different FGFRs. At this stage the vast majority of FGFR3 craniosynostosis is caused by a single mutation - Pro250Arg. The only other FGFR3 craniosynostosis mutations known are those which cause Crouzon syndrome with acanthosis nigricans. There has only been one craniosynostosis causing mutation found in FGFR1 so far. This is in contrast to FGFR2 where more than 30 different mutations have been characterised for a range of clinically recognised craniosynostosis syndromes.

The FGFR2 mutation detection work in this study contributed to the accumulation of known FGFR2 craniosynostosis mutations (Chapter 7). Three novel mutations were identified, G(-1)C of exon B in a Pfeiffer patient, T875A of exon U in a Crouzon/Jackson Weiss syndrome patient, and T797C of exon U in two related Crouzon patients. In total, FGFR2 mutations were identified in 12 unrelated Apert, 8 unrelated Crouzon and 3 unrelated Pfeiffer syndrome patients, and in 6 unrelated patients of mixed diagnosis. Knowledge of all known mutations should highlight which regions of the gene are more frequently mutated than others. This should eventually lead to a better understanding of the genotype-phenotype relationships in the FGFR craniosynostoses,

and potentially to a better understanding of the mechanisms that lead from FGFR mutations to craniosynostosis.

This and other studies have demonstrated there is not a one-to-one relationship between each mutation and a certain phenotype in the craniosynostosis disorders. There have been examples of a mutation giving differing phenotypes in different individuals, and also of different mutations leading to the same phenotype. The phenotypic variation arising from a single mutation can be sufficiently great as to lead to different clinical diagnoses. This has occurred between and even within families (Chapter 7). The finding of significant phenotypic variation between family members carrying the same mutation is not new, it was noted when the original Jackson Weiss syndrome family was described. The level of phenotypic variation found, and the lack of correspondence between the clinical and molecular genetic findings may lead to a new classification system for these disorders.

During the course of this study three polymorphisms were identified in the *MSX1* gene (Chapter 4), also two in the *FGFR3* gene (Chapter 5) and three in the *FGF8* gene (Chapter 8). In isolation these variants may be silent, since they were found in random blood donors and CEPH family members. However in combination with a mutation they may act to modify the phenotype. It is possible that these polymorphic variants may contribute to the phenotypic variability observed between those patients carrying the same mutation.

The advances made so far have enabled prenatal diagnosis to be offered to families which carry an identified mutation. To move towards some possible intervention in the pathogenesis of craniosynostosis requires a better understanding of the role of FGFR proteins in development and the effect that *FGFR* mutations have on this. As a step toward this, an attempt was made to distinguish wildtype and Pro250Arg FGFR3 proteins using high sensitivity immunofluoresence flow cytometry. The craniosynostosis mutations of the *FGFR* genes are postulated to cause ligand independent activation (constitutive activation) (De Moerlooze and Dickson, 1997). De Moerlooze and Dickson (1997) consider preventive treatment may be possible to help reduce the

phenotypic impact of FGFR mutations by administering some form of specific inhibitors of FGFR signalling to counter inappropriate activity.

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APPENDICES

Appendix 3.1: Hollway, G.E., Phillips, H.A., Ades, L.C., Haan, E.A. and Mulley, J.C. (1995) Localisation of craniosynostosis Adelaide type to 4p16. *Human Molecular Genetics* **4**, 681-684.

G.E. Hollway, H.A. Phillips, L.C. Adès, E.A. Haan and J.C. Mulley (1995)
Localization of craniosynostosis Adelaide type to 4p16.
Human Molecular Genetics, v. 4 (4), pp. 681-683, April 1995

Appendix 3.2: Ades, L.C., Senga, I.P., Morris, L.L., David, D.J. and Haan, E.A. (1994) Jackson Weiss Syndrome: Clinical and radiological findings in a large kindred and exclusion of the gene from 7p21 and 5qter. *American Journal of Medical Genetics* **51**, 121-130.

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Jackson-Weiss syndrome: Clinical and radiological findings in a large kindred and exclusion of the gene from 7p21 and 5qter.
American Journal of Medical Genetics, v. 51 (2), pp. 121–130, June 1994

Appendix 3.3: Lod Scores for Linkage Mapping in large South Australian Craniosynostosis Pedigree. Markers and distances are primarily taken from Weissenbach *et al.* (1992) and Gyapay *et al.* (1994) and the CEPH/NIH collaborative map.

