X-LINKED KALLMANN'S SYNDROME: A MOLECULAR GENETIC AND DEVELOPMENTAL ANALYSIS.

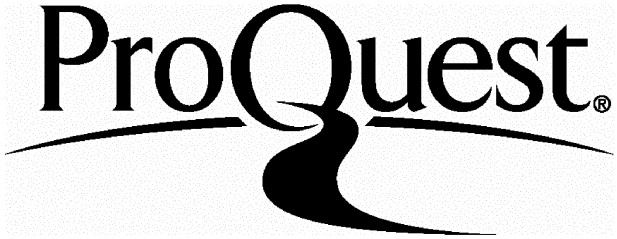
BY

VERONIQUE MICHAL DUKE B.Sc (Hons)

A thesis submitted to the University of London in fulfilment of the requirement for the degree of Doctor of Philosophy.

Academic Department of Medicine,
Division of Endocrinology
Royal Free Hospital School of Medicine,
Rowland Hill Street,
LONDON NW3 2QG.

1996



ABSTRACT

Kallmann's syndrome (KS) is defined as the association of hypogonadotrophic hypogonadism (IHH), caused by hypothalamic gonadotrophin releasing hormone (GnRH) deficiency, and anosmia, due to malformation of the olfactory bulbs and tracts. Patients most commonly present with delayed puberty and may also present in childhood with cryptorchidism. Renal agenesis seen in 40% and synkinesis observed in up to 90% of patients with X-linked KS (XKS) are likely to originate in the aberrant expression of *KAL* (Xp22.3) during early foetal development. Failure of the olfactory axons in the accessory olfactory nerves to project through the cribriform plate and establish synaptic contact with the developing olfactory bulb is thought to be central to the pathogenesis of these clinical features. GnRH neurons originating in the primitive nasal area consequently fail to migrate into the forebrain because of the absence of the "scaffolding" provided by these olfactory nerves.

This study has examined areas of KAL protein (680 amino acid protein containing a signal peptide but no membrane insertion or anchorage sequence, suggesting that this is a secreted protein) expression in the developing foetus and adult using anti-protein peptide antibodies generated in mice. A short sequence of the putative KAL sequence was used to synthesise a multiple antigenic peptide, which was then used as the antigen. Due to the non specificity of these antibodies the areas and stages of *KAL* gene expression were further investigated using *in situ* hybridisation and reverse transcriptase polymerase chain reaction (RT-PCR) on first trimester foetal tissue. This revealed *KAL* transcript in the olfactory bulbs, neuroretina and developing kidney. Patients with XKS and sporadic KS were investigated for mutations in *KAL* using established molecular biology techniques including PCR, direct DNA sequencing and single stranded conformational polymorphism (SSCP). Previously characterised mutations were confirmed and two new mutations identified. Using restriction fragment length polymorphism (RFLP) analysis, a methodology was established to successfully screen XKS carriers in a selection of patient pedigrees.

DEDICATION

This thesis is dedicated to the memory of my dear cousin Arieh "Alex" Ullmann who lost his brave and proud fight against lymphoma on 9th July 1995, aged 21.

ACKNOWLEDGEMENTS

Firstly I wish to thank Dr Pierre Bouloux, my PhD supervisor for all the guidance, encouragement and support he has given me during the three years I have worked for him. I am grateful for the opportunities he has given me both to put forward and present my own ideas and to collaborate with other scientists both within the Royal Free Hospital and outside. Your enthusiasm and commitment to your work has been a source of inspiration and encouragement to me, and at the same time your friendship, warmth and good humour has made the time spent working in your department very happy. Probably one of the most important lessons I have learnt from you as a young scientist is to be patient! I am particularly grateful for all the time you have spent with me in the preparation of this manuscript. I look forward to continuing to work with you and learning form you in the future.

I also wish to thank Prof Brian Cook (Biochemistry) for being my second PhD supervisor and for welcoming me into his department for seminars and group discussions .

My thanks go to Dr Phil Barnett for the time spent reading this manuscript and for his constructive suggestions. Good luck in your new job.

Thanks also to Dr Patricia Munroe for helping me settle into the lab and the Department of Medicine when I first arrived at the Royal Free Hospital.

I wish to thank Prof Richard Beagent for giving me the opportunity to spent some time in the Department of Clinical Oncology at the Royal Free, my thanks go to Will Nicholas, David Read and Geoff Boxer for their guidance and supervision.

I am most grateful to Prof Peter Thorogood, Department of Developmental Biology, Institute of Child Health for inviting me to work with Dr Adrian Woolf and Dr Paul Winyard on an exciting and worthwhile project, my time spent at the ICH was both an excellent learning experience and alot of fun!

In my time at the Royal Free I have had the opportunity to work with various people and made many friends. In particular Dr Richard Quinton, the most untidy person I know outside of my own family. Thanks for being so particular about collecting all our references and for photocopying them all so neatly. Working with you has been a laugh a minute and I look forward to carrying on our collaboration. My thanks go to Dr Prial De Zoysa for bringing

the computer facilities in our department into the twentieth century and for sorting out all their problems, large and small. I look forward to carrying on our work on Kallmann's Syndrome and solving together some of our unanswered questions.
To my fellow PhD student in the department Wohib Hassan, I wish you good luck in the completion of your studies, and in your future work.
My thanks to Patsy Coskeran the Endocrine Research nurse your friendship not to mention a bit of female company is always appreciated.
Thanks also to Joan and Maureen-the Endocrine secretaires for the use of their computers and printers not to mention all the sweets and biscuits. Thanks also to Jacky for always making me feel welcome on the Endocrine Investigations Unit.
My most grateful thanks go to all the patients who have given of their time and energy so willingly.

On a more personal note I wish to thank my parents for their love, support and encouragement not just in the last three years while I have been studying for my PhD but through all my student days and for being behind me in everything I do. Daddy when you read this work you will see I have not only been studying Hellmans mayonnaise! Mummy if some of what you read seem familiar don't be too surprised because as Marc and I always say "You have taught us all we know". Thanks mum for your help with sticking in all the figures! My thanks to my brother Marc (and cookie) for being such a good study partner, I am sorry our student days are now over! Good luck in your new job and enjoy the ironing!
To my very dear Andrew although at times your accountancy exams made us both lonely and misserabel, with every set of exams you sat another chapter of methods was written and now with your finals I have finished this thesis. Thanks for your encouragement and for the great love and respect you always show me and my work. I am proud to be your Mrs Dery Very B!

Finally I thank G-d for giving me the health and strength to complete this project. I pray that my colleagues, friends and family should have good health and success in all they do.

CONTENTS

LIST OF FIGURES.

LIST OF TABLES.

ABBREVIATIONS

BSA	Bovine serum albumin
cDNA	DNA complementary to mRNA
cm	centimetre
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide 5' triphosphates
DTT	Dithiothreitol
EtBr	Ethidium Bromide
g	gram
Kb	kilo base pairs
Kd	kilo daltons
L	litre
LB	luria broth
M	Molar ie. concentration in moles per litre
m	moles
mA	milliamperes-measure of electric current
min	minutes in time
ml	millilitre
mm	millimetre
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
OD	optical density measured in nano metres
O/N	over night
PEG	poly ethylene glycol
PFGE	pulsed field gel electrophoresis
RNA	ribonucleic acid
sec	second in time
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate buffer

Purines, Pyrimidines, Deoxy and dideoxy ribonucleotide nomenclature

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytosine triphosphate
dGTP	Deoxy-guanosine triphosphate
dTTP	Deoxy-thymine triphosphate
ddATP	Di-deoxy adenosine triphosphate
ddCTP	Di-deoxy cytosine triphosphate
ddGTP	Di-deoxy guanosine triphosphate
ddTTP	Di-deoxy thymine triphosphate

Amino acid code

A, Ala	alanine
C, Cys	cysteine
D, Asn	aspartic acid
E, Glu	glutamic acid
F, Phe	phenylanaline
G, Gly	glycine
H, His	histidine
I, Ile	iso-leucine
K, Lys	lysine
L, Leu	leucine
M, Met	methionine
N, Asn	asparagine
P, Pro	proline
Q, Gln	glutamine
R, Arg	arginine

S, Ser	serine
T, Thr	threonine
V, Val	valine
W, Trp	tryptophan
Y, Tyr	tyrosine

Symbols

α	alpha
β	beta
γ	gamma
δ	delta
λ	lambda, bacteriophage lambda
μ	mu

Chapter 1

KALLMANN'S SYNDROME - AN INTRODUCTION

1.1 Definition of Kallmann's Syndrome.

Kallmann's Syndrome (KS) or olfactogenital dysplasia is defined as the association of hypogonadotrophic hypogonadism (HH), the result of isolated gonadotrophin releasing hormone (GnRH) deficiency, and anosmia or hyposmia (absence of sense of smell) due to defective development of the olfactory bulb (Kallmann et al. 1944).