CHROMOSOME 1

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S160	-infini	-6.42	-3.06	-1.73	-0.60	-0.14	0.02
14cM							
D1S170*	1.20	1.18	1.09	0.98	0.72	0.44	0.15
11cM							
D1S199	-infini	-3.61	-1.61	-0.82	-0.17	0.07	0.11
10cM							
D1S234	-infini	-12.52	-6.31	-3.76	-1.49	-0.46	-0.03
~10cM							
CRTM*	-infini	-12.05	-6.91	-4.61	-2.35	-1.15	-0.42
4cM							
D1S164	-infini	-5.60	-2.75	-1.55	-0.50	-0.07	0.07
21cM							
MYCL1*	-infini	-7.35	-3.92	-2.51	-1.20	-0.55	-0.18
25cM							
D1S159	-infini	-11.42	-5.91	-3.66	-1.65	-0.71	-0.21
18cM							
D1S167*	-infini	-3.55	-1.43	-0.57	0.10	0.27	0.20
36cM							
D1S187	-infini	-8.97	-3.68	-1.66	-0.10	0.35	0.31
4cM							
D1S185	-infini	-2.07	-1.29	-0.91	-0.50	-0.27	-0.11
23cM							
APOA2*	-infini	-14.68	-7.75	-4.85	-2.19	-0.89	-0.23
~18cM							
D1S218	-infini	-10.23	-4.78	-2.60	-0.78	-0.1	0.06
~23cM							
F13B	-infini	-6.48	-3.14	-1.84	-0.74	-0.28	-0.07
~21cM							
Sg733	-infini	-7.82	-3.79	-2.20	-0.85	-0.30	-0.09
~36cM							
ACTN2*	-infini	-7.26	-3.12	-1.47	-0.18	0.18	0.15
19cM							
D1S102	-infini	-11.63	-6.52	-4.26	-2.07	-0.96	-0.33

* indicates the marker was genotyped by Dr L. Ades

CHROMOSOME 2

	0.0	0.01	0.05	0.1	0.2	0.3	0.4	
6cM								
D2S207	-infini		-12.61	-6.40	-3.84	-1.54	-0.50	-0.04
17cM								
D2S168	-infini		-16.21	-8.59	-5.42	-2.49	-1.05	-0.30
21cM								
D2S144	-infini		-9.23	-4.51	-2.64	-1.04	-0.36	-0.07
16cM								
D2S177	-infini		-1.09	-0.39	-0.10	0.13	0.18	0.13
13cM								
D2S123	-infini		-15.18	-8.15	-5.19	-2.45	-1.12	-0.41
21cM								
D2S145	-infini		-14.32	-7.51	-4.74	-2.24	-1.04	-0.40
23cM								
D2S113	-infini		-15.73	-8.23	-5.18	-2.41	-1.06	-0.34
14cM								
D2S121	-infini		-6.58	-3.73	-2.50	-1.29	-0.64	-0.24
18cM								
D2S114	-infini		-2.47	-1.01	-0.39	0.11	0.25	0.20
21cM								
D2S142	-infini		-8.95	-4.20	-2.31	-0.76	-0.20	-0.03
9cM								
D2S124	-infini		-7.11	-3.08	-1.51	-0.27	0.13	0.13
18cM								
D2S152	-infini		-9.40	-4.57	-2.59	-0.87	-0.14	0.10
16cM								
D2S155	-infini		-12.63	-6.34	-3.72	-1.37	-0.34	0.05
18cM								
D2S126	-infini		-16.87	-9.13	-5.84	-2.75	-1.20	-0.36
9cM								
D2S159	-infini		-12.67	-6.97	-4.51	-2.17	-0.97	-0.31
12cM								
D2S206	-infini		-13.21	-7.05	-4.50	-2.15	-0.97	-0.32

CHROMOSOME 3

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D3S1307	-infini	-17.05	-9.84	-6.67	-3.55	-1.85	-0.76
15cM							
D3S1304	-infini	-12.23	-6.10	-3.64	-1.54	-0.67	-0.29
25cM							
D3S1286	-infini	-5.80	-2.98	-1.80	-0.72	-0.23	-0.02
23cM							
D3S1260	-infini	-10.12	-5.12	-3.01	-1.12	-0.34	-0.06
9cM							
D3S1289	-infini	-8.31	-4.15	-2.43	-0.91	-0.23	0.03
colpvu	-infini	-7.11	-3.68	-2.28	-1.01	-0.42	-0.13
colalu	-infini	-8.34	-4.18	-2.48	-1.0	-0.35	-0.06
D3S1285	-0.39	-0.35	-0.24	-0.14	-0.02	0.04	0.04
D3S2	-infini	-13.41	-7.28	-4.78	-2.47	-1.26	-0.51
D3S1274	-infini	-3.02	-1.07	-0.36	0.14	0.22	0.12
22cM							
D3S1278	-infini	-9.19	-4.32	-2.32	-0.58	0.1	0.25
19cM							
D3S1292	-infini	-12.44	-6.24	-3.70	-1.44	-0.43	-0.01
22cM							
D3S1279	-infini	-6.71	-3.33	-1.96	-0.76	-0.23	-0.01
17cM							
D3S1282	-infini	-8.09	-4.54	-3.01	-1.53	-0.74	-0.27
18cM							
D3S1262	-infini	-8.38	-4.15	-2.39	-0.85	-0.21	0.01
23cM							
D3S1311	-infini	-2.37	-0.93	-0.33	0.12	0.20	0.12

D3S1289-D3S1285=22cM, D3S1285-D3S1274=19cM

CHROMOSOME 6

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
F13A1 ~13cM	-infini		-6.39	-3.54	-2.31	-1.13	-0.52	-0.18
D6S309 17cM	-infini		-15.29	-8.25	-5.27	-2.51	-1.17	-0.44
D6S289 21cM	-infini		-16.61	-9.05	-5.90	-2.95	-1.42	-0.51
D6S291 17cM	-infini		-11.23	-5.81	-3.64	-1.71	-0.82	-0.32
D6S282 14cM	-infini		-7.94	-4.74	-3.20	-1.61	-0.75	-0.25
D6S257 22cM	-infini		-9.87	-4.54	-2.47	-0.80	-0.19	-0.02
D6S275 13cM	-infini		-11.59	-5.90	-3.48	-1.33	-0.39	-0.02
D6S268 25cM	-infini		-5.65	-2.72	-1.46	-0.41	-0.07	-0.05
D6S292 18cM	-infini		-7.86	-3.22	-1.44	-0.04	0.39	0.37
D6S290 25cM	-infini		-1.11	-0.44	-0.19	0.01	0.07	0.06
D6S264 22cM	-infini		-5.83	-3.98	-2.95	-1.74	-0.98	-0.42
D6S281	-infini		-5.30	-2.02	-0.79	0.11	0.32	0.22
D6S202	-infini		-18.84	-10.32	-6.68	-3.26	-1.52	-0.53
DMDL	-infini		-4.69	-1.87	-0.74	0.14	0.34	0.20

CHROMOSOME 8

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
D8S200	-infini		-8.05	-4.50	-2.97	-1.50	-0.72	-0.26
D8S264 20cM	-infini		-16.36	-8.83	-5.72	-2.81	-1.30	-0.44
D8S265 20cM	-infini		-8.39	-4.82	-3.26	-1.73	-0.89	-0.35
D8S282 19cM	-infini		-2.88	-0.87	-0.13	0.34	0.33	0.11
D8S283 21cM	-infini		-16.47	-8.84	-5.66	-2.70	-1.23	-0.42
D8S260 14cM	-infini		-14.62	-7.79	-4.99	-2.42	-1.15	-0.43
D8S286 15cM	-infini		-11.27	-6.26	-4.08	-2.00	-0.92	-0.30
D8S257 12cM	-infini		-4.16	-1.48	-0.46	0.28	0.41	0.24
D8S281 26cM	-infini		-3.59	-2.10	-1.42	-0.74	-0.37	-0.14
D8S256 6cM	-infini		-9.20	-4.43	-2.52	-0.92	-0.29	-0.06
D8S274	-infini		-6.73	-2.17	-0.50	0.66	0.83	0.52

CHROMOSOME 9

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D9S178	-infini	-13.62	-7.79	-5.19	-2.63	-1.27	-0.45
23cM							
D9S157	-infini	-16.58	-9.29	-6.06	-2.94	-1.32	-0.42
17cM							
D9S169	-infini	-12.30	-6.71	-4.34	-2.11	-0.97	-0.33
21cM							
D9S175	-infini	-6.31	-2.87	-1.48	-0.30	0.13	0.20
17cM							
D9S152	-infini	-3.83	-1.23	-0.29	0.35	0.43	0.24
21cM							
D9S176	-infini	-3.51	-1.45	-0.63	0.01	0.19	0.15
20cM							
D9S154	-infini	-19.16	-10.69	-7.05	-3.56	-1.71	-0.62
22cM							
D9S179	had been ordered						
17cM							
D9S158	had been ordered						
D9S15	-infini	-5.51	-2.09	-0.76	0.22	0.42	0.28
D9S66	-0.35	-0.35	-0.35	-0.34	-0.29	-0.21	-0.11
D9S125	-infini	-2.14	-1.43	-1.11	-0.73	-0.45	-0.21

CHROMOSOME 10

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D10S249 18cM	-infini	-8.21	-4.69	-3.17	-1.68	-0.86	-0.34
D10S189 21cM	-infini	-16.51	-8.89	-5.71	-2.75	-1.27	-0.43
D10S191 16cM	-infini	-15.37	-8.24	-5.18	-2.32	-0.93	-0.24
D10S197 18cM	-infini	-6.75	-3.14	-1.61	-0.31	0.12	0.12
D10S220 16cM	-infini	-10.85	-5.28	-2.99	-1.01	-0.22	0.01
D10S210 15cM	-infini	-8.41	-4.84	-3.29	-1.75	-0.90	-0.36
D10S607 17cM	-infini	-9.71	-5.79	-3.92	-2.01	-0.97	-0.34
D10S185 22cM	-infini	-16.92	-9.67	-6.49	-3.39	-1.74	-0.71
D10S187 2cM	-infini	-10.79	-5.88	-3.80	-1.88	-0.93	-0.37
D10S190 3cM	-infini	-7.24	-3.28	-1.77	-0.60	-0.20	-0.08
D10S209 7cM	-infini	-12.11	-7.04	-4.79	-2.56	-1.31	-0.52
D10S587 1cM	-infini	-4.80	-2.72	-1.84	-0.99	-0.52	-0.21
D10S216 5cM	-0.07	-0.07	-0.05	-0.02	0.00	0.01	0.01
D10S575 0cM	-infini	-6.76	-3.80	-2.48	-1.19	-0.52	-0.16
D10S214 2cM	-infini	-2.04	-1.27	-0.90	-0.50	-0.26	-0.11
D10S186 2cM	-infini	-3.03	-0.97	-0.19	0.31	0.31	0.11
D10S217 13cM	-infini	-5.58	-2.73	-1.54	-0.53	-0.12	0.02
D10S212	-infini	-6.63	-3.21	-1.84	-0.70	-0.25	-0.07