1.2 History of Kallmann's Syndrome

The first description of a patient with both hypogonadism and anosmia was made by the Spanish pathologist, Maestre de San Juan in 1856. The first report of a possible syndrome associating these two clinical features was made by Weidenreich in 1914; in a review of nine cases of olfactory lobe agenesis, genital hypoplasia was noted in three. In a report of several cases of familial hypogonadism and eunuchoidism (partial androgen deficiencies) Kallmann et al., in 1944 were the first to suggest a genetic aetiology to this syndrome.

Looking back in more detail on studies performed on eunuchs, Sainton in 1902 (Sainton, 1902) described the autopsy findings of familial testicular atrophy in a mentally defective eunuchoid patient with two normal sisters and a normal brother, but with two brothers, one maternal uncle and two maternal grand-uncles affected by the same hypogonadal anomalies. Goldstein in 1912 described two eunuchoid sibships consisting of two pairs of brothers with various degrees of mental deficiency and "adiposogenital dystrophy". Furno in 1931 documented a pedigree of twenty six members spanning three generations, with seven eunuchs and three non-gonadal endocrinopathies. Patterson, Bonnier and Moebius subsequently reported similar pedigrees (Patterson and Bonnier, 1937). These reports suggested a genetic aetiology to eunuchoidism, but also emphasised that the number of affected males far exceeded the number of affected females.

The association of eunuchoidism and anosmia in patients has been reported by several investigators including Weindenrich in (1914),(Weidenreich, 1914; Oldberg ST, 1932;

Kanai, 1940) Oldberg and Kanai. Kanai reported nineteen cases of olfactory lobe defects and in six of these cases the anomaly was found to be associated with eunuchoidism. Kanai's interpretation was that, this was due to a common ectodermal defect in the development of the base of the brain. Peritz and Maas (1910) also described a mentally defective eunuchoid who had "lost his sense of smell".

Kallmann et al., (Kallmann et al. 1944) investigated the tendency of primary gonadal defects to occur as a familial trait and established the particular clinical and genealogical conditions under which this occurred. In all, 42 families were investigated in which 48 eunuchoid patients comprising 12 female and 36 males were described. (Ten of the index patients belonged to kinships with more than one affected person). In the investigation of the index cases and their families, the dominant manifestations were hypogonadism, developmental defects, mental anomalies, diabetes, motor disorders, sex reversal, cerebral lesions, colour blindness and disturbance of olfaction. No sex-linked anomalies were found other than colour blindness. More significantly, eunuchoidism occurred together with colour blindness only in cases where hypogonadism was also present as a family trait. Synkinesis (congenital mirror movements of the hands) and mental defects were found only in conjunction with primary eunuchoidism. Of twelve female cases, none was seen to have either anosmia or colour blindness. In three other pedigrees, there was evidence of a wider spectrum of clinical defects, besides hypogonadotrophic hypogonadism and anosmia (Kallmann et al. 1944). Kallmann et al., were able to ascertain primary hypogonadism in 3 out of 42 families, eunuchoidism being present in one female and eleven males. The number of non-eunuchoid members was 27 females to 31 males. In the families where primary hypogonadism was seen, eunuchoidism was associated with colour blindness, anosmia, synkinesis and mental defects. Kallmann first hypothesised a common genetic basis for these clinical features forming the basis of the syndrome eponymously named after him. He described it in both males and females, and recognised the association with other congenital anomalies.

A connection between anosmia and agenesis of olfactory lobes was first made by DeMorsier in 1954 (De Morsier, 1954). The hypogonadism in KS was later attributed to hypogonadotropism by Nawakowski and Lenz in 1959 (Nowakowski and Lenz, 1961). Prior to the discovery of gonadotrophin releasing hormone (GnRH) in 1971 it was not possible to determine whether the defect in patients with KS was hypothalamic or pituitary gland

in origin (Crowley, Jr. and Jameson, 1992). It has since been established that in all patients, hypogonadism originates in the hypothalamus, since exogenous pulsatile injections of GnRH can induce gonadotrophin release and pubertal development in the majority of patients.

1.3 Clinical presentation and diagnosis.

Kallmann's Syndrome comprises two principal features: anosmia (inability to smell) and isolated hypogonadotrophic hypogonadism (IHH). The anosmia differentiates KS from other forms of HH and is essential for establishing the diagnosis. Diagnosis in males is sometimes made during early infancy (Evain-Brion et al. 1982; Conrad et al. 1978) or childhood because of cryptorchidism or rarely because of micropenis, but is more commonly made later in life as patients present with pubertal delay, arrested puberty or even primary infertility. Characteristically, the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are inappropriately low for a circulating sex steroid. Occasional individuals with KS have been described in whom pubertal regression occurred, with shrinkage of testicular volume late in puberty and the appearance of a hypogonadotrophic hypogonadal state (Crowley, Jr. and Jameson, 1992).

1.4 Inheritance and Incidence.

KS shows genetic heterogeneity, occurring as X-linked recessive (Frawley and Boockfor, 1991) (XKS), autosomal recessive (Santen and Paulsen, 1973) and autosomal dominant trait (White et al. 1983). It occurs most commonly in a sporadic form (SKS) however. Genetic analysis is complicated by the occurrence of infertility in patients (unless successfully treated). Early studies suggested that KS affected 1 in 10,000 males and 1 in 50,000 females (Jones and Kemmann, 1976). These figures were based on a survey performed on French army conscripts. A later study looking at HH and anosmia over a six year period (Pawlowitzki et al. 1987) to determine the mean age of diagnosis and prevalence found that out of 791 males referred with HH, 19 had KS ie.1 in 46 and that the mean age at presentation was 24.8 years, 12 years after the onset of normal puberty. The five fold excess of affected males over females (Jones and Kemmann, 1976) suggests that the X-linked form is the most frequent inherited form of the disease. In the majority of cases no family history of KS is elicited. (SKS)

The autosomal recessive form of KS shows incomplete penetrance since the carriers of the somatic abnormality demonstrate little if any signs of being carriers (for example reduced sense of smell or abnormal levels of gonadotrophin). There is a less than 50% incidence of KS in each generation of offspring of known carriers and neither is there an equal male to female occurrence of KS (Santen and Paulsen, 1973).

1.5 Morphological abnormalities associated with Kallmann's Syndrome

The HH and anosmia of KS are often associated with other defects, both within and outside the central nervous system.

1.5.1 Olfactory system

Anosmia can be easily overlooked if the patient is not specifically questioned about his or her ability to detect odours. Present since birth, it is often not recognised as a problem by the patient. The anosmia in these patients is due to defective development of the rhinencephalon (Lieblich et al. 1982), ie. absence or under development of olfactory bulbs, tracts and olfactory sulci. Traditionally, olfaction has been tested clinically by using seven odorants (Rosen et al. 1979). Recently, more refined techniques have been introduced to improve the accuracy of olfaction testing. These new tests require the patient to scratch a patch on a card, causing release of an odorant. The patient is then given four possible options as to the identity of the odorant and he marks his or her response on a multiple choice score card. The responses provided by the patient will allow the degree of anosmia ie. complete or partial to be scored.

Histologically, the olfactory mucosa in KS is abnormal in appearance. Olfactory neurones lack ciliation, and there is disordered axonal growth . These findings resemble the long term changes seen in animals following experimental olfactory bulbectomy (Monti-Graziadei and Graziadei, 1992), and perhaps suggest that the primary defect does not reside in the olfactory mucosa.

1.5.2 Gonadotrophin and gonadal steroids

All patients with KS have low levels of serum sex steroids with inappropriately low FSH, LH and urinary gonadotropins (Lieblich et al. 1982; Males et al. 1973), confirming the

presence of hypogonadotrophic hypogonadism. Reports have been made of patients who appear normal and then regress during or after puberty to a hypogonadal state.

1.5.3 Central nervous system (CNS)

Defects in the CNS of KS patients are most commonly associated with the X-linked form of the syndrome. Associated neurological abnormalities include synkinesis (congenital mirror movements of the hands). This is an involuntary mirror movement of the opposite limb to that which is undergoing voluntary movement. These type of movements have to be distinguished from acquired symptomatic synkineses due to lesions of the extra pyramidal system (Conrad et al. 1978). The abnormality is limited mainly to the lower arms and hands, and observed particularly when skilled movements of the hands are undertaken including writing, rapid finger tapping, wrist flexing and extension and fist clenching. All these movements when executed on one side are simultaneously and involuntarily mirrored by the contra lateral limb in a non-suppressible manner. Synkinesis is thought to result from a midline developmental defect during formation of the neural tube, with incomplete pyramidal tract decussation. Normally 95% of cortical signals decussate at the pyramids (signal from the left hemisphere of the brain controls movement on the right side of the body and vice versa) with only 5% of cortico-spinal tracts remaining ipsilateral (eg. signal for movement on one side of body originating from the brain hemisphere on the same side). In some stroke patients, the normally dormant ipsilateral pathways become activated. This may also occur in XKS patients. It is possible that during development failure of pyramidal tract decussation in the medulla oblongata could result in mirror movements.