CHROMOSOME 11

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
2cM							
D11S922	-infini	-16.62	-8.89	-5.62	-2.58	-1.08	-0.30
25cM							
D11S899	-infini	-11.43	-6.36	-4.14	-1.99	-0.89	-0.28
18cM							
D11S907	-infini	-10.88	-5.39	-3.14	-1.16	-0.28	0.06
13cM							
D11S903	-infini	-6.71	-3.33	-1.96	-0.76	-0.23	-0.01
11cM							
D11S913	-infini	-6.09	-2.62	-1.22	-0.12	0.20	0.16
11cM							
D11S916	-infini	0.45	1.60	1.87	1.76	1.30	0.64
6cM							
D11S937	-infini	-8.30	-3.48	-1.56	-0.03	0.42	0.33
14cM							
D11S931	-infini	-6.85	-3.31	-1.82	-0.54	-0.05	0.08
35cM							
D11S933	-infini	-6.32	-3.90	-2.72	-1.46	-0.74	-0.29
16cM							
D11S910	-infini	-9.78	-4.87	-2.83	-1.03	-0.27	0.01
9cM							
D11S968	-infini	-7.27	-3.69	-2.16	-0.77	-0.16	0.06

CHROMOSOME 12

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D12S94	-infini	-5.87	-2.55	-1.28	-0.27	0.06	0.11
22cM							
D12S98	-infini	-12.74	-6.55	-4.02	-1.75	-0.68	-0.15
28cM							
D12S87	-infini	-13.01	-6.28	-3.61	-1.34	-0.39	-0.02
12cM							
D12S96	-infini	-4.85	-2.19	-1.16	-0.34	-0.06	0.01
13cM							
D12S80	-infini	-10.84	-5.33	-3.07	-1.09	-0.24	0.07
18cM							
D12S101	-infini	-10.16	-4.79	-2.68	-0.92	-0.21	0.03
18cM							
D12S105	-infini	-11.98	-5.92	-3.50	-1.43	-0.53	-0.14
17cM							
D12S76	-infini	-7.11	-3.16	-1.66	-0.48	-0.07	0.03
32cM							
D12S97	-infini	-6.38	-3.02	-1.69	-0.56	-0.09	0.06

CHROMOSOME 13

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
D13S175 21cM	-infini		-9.33	-4.54	-2.58	-0.86	-0.13	0.12
D13S171 22cM	-infini		-7.28	-3.71	-2.20	-0.86	-0.28	-0.05
D13S155 23cM	-infini		-11.83	-5.79	-3.38	-1.32	-0.43	-0.05
D13S170 16cM	-infini		-10.15	-5.13	-3.00	-1.08	-0.25	0.06
D13S159 18cM	-infini		-9.89	-4.90	-2.79	-0.93	-0.19	0.02
D13S173 22cM	-infini		-15.54	-8.41	-5.35	-2.49	-1.09	-0.35

D13S293-had been ordered

CHROMOSOME 16

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S291	-infini		-1.00	-0.26	0.04	0.27	0.29	0.19
D16S292	-infini		-6.61	-3.17	-1.78	-0.60	-0.15	-0.01
D16S287	-infini		-10.55	-5.99	-3.95	-1.96	-0.92	-0.32
D16S298	-infini		-4.72	-2.02	-0.98	-0.24	-0.05	-0.01
D16S300	-infini		-4.14	-2.06	-1.21	-0.47	-0.16	-0.03
D16S265	-infini		-16.36	-9.25	-6.16	-3.17	-1.56	-0.58
D16S266	-infini		-4.70	-1.98	-0.92	-0.12	0.09	0.06
D16S304	-infini		-16.58	-8.87	-5.63	-2.64	-1.19	-0.43
D16S303	-infini		-6.72	-3.35	-2.01	-0.86	-0.35	-0.12

CHROMOSOME 17

		0.0	0.01	0.05	0~1	0.2	0.3	0.4
D17S849	-infini		-15.21	-7.77	-4.77	-2.13	-0.94	-0.35
20cM								
D17S804	-infini		-9.96	-5.20	-3.27	-1.51	-0.66	-0.21
27cM								
D17S793	-infini		-9.93	-5.08	-3.07	-1.25	-0.42	-0.05
21cM								
D17S806	-infini		-8.15	-3.51	-1.72	-0.29	0.19	0.25
D17S520	-infini		-20.57	-11.41	-7.49	-3.75	-1.79	-0.64
NF1alu	-infini		-4.02	-1.90	-1.04	-0.32	-0.06	0.01
D17S794	-infini		-9.94	-4.52	-2.38	-0.62	0.04	0.18
18cM								
D17S801	-infini		-15.91	-8.31	-5.16	-2.28	-0.91	-0.22
14cM								
D17S784	-infini		-11.17	-5.70	-3.48	-1.54	-0.66	-0.21

D17S806-D17S794=21cM

CHROMOSOME 18

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
D18S59	-infini		-21.24	-11.61	-7.60	-3.83	-1.86	-0.68
18cM								
D18S62	-infini		-9.35	-4.63	-2.75	-1.13	-0.43	-0.11
23cM								
D18S53	-infini		-20.35	-10.79	-6.88	-3.30	-1.54	-0.56
21cM								
D18S57	-infini		-12.14	-6.57	-4.22	-2.03	-0.93	-0.31
21cM								
D18S64	-infini		-3.81	-2.31	-1.63	-0.91	-0.49	-0.21
22cM								
D18S61	-infini		-22.20	-13.46	-9.52	-5.45	-3.03	-1.32
23cM								
D18S70	-infini		-18.83	-10.39	-6.80	-3.39	-1.63	-0.59

CHROMOSOME 20

		0.0	0.01	0.05	0.1	0.2	0.3	0.4
D20S103	-infini		-12.68	-7.02	-4.61	-2.32	-1.12	-0.40
2cM								
D20S117	-infini		-14.73	-8.23	-5.38	-2.62	-1.20	-0.39
14cM								
D20S95	-infini		-16.73	-9.11	-5.93	-2.94	-1.39	-0.49
19-25cM								
D20S66	-infini		-21.16	-11.97	-8.00	-4.16	-2.08	-0.79
3-9cM								
D20S104	-infini		-6.79	-4.26	-2.97	-1.59	-0.79	-0.30
23cM								
D20S107	-infini		-12.52	-7.34	-5.00	-2.64	-1.34	-0.52
21cM								
D20S109	-infini		-20.27	-11.13	-7.24	-3.54	-1.64	-0.56
12cM								
D20S102	-infini		-1.73	-1.04	-0.74	-0.42	-0.22	-0.08

CHROMOSOME 21

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D21S11 6.5cM	-infini	-13.25	-6.86	-4.15	-1.70	-0.62	-0.16
D21S214 29.9cM	-infini	-14.14	-7.69	-4.91	-2.31	-1.03	-0.34
D21S167 12.6cM	-infini	-11.41	-5.89	-3.61	-1.56	-0.60	-0.14
D21S212	-infini	-3.44	-0.80	0.16	0.76	0.77	0.49

CHROMOSOME22

	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D22S25	-infini	-16.51	-8.87	-5.67	-2.69	-1.20	-0.38
F8VWFP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D22S303	-infini	-5.39	-2.60	-1.45	-0.45	-0.04	0.08
D22S315 7cM	-infini	-6.46	-2.95	-1.53	-0.36	0.05	0.11
D22S275 8cM	-infini	-14.56	-7.63	-4.76	-2.12	-0.86	-0.22
D22S278 10cM	-infini	-6.01	-3.08	-1.81	-0.65	-0.14	0.05
D22S279 6cM	-infini	-13.26	-6.50	-3.80	-1.46	-0.45	-0.03
D22S274	-infini	-4.58	-2.45	-1.54	-0.68	-0.27	-0.06

Appendix 4.1: Hollway, G.E. and Mulley, J.C. Polymorphic variants within the homeobox gene *MSX1*. *Clinical Genetics* - accepted for publication.

Short Report on DNA Markers at Candidate Locus

Polymorphic Variants within the Homeobox Gene *MSX1*

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Women's and Children's Hospital, Adelaide, Australia

Key words: *MSX1*, craniosynostosis, polymorphism

Running title : *MSX1* polymorphisms

G.E. Hollway and J.C. Mulley (1998) Polymorphic variants within the homeobox gene MSX1: a candidate gene for developmental disorders.
Clinical Genetics, v. 54 (2), pp. 152–154, August 1998

Appendix 5.1: Muenke, M., Gripp, K.W., McDonald-McGinn, D.M., Gaudenz, K., Whitaker, L.A., Bartlett, S.P., Markowitz, R.I., Robin, N.H., Nwokoro, N., Mulvihill, J.J., Losken, H.W., Mulliken, J.B., Guttmacher, A.E., Wilroy, R.S., Clarke, L.A., Hollway, G., Ades, L.C., Haan, E.A., Mulley, J.C., Cohen Jr, M.M., Bellus, G.A., Francomano, C.A., Moloney, D.M., Wall, S.A., Wilkie, A.O.M. and Zackai, E.H. (1997) A unique point mutation in the Fibroblast Growth Factor Receptor 3 gene (*FGFR3*) defines a new craniosynostosis syndrome. *American Journal of Human Genetics* **60**, 555-564.