Lack of inhibitory fibres within the corpus callosum has been proposed as another mechanism to explain synkinesis. Inhibitory transcallosal fibres are thought to originate in one hemisphere, pass through the corpus callosum, and inhibit the contra lateral uncrossed pyramidal tract. Defects in this pathway might, in theory, result in a disinhibition of the contra lateral cortical motor neurons.

Abnormal visuospatial attention, ocular motor abnormalities, sensory neural hearing loss, cerebellar dysfunction and pes cavity deformities (Schott and Wyke, 1981) have also been found in association with KS, though this has not been confirmed by other workers.

1.5.4 Urogenital system

Unilateral renal agenesis (absence of one kidney) has been reported in almost 50% of XKS patients (Wegenke et al. 1975; Kirk et al. 1994). Bilateral renal agenesis has been reported in KS in occasional pedigrees with XKS. These reports have been made in neonates who failed to survive. Micropenis and cryptorchidism are also features of KS, cryptorchidism reported in almost 90% of X-linked patients. The vas deferens has been shown to be absent in some patients (Prager, 1993; Lieblich et al. 1982).

In the developing human, the genital and urinary systems arise in close proximity to each other. Primordial germ cells migrate form the yolk sac in the fifth week of gestation to the area of the future gonads were they proliferate to form a pair of genital ridges. The mesonephros and coelomic epithelium invade the mesenchyme in the region of the presumptive gonads by week six to form the presumptive sex cords. By week six the Mullerian ducts begin to form lateral to the mesonephric ducts in both males and females. In both sexes, germ cells and sex cords are present in both cortical and medullary regions of the presumptive gonads.

Testis determining factor (TDF) is thought to be encoded on the sex determining region of the Y chromosome (SRY gene)(Sinclair, A. H et al. 1990; Hawkins et al. 1992). When SRY is synthesised in the undifferentiated sex cord cells of the presumptive gonads, male development is triggered. If SRY is absent or defective, female development will occur. Under the influence of SRY cells in the medullary region of the sex cord begin to differentiate into Sertoli cells. The cortical sex cord degenerates at this point. During the seventh week of embryogenesis, the differentiating Sertoli cells form the testis cord (this region gives rise to seminiferous tubules during puberty). The testis cords distal to the germ cell region develop lumina and differentiate into ducts called the rete testis. The tubules of the rete testis will become connected to residual mesonephric ducts which give rise to the vas deferens. During week seven the testis will round up and reduce its contact with the mesonephros. The close embryonic proximity of the mesonephros and metanephros during development, may form the basis for occasional reports of unilateral renal agenesis and absent vas deferens observed in males (Larsen William James, 1993).

Renal ultrasound should be carried out in XKS patients because of the strong association of unilateral renal agenesis with XKS.

1.5.5 Other clinical features associated with KS

Cleft lip and palate, cafe au lait pigmentation, mild hearing loss, short fourth metacarpal (Bardin, 1971), mild red-green colour blindness, gynaecomastia (Oppermann et al. 1987; Sunohara et al. 1985) and mental retardation have also been described in patients with KS. These associations, although weak, have been widely quoted in the literature even though the number of patients in which they have been seen is small.

Single case reports of patients with KS have described numerous associations including cardiovascular anomalies (Randall Moorman et al. 1984), atrial septal defect, mitral valve prolapse and a large intracranial cyst. A study by Cowen and Green (Fantini et al. 1992) recently attributed a variant of schizophrenia to KS by comparing the various features of schizophrenia to those of KS. Rarer associations include KS with ulcerative colitis (Turnbull et al. 1988), midline cerebral defects (Parr, 1988) and bilateral choanal atresia (Klein et al. 1987). If these clinical features are in fact associated with KS they allude to the pleitropic effect of KAL in human development.

1.6 Diagnosing KS

1.6.1 Magnetic resonance imaging in Kallmann's Syndrome

Magnetic resonance imaging (MRI) has been used to show morphological abnormalities in olfactory sulci and bulbs in KS. Klingmuller et al., (Klingmuller et al. 1987) hypothesised that the clinical features of KS were secondary to defective development of the rhinencephalon and the hypothalamic site of GnRH secretion. Transverse MRI brain sections of five patients with IHH and four with KS demonstrated that patients with KS all had either absent or underdeveloped olfactory sulci, whereas normal men or men with IHH had normal sulci. They failed to demonstrate any morphological changes in the hypothalamus of any of the men studied. Truwit studied nine patients with clinical and laboratory evidence of KS (Truwit et al. 1993). All had abnormal olfactory sulci, three demonstrated bilateral aplasia of the anterior olfactory sulci, while rudimentary sulci were detected in four patients. The olfactory bulbs and tracts appeared hypoplastic in five patients and absent in one. The hypothalamus was found to be normal in all subjects. Yousem (Yousem et al. 1993) studied two males (one black and one white) with KS. Neither had olfactory bulbs or tracts. One had

unilateral and the other bilaterally absent olfactory sulci. Their temporal lobe size and volume were equivalent to normal controls (Yousem et al. 1993). Of the six patients reported by Knorr (1993) three had absent olfactory tracts, one had normal olfactory sulci and the other five had hypoplastic or aplastic sulci. All had normal pituitary glands. Three were cryptorchid and only one had a normal penis (Knorr et al. 1993). Further studies (Vogl et al. 1994; Takeda et al. 1992; Birnbacher et al. 1994) using MRI have confirmed the hypoplasia of the olfactory sulci which is more pronounced anteriorly. The olfactory bulbs were shown to be either rudimentary or absent and the olfactory tracts were either absent or hypoplastic in KS patients as opposed to the normal appearance in patients with IHH. Again the hypothalamus was seen to be unchanged in these KS patients as well as in the patients with IHH.

MRI can therefore be used as an aid in the diagnosis of KS, to confirm hypoplasia of the olfactory sulci and absence of the olfactory bulbs and tracts.

1.6.2 Hormone profiles in KS

Patients should have a simple blood test to examine, testosterone, luteinizing hormone(LH) and follicle stimulating hormone(FSH) levels. In a patient with KS, gonadotrophin and sex hormones would be expected to be low, confirming the diagnosis of hypogonadotrophic hypogonadism. Cortisol, T4, and TSH measures are indicated to exclude other anterior pituitary deficiencies, although in a patient with complete anosmia the latter tests are rarely contributory.

1.6.3 Olfactory testing

The patients olfaction would be tested as described above (1.5.1), and the results together with the hormone profile, MRI and ultrasound results and family history enable a definite diagnosis of KS to be made.(J.M.W.Kirk et al. 1994)

Chapter 2

THE GENETICS OF KALLMANN'S SYNDROME

Kallmann's Syndrome (KS) is inherited in a number of ways. Given that females are affected (Tagatz et al. 1970)(Levy and Knudtzon, 1993)(Jones and Kemmann, 1976) and the evidence of father to son transmission (Merriam et al. 1977) together with the presence of families showing autosomal inheritance (Hermanussen and Sippell, 1985) it is probable that one or more defective autosomal genes may be responsible for KS. The identity of these autosomal loci is currently unknown. The gene for the X-linked form of KS (XKS) has been identified (*KAL*) and characterised. Most patients presenting clinically do not however have a demonstrable family history of the condition. Such isolated cases are termed sporadic (SKS).

2.1 Autosomal Kallmann's Syndrome

2.1.1 The female Kallmann patient

HH and anosmia in females has been called "Olfacto genital dysplasia" and occurs rarely. Tagatz et al (Tagatz et al. 1970) reported three affected females with primary amenorrhoea, infantile sexual development, anosmia and a normal 46XX karyotype. No other physical abnormality was recorded. In all cases, the females responded well to ovulation induction by gonadotropins and two achieved pregnancies and had normal babies. The association of anosmia and HH in these patients was probably due to a genetic lesion much the same as that described by Kallmann et al (Kallmann et al. 1944).

Levy et al (Levy and Knudtzon, 1993) documented two sisters showing all the clinical features of KS. However, their father did not show any of the signs of KS. He did have duplicate left sided ureters similar to his daughters. The fact that both daughters had the same symptoms suggested a dominant form of inheritance. The father may have carried the gene but with no penetrance.

A female with KS has been reported to have a monozygotic twin sister showing no pubertal delay, although both sisters were anosmic. The only other member in this family to have any abnormality was the great-grandmother who was said to be anosmic (Hermanussen and

Sippell, 1985). It is difficult to ascertain the type of inheritance involved, although it is clearly not X-linked. Further genetic studies on these females and the modes of inheritance of their disorder must await the isolation and characterisation of the autosomal gene(s).

2.1.2 Father to son transmission of KS

Merriam et al (Merriam et al. 1977) described a man with KS who underwent fertility treatment and fathered three children. The first two children (a daughter and a son) were normal but the third child a son had KS. This family showed an autosomal dominant mode of inheritance.

2.1.3 Further family studies of KS

Santen and Paulsen studied six kindreds, (17 affected males, 9 affected females)(Santen and Paulsen, 1973) and concluded that the most likely mode of inheritance in their cohort was autosomal dominant with incomplete penetrance. They suggested incomplete penetrance since less than 50% of offspring inherited KS and there was not an equal number of affected males to females, ie. deviating from the 1:1 sex expectation suggesting the autosomal form of KS is subject to sex limitation, far more males being affected than females (Santen and Paulsen, 1973). Hipkin et al (Hipkin et al. 1990) reported a set of monozygotic male twins. One had definite KS and the other was completely normal as were his mother, father and sister. Families such as these, where there are no other affected members are suggestive of autosomal recessive inheritance (White et al. 1983; Lieblich et al. 1982).

A survey of the literature reveals that the most likely forms of inheritance of KS are either X-linked or sex limited autosomal dominant (Menon et al. 1984). The cohort of patients studied at the Royal Free Hospital however, showed sporadic cases to be the most frequent, followed by X-linked patients (XKS).

2.2 X-linked Kallmann's Syndrome.

There is strong evidence that the X-linked form KS occurs more frequently than the autosomal forms.

2.2.1 Cloning of the X-linked gene.

In 1991 two groups, from Texas and Paris, both successfully cloned a candidate gene for

the XKS. Each approached the task in a different way, using DNA from the same patients, but arriving at the identical putative cDNA sequence.

Andrea Ballabio in Baylor College of Medicine, Texas, USA had previously assigned the locus for the XKS to Xp22.3 using deletion mapping and linkage analysis in families with isolated KS (Ballabio et al. 1987; Ballabio et al. 1989; Meitinger et al. 1990). The critical region spanned the breakpoint of an X/Y translocation patient with KS t(X;Y)1 to the breakpoint of another X/Y translocation t(X;Y)2 in a patient without KS. DNA probes derived from this region of the genome recognised different size fragments in the two patients, defining loci in the proximal and distal limits of the KS interval. Directional chromosomal walking towards the KS locus was initiated using a YAC (yeast artificial chromosome) system. Two of the YACs used had X specific DNA and two had Y specific DNA homologous to the X region within the defined loci. Evolutionary conserved sequences were sought within the YAC clones; YACs were subcloned into lambda phage vector and overlaps between the clones determined (contigs).

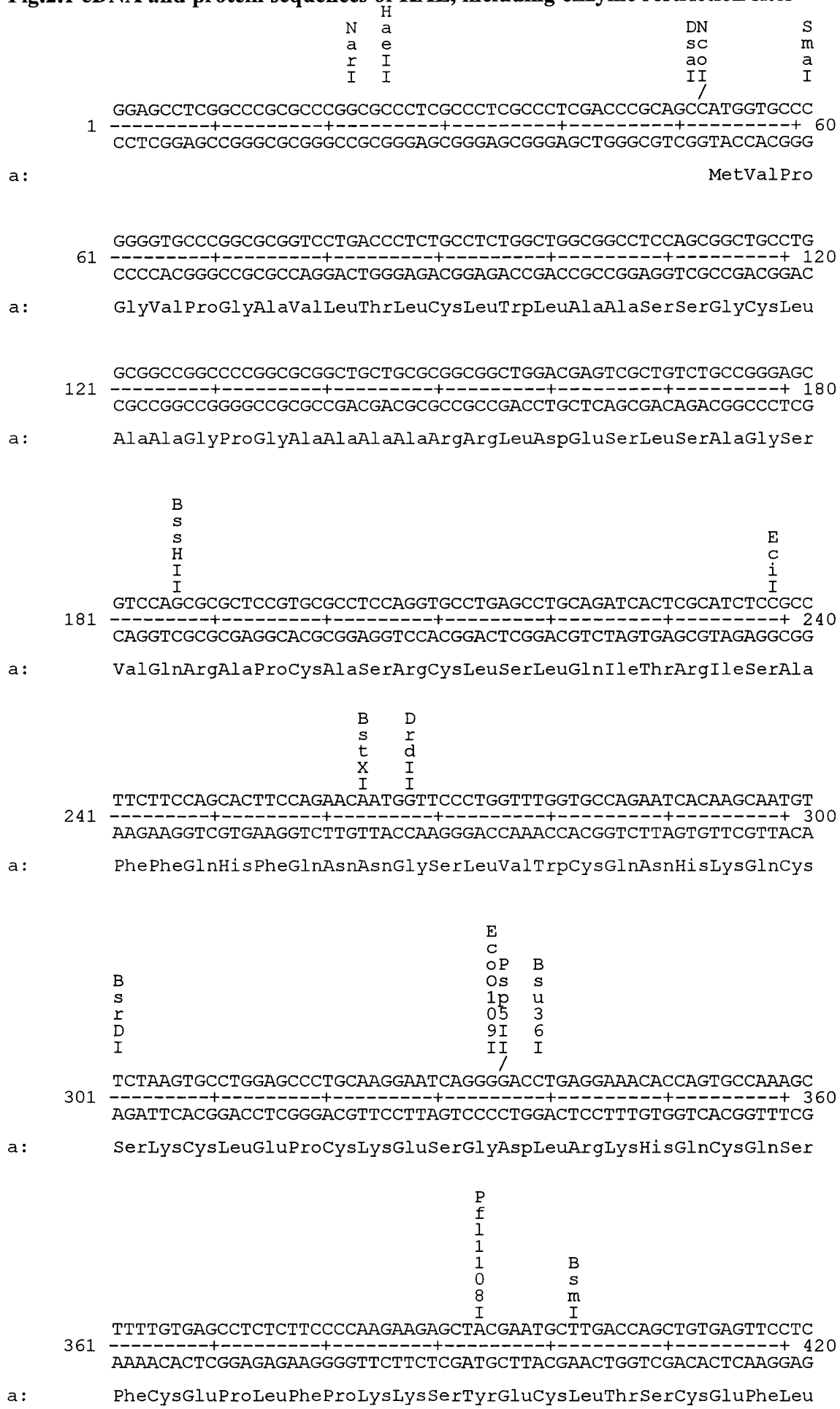
Phage clones found to be evolutionarily conserved by hybridisation to zoo blots (membranes bearing DNA from different animal species) of DNA from distantly related species were then used to screen the cDNA libraries from three different RNA sources (human foetal brain, total embryo and teratocarcinoma tissue). Several clones corresponding to a gene in the KS region were isolated. The longest of these clones was used to probe a foetal brain cDNA library. The cDNA isolated from this was then sequenced. Mapping of the YAC contig (yeast artificial chromosomes containing large lengths of chromosomal material in contiguous series) allowed them to establish that KALIG1 spans 210Kb of human genomic DNA.