A Unique Point Mutation in the Fibroblast Growth Factor Receptor 3 Gene (*FGFR3*) Defines a New Craniosynostosis Syndrome

M. Muenke, K. W. Gripp, D. M. McDonald-McGinn, K. Gaudenz, L. A. Whitaker, S. P. Bartlett, R. I. Markowitz, N. H. Robin, N. Nwokoro, J. J. Mulvihill, H. W. Losken, J. B. Mulliken, A. E. Guttmacher, R. S. Wilroy, L. A. Clarke, G. Hollway, L. C. Adès, E. A. Haan, J. C. Mulley, M. M. Cohen, Jr., G. A. Bellus, C. A. Francomano, D. M. Moloney, S. A. Wall, A. O. M. Wilkie, and E. H. Zackai

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Muenke M., Gripp K.W., McDonald-McGinn D.M., Gaudenz K., Whitaker L.A., Bartlett S.P., Markowitz R.I., Robin N.H., Nwokoro N., Mulvihill J.J., Losken H.W., Mulliken J.B., Guttmacher A.E., Wilroy R.S., Clarke L.A., Hollway G., Adès L.C., Haan E.A., Mulley J.C., Cohen M.M. Jr, Bellus G.A., Francomano C.A., Moloney D.M., Wall S.A., Wilkie A.O. and Zackai E.H. (1997) A unique point mutation in the fibroblast growth factor receptor 3 gene (FGFR3) defines a new craniosynostosis syndrome.
American Journal of Human Genetics, v. 60 (3), pp. 555-564, March 1997

Appendix 7.1: Hollway, G.E., Suthers, G.K., Haan, E.A., Thompson, E., David, D.J., Gecz, J. and Mulley, J.C. (1997) Mutation detection in *FGFR2* craniosynostosis syndromes. *Human Genetics* **99**, 251-255.

Hollway, G.E., Suthers, G.K., Haan, E.A., Thompson, E., David, D.J., Gecz, J. and Mulley, J.C. (1997) Mutation detection in FGFR2 craniosynostosis syndromes. *Human Genetics, v. 99 (2), pp. 251-255, February 1997*

Appendix 8.1

Genomic sequence of *FGF8*, including exons 1A, 1B, 1C, 1D, 2 and 3 (Gemel *et al.*, 1996). Accession Numbers: U47009, U47010 and U47011. Primer sequences are underlined, with their directions indicated by arrows. The exonic sequences are in blue italic type, the intronic and 5' untranslated sequences are not.

```
   1 gcggcgcggc gagcacgacg ttccacggga cccgcggagc cgcgtcgtga tcgccgccgg
                          FGF8-11 →
  61 cctcccgcac ccgcaccctc tccgctcgcg ccctgctcag cgcgtcctcc cgcggcggcc
 121 cgcgggacgg cgtgacccgc cgggctctcg gtgccccggg gccgcgcgcc atgggcagcc
 181 cccgctccgc gctgagctgc ctgtgagtac cgcgccgccc tgccccgcca cccgcccccc
 241 gcgcccctcg cctcacccgc gcctctctct cccgcccgct tttgtctccc acaggctgtt
                          FGF8-1BF →                      ← FGF8-12
 301 gcacttgctg gtcctctgcc tccaagccca ggtgaggagg ggtgcgcgga ggcgggggcc
 361 gggcgcgccg gtgtgagacc cgggtgggca gcgccggtcg ggggcaccgg gactgactct
 421 gcggccggcg cgggagggct gagggcacct tagaaaccca gccccgagcc accccgagga
 481 gggagctgag gcacagagag gtagcacccc tcctgaggtc acacagcgag tgagtggcca
 541 ggatagaaac gaaggtctcg gagcccagca ctgtccccca tgcatcctcg ccggctgggg
                ← FGF8-1BR
 601 gcatagggaa cacccagccg ccgaggaagg gggcagccgc ggccagggga tggatgttcg
 661 atgccagggg aagccgggtg actgcagcag agaccctctc agcgcccctc gggggaggct
 721 tgtggccgat ttggcccaat gatcgggtgc ccaggttccc tgcactctca acatttgctc
 781 cgtaaatttg tctttataaa tgtcaggggt cggggagggc tgcctcccta cttaaaagcg
                                      FGF8-1CF →
 841 ccctgctcct ctaggaaggc ccgggcaggg gccctgcgct gggcagggag ctcgcttccc
 901 tgttccgggc tggccgggag ccccagggtg tctcccaaca ggtgggtcca gcttctccct
 961 ggggctcgtt catcctgggc tgggtctgcc cgacttgcgt gggtgggggа tggtggcctg
          ← FGF8-1CR
1021 ggctggcatg ttgggggaac ccagcacctg ctgcggcttg gggcagtgag ggggacgcag
1081 ggtgatgggg ccactcgggc cctgggcgga gtagcattat aatggtgtat tgtgtatttt
                          FGF8-1DF →
1141 tcaatttcct aaaggtaact gttcagtcct cacctaattt tacacagcat gtgagggagc
1201 agagcctggt gacggatcag ctcagccgcc gcctcatccg gacctaccaa ctctacagcc
1261 gcaccagcgg gaagcacgtg caggtcctgg ccaacaagcg catcaacgcc atggcagagg
1321 acggcgaccc cttcggtaag gcgcgctgaa ggtagcttgt gggatgcgcg gcgcctggac
1381 caccagtccc aactgcactg gccgggggcc tgcagggtcc ccagctcaga agggaaggag
          ← FGF8-13
1441 ttaaggcaga aaggtgtctt gagggtcagc tggggaagag agggctaggg aaggggcctc
1501 ggggagtact tggccacagg tgccccttc cctgcccagc aaatgaatga atctaggaat
1561 ctcccttac acctcccccc ttcatacact cctccccagc tgcaggtgga ggcagggggt
1621 tcaggggggag ggggcctagg tttaaggcaa gcgcttgaaa gaggatatgt cgccagggcc
1681 cacaaaagga gagtgggaaa gagaagggag agaaatcccc tcaactctcc cagttggaaa
1741 aggaggttgc tgggaaccct taaatacttt aatcggatgg gtcaatttac acgtggaaac
1801 tgggcttgga agggggctct ccctgctcca ttcctgggga acccaggcgg ctggggcagg
1861 gctcccggct ccctgtgggg tggcaagtgc ggagagctac tgccggcaga gaggctgagg
```