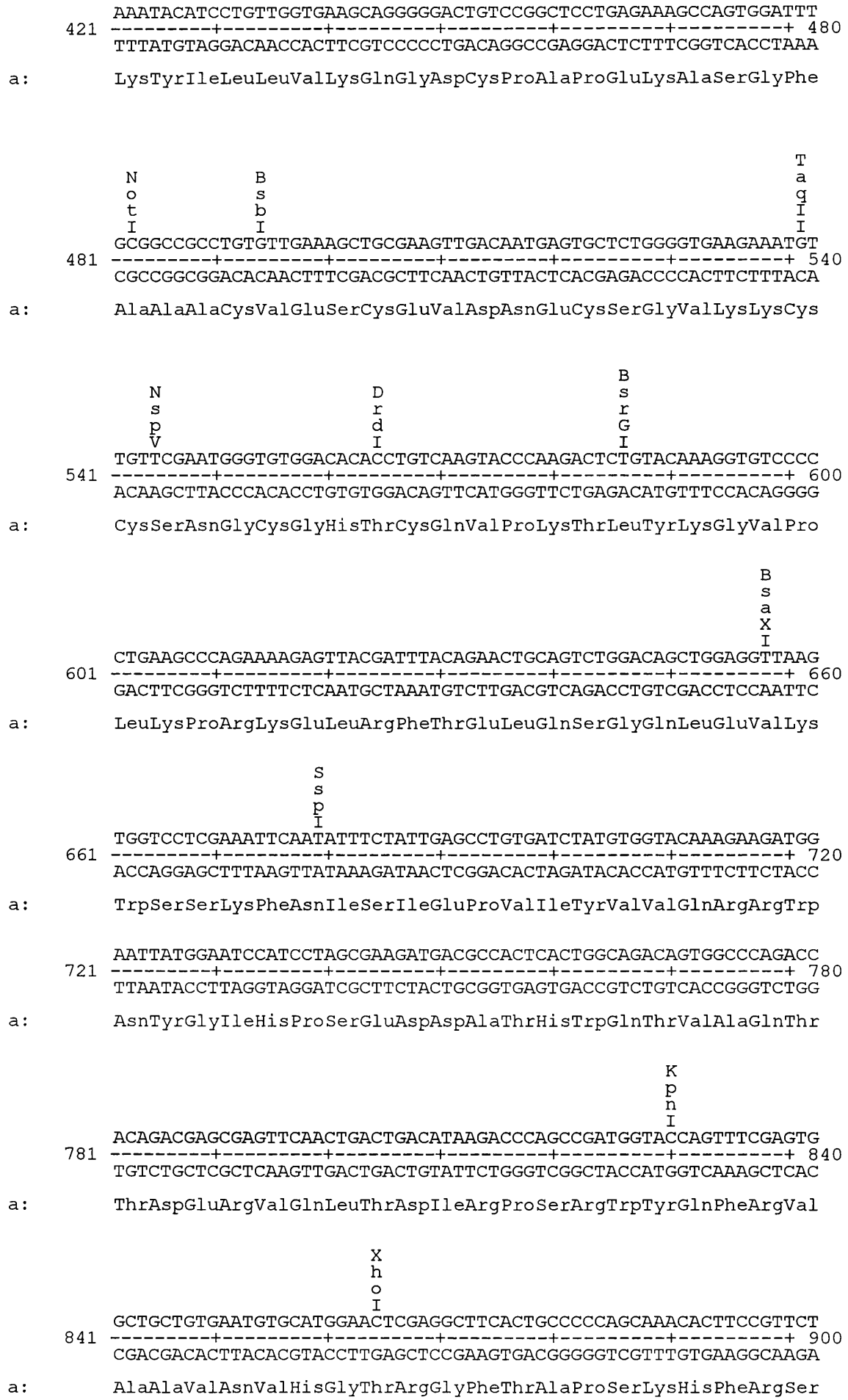
Probes corresponding to both the 3' and the 5' ends of the KALIG1 region were used to probe EcoR1 digests of DNA from translocation patients with and without KS. All the bands detected male specific bands suggesting the presence of a Y homologue. Analysis of patients with partial deletions made it possible to orientate the gene with respect to the short arm of the X chromosome the 5' end being located towards the centromere and the 3' end towards the telomere. The entire cDNA sequence of 4,093bp contained one continuous open reading frame with a start site at nucleotide 64 and a termination codon at nucleotide

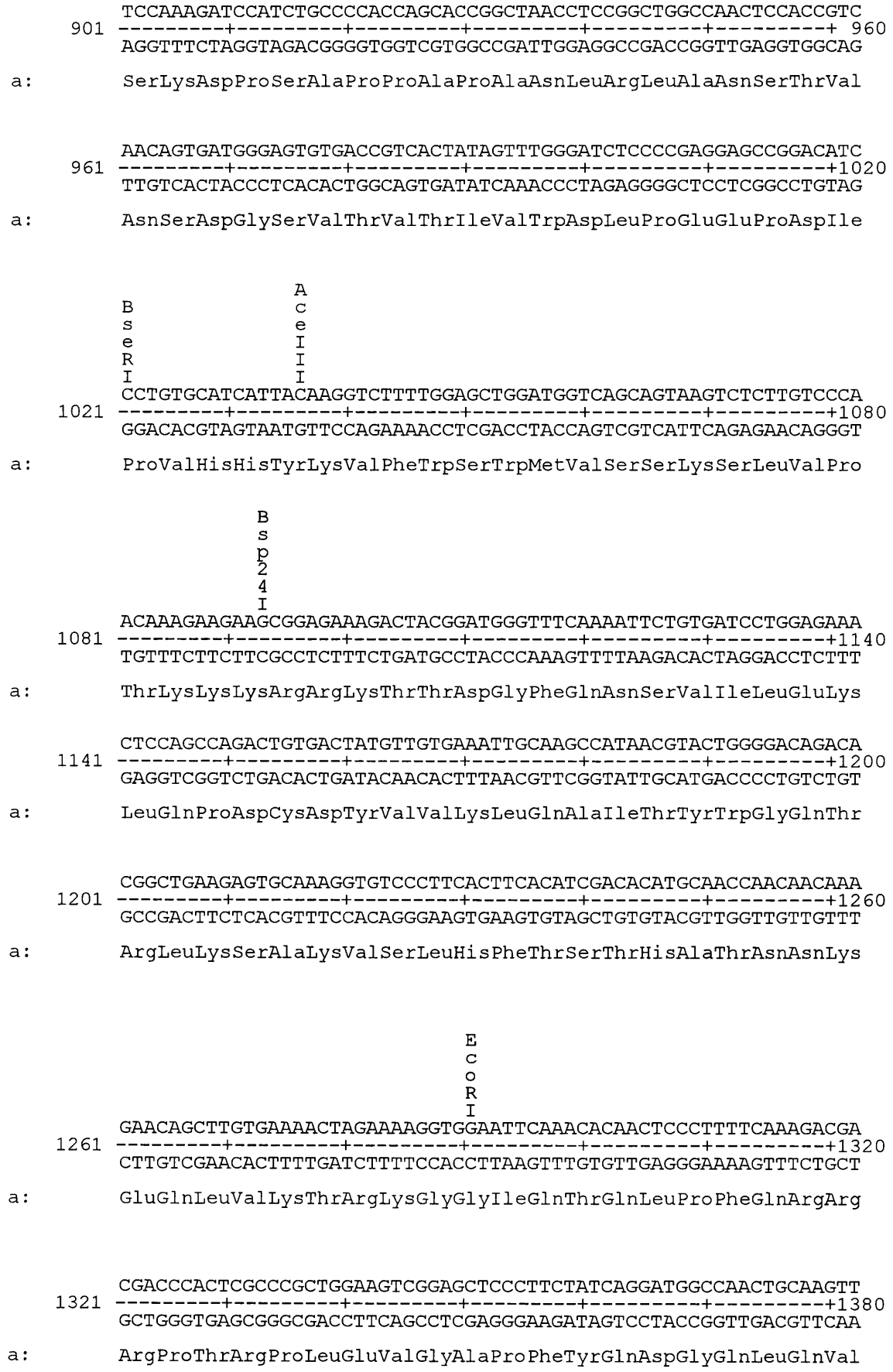
2,104 coding for a predicted protein product of 680 amino acids.