1921 actgattgat ttctaaaaca ttcaatattc ctgccttgaa acggggccaa tttcagagtc
1981 ctggatgtac caagagaaaa cgcaagcctc tgggcggctg gtgttgtttt tagagagcga
2041 ttattgtgag tttaaaaacc tttaaataat gaccacgtta aacaagcatg aaaggagagg
2101 ggaagactct attaaaatgt aataaaaagg tgaggcattt taagagctaa gaagaaaaat
2161 gaagaatgat ataaaacaga aaaagaacga gctttttttc agatgtggag tttgaaagga
2221 gttgcaagct gtaaatgtta aaaaagaggt tcaagattgt tctctctgaa tctctggaga
2281 gacccctgcg tctggcagag cgattaaaac tcttcttact ttcacctttt ttaacatttc
2341 ctgaatactt tctcctcttc ttgagccagt ttgggggatc gagtatgtct cgctgcattt
2401 tttctgcatc gacgtagctg acttcccttc aaagctggag gagacataat tttcctggga
2461 taaatctgtc actggggtgg aagaataggt ttgaaaatgt taagctttcc aaaagcccgg
2521 tcgggagagc tgccgtaaac tgcggcccca gctccccaga tcggagcaga ttcaaccgac
2581 atgggagaga gcgggctttc tggggtgggg gtgggggcgg cctgcctggt ggccgagctc
2641 ccttactggg ctggcagagc aggcatacca tccctttgtc cctccaaatt agctgcttgc
2701 tgcccttctc agcttctaag gagaggagac gaatgcctgg ctcttccagg gtttgctctg
2761 tcagagcaaa tggcccagca tcccttggat ttcggggagg gaagcagaag tcaagctggt
2821 gtgggggaat tc

exon 2 FGF8, Accession U47010

1 ggatcctcgg agatcttttg attagctatg gaactctgat tcaattaaat tttacttaga
61 tcctcaatct aagagacatc actgtctggt ttaaaaagag ggtggaagga atggcacttc
121 agccttttca atttgatagt cccaagacct ctcttgagaa cccagtttga aaaccataat
181 cccgtccact gccctctgta aacccagggg tggggcccga ggtccagaaa agccctttct
241 ctgggttgca cagcatgtta gtagctgggc tggagctgga gtccatgttt tgggttcctg
301 gtctttggag cagttgctgc tgggctgtga caagtgtgtg ggccagctgg ggtcagggat
FGF8-14
361 ctgccaatag ccatcctgct accttctctg acag*caaagc tcatcgtgga gacggacacc*
421 tttggaagca gagttcgagt ccgaggagcc gagacgggcc tctacatctg catgaacaag
*481 aaggggaagc tgatcgccaa g*gtgaggccc cagcaggcag gtggggtccg cgggacctag
541 ggagtggctt gggctggctg gactcctcca agacagtcct gggctctgat cccaacaagg
FGF8-15
601 taggcagaag ccacagggcc atcttggggt tacctcacga ttgggtgcag gagtgaggga
661 gaaggaagct gtttgagaga tgaaaactat aaaaggcgaa ggaggaagaa tgaaggctag
721 ggaggggctg gggggtggtg gttggtaaat tccatatatc tttttaacaa atcgccaaat

exon 3, Accession: U47011.