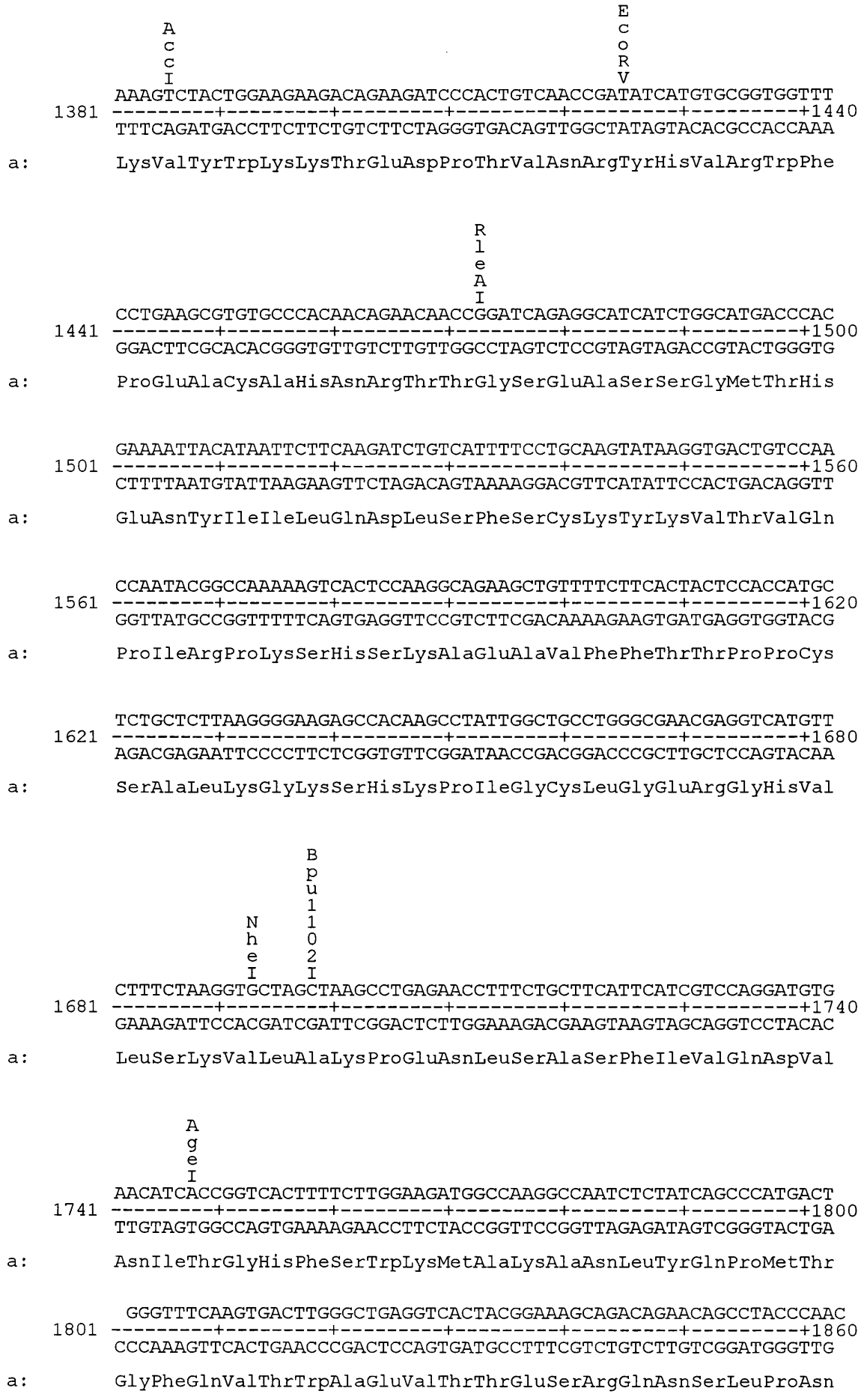
At the same time, Christine Petit at the Pasteur Institute in Paris isolated and characterized a YAC encompassing the KAL interval using pulse field gel electrophoresis (PFGE) and the DNA from the same two patients used by Ballabio et al., one with a terminal deletion of the short arm of the X chromosome and the other with an X/Y translocation. Both the patients were affected with other diseases previously mapped to the distal part of the X chromosome: short stature, recessive chondrodyspalsia punctata, mental retardation and ichthyosis. However, one patient was affected with KS, the other not. CRI-S232 was the only probe to recognize a locus in this region, and was used to identify 13 different YACs from a library. Several clones were derived from these YACs by PCR and hybridised to a panel of deletion patients. The data from this screen together with the CRI-S232 results, allowed the KAL interval to be reduced to the distance between the locus detected by CRI-S232 and the deletion patient breakpoint, an interval not exceeding 125Kb.
The region was narrowed down yet further to 67 Kb by performing restriction digests of genomic DNA of the deletion and translocation patients with both EcoR1 and Hind111 and probing them with EcoR1 fragments of the lambda phage inserts prepared from the YACs. The entire 67Kb interval was sequenced and analyzed by computer to identify coding and non-coding regions. A total of 19 putative exons were identified; these were amplified by PCR and used to screen zoo blots. Only two probes identified unique sequences in other mammals. A cDNA library was generated from a 36 day old Macaccus fascicolaris fetus; this was screened together with two human foetal brain libraries with the same two probes. 7 overlapping clones were isolated and a 6.3Kb cDNA sequence was obtained and named ADMLX (adhesion molecule like from the X chromosome), due to the homologies of the predicted protein to other proteins. It contained a single open reading frame of 679 amino acids. The first ATG was at 153 nucleotide downstreeam from the 5' extremity and the TAA stop codon at nucleotide 2104. The 3' untranslated region is unusually large-4123 nucleotides.

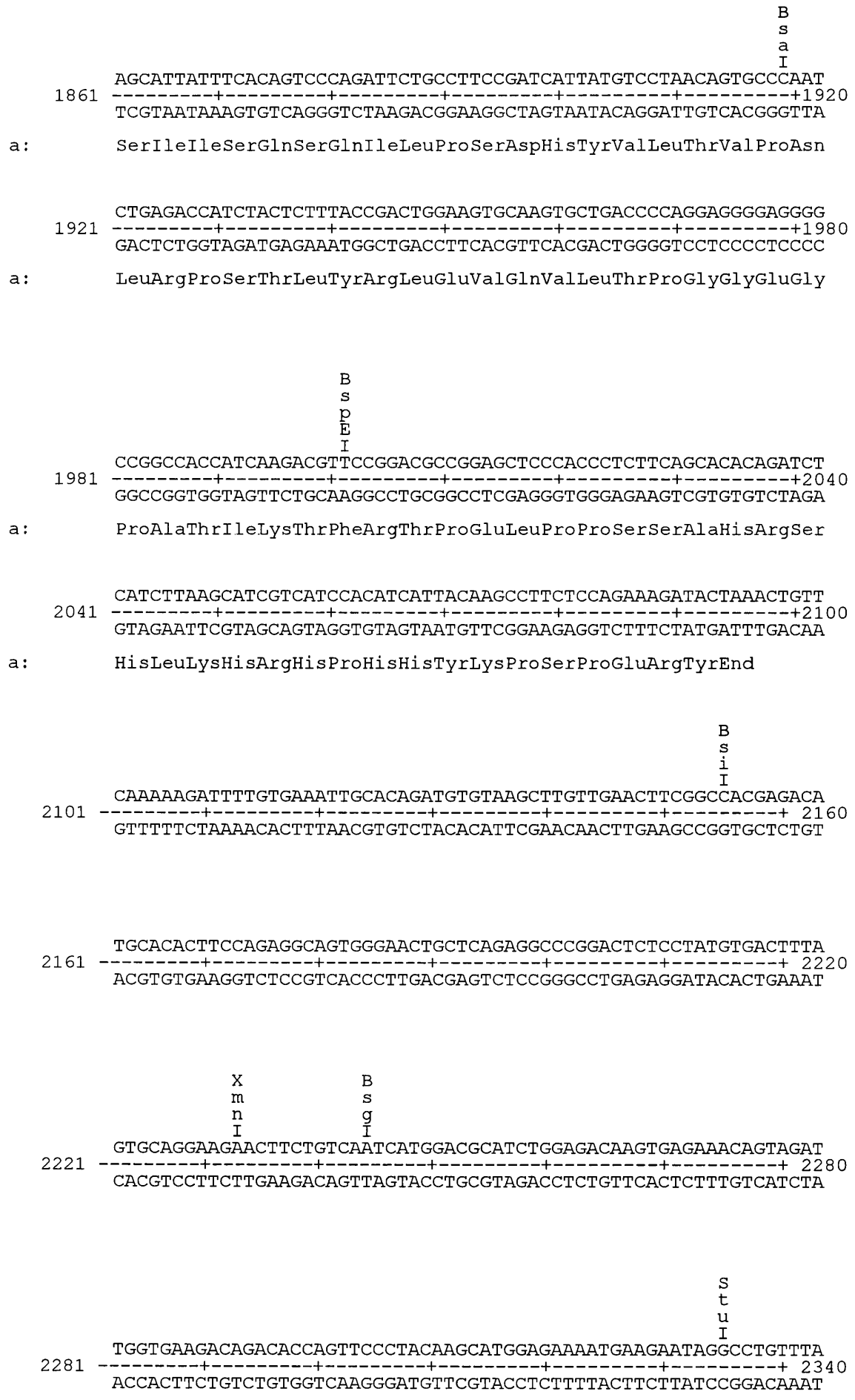
Fig.2.1 cDNA and protein sequences of KAL, including enzyme restriction sites




AAATACATCCTGTTGGTGAAGCAGGGGGACTGTCCGGCTCCTGAGAAAGCCAGTGGATTT
421
TTTATGTAGGACAACCACTTCGTCCCCCTGACAGGCCGAGGACTCTTTCGGTCACCTAAA
a: LysTyrIleLeuLeuValLysGlnGlyAspCysProAlaProGluLysAlaSerGlyPhe
NotI
BsbI
TaqII
GCGGCCGCCTGTGTTGAAAGCTGCGAAGTTGACAATGAGTGCTCTGGGGTGAAGAAATGT
481
CGCCGGCGGACACAACTTTCGACGCTTCAACTGTTACTCACGAGACCCCACTTCTTTACA
a: AlaAlaAlaCysValGluSerCysGluValAspAsnGluCysSerGlyValLysLysCys
NspV
DrdI
BsrGI
TGTTCGAATGGGTGTGGACACACCTGTCAAGTACCCAAGACTCTGTACAAAGGTGTCCCC
541
ACAAGCTTACCCACACCTGTGTGGACAGTTCATGGGTTCTGAGACATGTTTCCACAGGGG
a: CysSerAsnGlyCysGlyHisThrCysGlnValProLysThrLeuTyrLysGlyValPro
BsaXI
CTGAAGCCCAGAAAAGAGTTACGATTTACAGAACTGCAGTCTGGACAGCTGGAGGTTAAG
601
GACTTCGGGTCTTTTCTCAATGCTAAATGTCTTGACGTCAGACCTGTCGACCTCCAATTC
a: LeuLysProArgLysGluLeuArgPheThrGluLeuGlnSerGlyGlnLeuGluValLys
SspI
TGGTCCTCGAAATTCAATATTTCTATTGAGCCTGTGATCTATGTGGTACAAAGAAGATGG
661
ACCAGGAGCTTTAAGTTATAAAGATAACTCGGACACTAGATACACCATGTTTCTTCTACC
a: TrpSerSerLysPheAsnIleSerIleGluProValIleTyrValValGlnArgArgTrp
AATTATGGAATCCATCCTAGCGAAGATGACGCCACTCACTGGCAGACAGTGGCCCAGACC
721
TTAATACCTTAGGTAGGATCGCTTCTACTGCGGTGAGTGACCGTCTGTCACCGGGTCTGG
a: AsnTyrGlyIleHisProSerGluAspAspAlaThrHisTrpGlnThrValAlaGlnThr
KpnI
ACAGACGAGCGAGTTCAACTGACTGACATAAGACCCAGCCGATGGTACCAGTTTCGAGTG
781
TGTCTGCTCGCTCAAGTTGACTGACTGTATTCTGGGTCGGCTACCATGGTCAAAGCTCAC
a: ThrAspGluArgValGlnLeuThrAspIleArgProSerArgTrpTyrGlnPheArgVal
XhoI
GCTGCTGTGAATGTGCATGGAACTCGAGGCTTCACTGCCCCCAGCAAACACTTCCGTTCT
841
CGACGACACTTACACGTACCTTGAGCTCCGAAGTGACGGGGGTCGTTTGTGAAGGCAAGA
a: AlaAlaValAsnValHisGlyThrArgGlyPheThrAlaProSerLysHisPheArgSer


TCCAAAGATCCATCTGCCCCACCAGCACCGGCTAACCTCCGGCTGGCCAACTCCACCGTC
901
AGGTTTCTAGGTAGACGGGGTGGTCGTGGCCGATTGGAGGCCGACCGGTTGAGGTGGCAG
a: SerLysAspProSerAlaProProAlaProAlaAsnLeuArgLeuAlaAsnSerThrVal
AACAGTGATGGGAGTGTGACCGTCACTATAGTTTGGGATCTCCCCGAGGAGCCGGACATC
961
TTGTCACTACCCTCACACTGGCAGTGATATCAAACCCTAGAGGGGCTCCTCGGCCTGTAG
a: AsnSerAspGlySerValThrValThrIleValTrpAspLeuProGluGluProAspIle
BseRI
AceIII
CCTGTGCATCATTACAAGGTCTTTTGGAGCTGGATGGTCAGCAGTAAGTCTCTTGTCCCA
1021
GGACACGTAGTAATGTTCCAGAAAACCTCGACCTACCAGTCGTCATTCAGAGAACAGGGT
a: ProValHisHisTyrLysValPheTrpSerTrpMetValSerSerLysSerLeuValPro
Bsp24I
ACAAAGAAGAAGCGGAGAAAGACTACGGATGGGTTTCAAAATTCTGTGATCCTGGAGAAA
1081
TGTTTCTTCTTCGCCTCTTTCTGATGCCTACCCAAAGTTTTAAGACACTAGGACCTCTTT
a: ThrLysLysLysArgArgLysThrThrAspGlyPheGlnAsnSerValIleLeuGluLys
CTCCAGCCAGACTGTGACTATGTTGTGAAATTGCAAGCCATAACGTACTGGGGACAGACA
1141
GAGGTCGGTCTGACACTGATACAACACTTTAACGTTCGGTATTGCATGACCCCTGTCTGT
a: LeuGlnProAspCysAspTyrValValLysLeuGlnAlaIleThrTyrTrpGlyGlnThr
CGGCTGAAGAGTGCAAAGGTGTCCCTTCACTTCACATCGACACATGCAACCAACAACAAA
1201
GCCGACTTCTCACGTTTCCACAGGGAAGTGAAGTGTAGCTGTGTACGTTGGTTGTTGTTT
a: ArgLeuLysSerAlaLysValSerLeuHisPheThrSerThrHisAlaThrAsnAsnLys
EcoRI
GAACAGCTTGTGAAAACTAGAAAAGGTGGAATTCAAACACAACTCCCTTTTCAAAGACGA
1261
CTTGTCGAACACTTTTGATCTTTTCCACCTTAAGTTTGTGTTGAGGGAAAAGTTTCTGCT
a: GluGlnLeuValLysThrArgLysGlyGlyIleGlnThrGlnLeuProPheGlnArgArg
CGACCCACTCGCCCGCTGGAAGTCGGAGCTCCCTTCTATCAGGATGGCCAACTGCAAGTT
1321
GCTGGGTGAGCGGGCGACCTTCAGCCTCGAGGGAAGATAGTCCTACCGGTTGACGTTCAA
a: ArgProThrArgProLeuGluValGlyAlaProPheTyrGlnAspGlyGlnLeuGlnVal


AccI
EcoRV
AAAGTCTACTGGAAGAAGACAGAAGATCCCACTGTCAACCGATATCATGTGCGGTGGTTT
1381
TTTCAGATGACCTTCTTCTGTCTTCTAGGGTGACAGTTGGCTATAGTACACGCCACCAAA
a: LysValTyrTrpLysLysThrGluAspProThrValAsnArgTyrHisValArgTrpPhe
RleAI
CCTGAAGCGTGTGCCCACAACAGAACAACCGGATCAGAGGCATCATCTGGCATGACCCAC
1441
1500
GGACTTCGCACACGGGTGTTGTCTTGTTGGCCTAGTCTCCGTAGTAGACCGTACTGGGTG
a: ProGluAlaCysAlaHisAsnArgThrThrGlySerGluAlaSerSerGlyMetThrHis
GAAAATTACATAATTCTTCAAGATCTGTCATTTTCCTGCAAGTATAAGGTGACTGTCCAA
1501
1560
CTTTTAATGTATTAAGAAGTTCTAGACAGTAAAAGGACGTTCATATTCCACTGACAGGTT
a: GluAsnTyrIleIleLeuGlnAspLeuSerPheSerCysLysTyrLysValThrValGln
CCAATACGGCCAAAAAGTCACTCCAAGGCAGAAGCTGTTTTCTTCACTACTCCACCATGC
1561
1620
GGTTATGCCGGTTTTTCAGTGAGGTTCCGTCTTCGACAAAAGAAGTGATGAGGTGGTACG
a: ProIleArgProLysSerHisSerLysAlaGluAlaValPhePheThrThrProProCys
TCTGCTCTTAAGGGGAAGAGCCACAAGCCTATTGGCTGCCTGGGCGAACGAGGTCATGTT
1621
1680
AGACGAGAATTCCCCTTCTCGGTGTTCGGATAACCGACGGACCCGCTTGCTCCAGTACAA
a: SerAlaLeuLysGlyLysSerHisLysProIleGlyCysLeuGlyGluArgGlyHisVal
NheI
Bpu1102I
CTTTCTAAGGTGCTAGCTAAGCCTGAGAACCTTTCTGCTTCATTCATCGTCCAGGATGTG
1681
1740
GAAAGATTCCACGATCGATTCGGACTCTTGGAAAGACGAAGTAAGTAGCAGGTCCTACAC
a: LeuSerLysValLeuAlaLysProGluAsnLeuSerAlaSerPheIleValGlnAspVal
AgeI
AACATCACCGGTCACTTTTCTTGGAAGATGGCCAAGGCCAATCTCTATCAGCCCATGACT
1741
1800
TTGTAGTGGCCAGTGAAAAGAACCTTCTACCGGTTCCGGTTAGAGATAGTCGGGTACTGA
a: AsnIleThrGlyHisPheSerTrpLysMetAlaLysAlaAsnLeuTyrGlnProMetThr
GGGTTTCAAGTGACTTGGGCTGAGGTCACTACGGAAAGCAGACAGAACAGCCTACCCAAC
1801
1860
CCCAAAGTTCACTGAACCCGACTCCAGTGATGCCTTTCGTCTGTCTTGTCGGATGGGTTG
a: GlyPheGlnValThrTrpAlaGluValThrThrGluSerArgGlnAsnSerLeuProAsn