1 ctcgagctcc ccacttcctg ggcttctggg gctggggtct tagcatcttc tcccaggcct
61 cccctccccc ataggtggct gccctggggc cagggaaccg aagtcctggg ggggtgagag
121 gggcaggtgg ggagacgggt ggccagactg gtgggcagga ggccagagca ggccaggctc
181 tgggcccctc tctctgtctt tctgcgttgg ggcccagccc tccgtagaca accatgtgtc
241 actgctgcct gggaaggaca ggaagttgcc gggtgggctg cgagttgtga gggattagag
FGF8-16
301 agcgggtgcc caggcagggg ggtggggctg cggctcctgc ccacctcgcc atctgctggg
361 gtgcccacct gctgtctggg gccgctcgcc ctctgcctct gctgggggg ctctgtaacg
421 tggtgtctgg ctcccctacc tg*cagagcaa cggcaaaggc aaggactgcg tcttcacgga*
FGF8-3F

481 *gattgtgctg gagaacaact acacagcgct gcagaatgcc aagtacgagg gctggtacat*

FGF8-17

541 *ggccttcacc cgcaagggcc ggccccgcaa gggctccaag acgcggcagc accagcgtga*

601 *ggtccacttc atgaagcggc tgccccgggg ccaccacacc accgagcaga gcctgcgctt*

661 *cgagttcctc aactacccgc ccttcacgcg cagcctgcgc ggcagccaga ggacttgggc*

721 *ccccgagccc cgatag*tgct gcctggccct ccccacaatg ccagaccgca gagaggctca

FGF8-3R

781 tcctgtaggg cacccaaaac tcaagcaaga tgagctgtgc gctgctctgc aggctgggga

841 ggtgctgggg gagccctggg ttccggttgt tgatattgtt tgctgttggg tttttgctgt

901 ttttttttt ttttttttt ttaaaacaaa agagaggctc tatttttgta ttccacttgg

961 ctgtggtgtc tgtcttctta actctcagaa agctccatta gtggcctaga ctgggattcc

1021 ggctgggggt ttgcgggggt ggggggcttt ctctagcctg tgctgctgag gccccagtac

1081 ctccagggcc agttggctgg gcagccaggg actccactgc acccccaggt ggggcaggga

1141 ggaaaggact gtgacatagg gcagtcctct tagaagtggg tatcagactg gtggctatta

1201 aatgattgaa atatttattt aacttgcata ttaaaaatgt gtgctggaga gtgagtcctg

1261 ccggggtcag cccctccctc caaccttgcc ccagctggtg ggcggctggg agacgcagat

1321 gaccaggtgc cagctctgac cacagcctcc ctccagccta aagacacctg cctgtcaacc

1381 atccccatca ctgtcacttg aggggttttc ctgcaaggac agaagcaggg aaaggggcaa

1441 gaagaggctc ttagctagtc cttggagctc tcagatgtgt acctcctagc actttacaga

1501 ggtcattgct aacacttccc caggccacct cagggccaga aataatggat gtgctagggc

1561 tagagctgta atcatggatt taatcctctt aaaaagtgct tctctgagtg cctaggtcca

1621 tgtgggagac aggttggaga ttccagaact tgctctttct gagactcagg ctccagaaaa

1681 tgaaagaaaa gagcagctgc cagggtccaa ggtgggggca tattggaggg ggaccaccaa

1741 gactggtgtt gacaatggtg atgtgggaca agtgttaacc ttgggtgata tggtgagata

1801 gctgtgggca gaaagcactg agctgaggtg cggcgaggag cctggggaac tgtcttccag

1861 gaagaggctg cccacctcgg aggatgggct ggcgggagag gagctgggca ccggatggca

1921 ccagaaggga agctcatagg cctagcgcag aactaaaggc agtcatagcc ttggggagaa

1981 gcaggaggcc gtatgtggag ggagggaggg ctgctgtggg agtggtggag caggtcatgg

2041 tgtgggcaga gaagggaatg ggcaagggtg caggtgtgtg tttgcgtgtg gactggtgag

2101 actggtgtcc tgccacaccg agggagagcc caggccccac ggcagtttcc tgagtgcaga

2161 gctggcccag gcttcatcgc tgaggcctcc cattagggct gctcctgctt ccttccttgt

2221 ggatgccctg ggctggtccc acagcccagc tactgagcca gtctaga

Appendix 9.1: Hollway, G.E., Suthers, G.K., Battesse, K.M., Turner, A.M., David, D.J. and Mulley, J.C. (1998) Deafness due to Pro250Arg mutation of *FGFR3*. *The Lancet* **351**, 877-878.

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