BsaI
AGCATTATTTCACAGTCCCAGATTCTGCCTTCCGATCATTATGTCCTAACAGTGCCCAAT
1861 ---------+---------+---------+---------+---------+---------+1920
TCGTAATAAAGTGTCAGGGTCTAAGACGGAAGGCTAGTAATACAGGATTGTCACGGGTTA
a: SerIleIleSerGlnSerGlnIleLeuProSerAspHisTyrValLeuThrValProAsn
CTGAGACCATCTACTCTTTACCGACTGGAAGTGCAAGTGCTGACCCCAGGAGGGGAGGGG
1921 ---------+---------+---------+---------+---------+---------+1980
GACTCTGGTAGATGAGAAATGGCTGACCTTCACGTTCACGACTGGGGTCCTCCCCTCCCC
a: LeuArgProSerThrLeuTyrArgLeuGluValGlnValLeuThrProGlyGlyGluGly
BspEI
CCGGCCACCATCAAGACGTTCCGGACGCCGGAGCTCCCACCCTCTTCAGCACACAGATCT
1981 ---------+---------+---------+---------+---------+---------+2040
GGCCGGTGGTAGTTCTGCAAGGCCTGCGGCCTCGAGGGTGGGAGAAGTCGTGTGTCTAGA
a: ProAlaThrIleLysThrPheArgThrProGluLeuProProSerSerAlaHisArgSer
CATCTTAAGCATCGTCATCCACATCATTACAAGCCTTCTCCAGAAAGATACTAAACTGTT
2041 ---------+---------+---------+---------+---------+---------+2100
GTAGAATTCGTAGCAGTAGGTGTAGTAATGTTCGGAAGAGGTCTTTCTATGATTTGACAA
a: HisLeuLysHisArgHisProHisHisTyrLysProSerProGluArgTyrEnd
BsiI
CAAAAAGATTTTGTGAAATTGCACAGATGTGTAAGCTTGTTGAACTTCGGCCACGAGACA
2101 ---------+---------+---------+---------+---------+---------+ 2160
GTTTTTCTAAAACACTTTAACGTGTCTACACATTCGAACAACTTGAAGCCGGTGCTCTGT
TGCACACTTCCAGAGGCAGTGGGAACTGCTCAGAGGCCCGGACTCTCCTATGTGACTTTA
2161 ---------+---------+---------+---------+---------+---------+ 2220
ACGTGTGAAGGTCTCCGTCACCCTTGACGAGTCTCCGGGCCTGAGAGGATACACTGAAAT
XmnI
BsgI
GTGCAGGAAGAACTTCTGTCAATCATGGACGCATCTGGAGACAAGTGAGAAACAGTAGAT
2221 ---------+---------+---------+---------+---------+---------+ 2280
CACGTCCTTCTTGAAGACAGTTAGTACCTGCGTAGACCTCTGTTCACTCTTTGTCATCTA
StuI
TGGTGAAGACAGACACCAGTTCCCTACAAGCATGGAGAAAATGAAGAATAGGCCTGTTTA
2281 ---------+---------+---------+---------+---------+---------+ 2340
ACCACTTCTGTCTGTGGTCAAGGGATGTTCGTACCTCTTTTACTTCTTATCCGGACAAAT

```
          ATGCTAAATTTTGTTTTCATGTATGGTGTCGCTCATTTCTATTG
2341 ---------+---------+---------+---------+---- 2384
          TACGATTTAAAACAAAAGTACATACCACAGCGAGTAAAGATAAC
```

Enzymes that do cut and were not excluded:

AccI AceIII AgeI Bpu1102I BsaI BsaXI BsbI BseRI BsgI BsiI BsmI
Bsp24I BspEI BsrDI BsrGI BssHII
BstXI Bsu36I DrdI DrdII DsaI EciI EcoO109I EcoRI
EcoRV HaeII KpnI NarI NcoI NheI NotI NspV
Pfl1108I Psp5II RleAI SmaI SspI StuI TaqII XhoI
Xmn

Enzymes that do not cut:

AatII AflIII Alw44I ApaI ApaBI AscI AvrII BaeI
BamHI Bce83I BclI BglI BsaAI BsaBI BscGI BsmBI
BspLU11I BspMI BsrBI Bst1107I BstEII ClaI DraI DraIII
Eam1105I Eco47III EcoNI FspI HgiEII HpaI MluI MunI
NdeI NruI NsiI PacI PmeI PmlI PshAI Psp1406I
PvuI RcaI RsrII SacII SalI ScaI SexAI SfiI
SgfI SgrAI SnaBI SpeI SphI SrfI Sse8387I SunI
SwaI Tth111I VspI XbaI

Enzymes excluded; MinCuts: 1 MaxCuts: 1

AciI AflII AluI AlwI Alw21I AlwNI ApoI AvaI
AvaII BanI BanII BbsI BbvI BccI BcefI BcgI
BcgI BfaI BglII BmgI BpmI Bpu10I BsaHI BsaJI
BsaWI BsiEI BslI BsmAI BsmFI BsoFI Bsp24I Bsp1286I
BspGI BsrI BsrFI BstYI Cac8I CjeI CjeI CjePI
CjePI CviJI CviRI DdeI DpnI EaeI EagI EarI
Eco57I EcoRII FauI FokI FseI GdiII HaeI HaeIII
HgaI HhaI Hin4I HincII HindIII HinfI HphI MaeII
MaeIII MboII MmeI MnlI MscI MseI MslI MspI
MspA1I MwoI NciI NgoAIV NlaIII NlaIV NspI PflMI
PleI PstI PvuII RsaI SacI SapI Sau96I Sau3AI

ScrFI SfaNI SfcI StyI TaqI TfiI ThaI TseI

Tsp45I Tsp509I Tth111II UbaJI XcmI

2.2.2 Expression of the *KAL* gene

Ubiquitous expression of the KALIG1 gene was suggested by reverse transcriptase polymerase chain reaction (RT-PCR) using RNA samples from several tissues from an 18 week old female foetus, including brain, muscle, kidney, liver and intestine as well as adult brain, liver, kidney, skeletal muscle, lymphoblastoid cells and teratoma cells. In foetal tissue, expression was seen in all tissues other than kidney. In the adult, all tissues tested showed expression of KALIG 1/ADMLX. Northern blot analysis gave no detectable signal probably due to the low copy number of this transcript.

This thesis describes work investigating the areas of *KAL* expression in the earlier developing human embryo and foetus using both RT-PCR and *in situ* hybridisation.

2.2.3 Validation of KALIG1 / ADMLX

Final validation of the gene's position and sequence required the identification of affected individuals with point mutations or small intragenic deletions in the gene. This search was performed in a group of 20 unrelated KS patients (Hardelin et al. 1992). Using Southern blotting, PCR and DNA sequencing, two complete gene deletions were identified, (Hardelin et al. 1993b) as well as four other patients harbouring point mutations in exons 5 and 6 of *KAL*. Of the four patients, three had a base transition resulting in a STOP codon and the fourth had a single base deletion causing a frame shift mutation. The remaining 14 patients had no obvious mutations. Further studies of a similar nature have been undertaken to both further validate the gene for the X-linked form of KS, and at the same time to identify mutational hot spots. By amplifying and sequencing each of the 14 exons of the remaining 14 patients who appeared to have no mutation on the preliminary screen using Southern blotting, two sequence variations (one in exon 11 coding for an amino acid change and one in exon 12 resulting in the introduction of a restriction site but no amino acid change) were identified. Nine different point mutations were also identified. The base transitions were seen in exons 5,6,7 and 9 (Hardelin et al. 1993a).

All of these data further suggest that the inheritance of KS is heterogenous since mutations in *KAL* are not found frequently and there have been no reports to date of the same mutation being identified in two unrelated individuals. However, there have been sufficient mutations reported to validate KALIG 1/ ADMLX as the gene responsible for XKS.

A very recent study however (Giancarlo Perenti et al. 1995) suggests that XKS can show

variable penetrance. This was demonstrated in three sibs who had the same partial deletion of KAL (only the first exon was present) but showed great variation in their clinical features. This would also suggest that KAL may be compensated for by some other gene product, as yet unidentified. Work described in this thesis has shed further light on the nature of mutations responsible for XKS and sporadic KS.

2.2.4 X-linked Kallmann's Syndrome as part of a contiguous gene defect.

Large terminal or interstitial deletions of the Xp22.3 region (Lee et al. 1993) have been described in patients affected by several monogenic diseases known as contiguous gene defects including: X-linked short stature, chondrodysplasia punctata, mental retardation, ichthyosis and KS (Petit et al. 1990; Ballabio et al. 1988; Anthony C.Casamassima et al. 1993; Lyle G Best et al. 1990). Deletion mapping of a panel of patients with one or more of these defects has enabled an order of these diseases to be established from telomere to centromere as follows: Short stature, chondrodysplasia punctata (holes in the bones),(Klink et al. 1994) mental retardation, X-linked ichthyosis due to steroid sulphatase deficiency (Ballabio et al. 1986; Andria et al. 1984; Ballabio et al. 1985) and XKS (Ballabio et al. 1989; Ballabio et al. 1987). These disorders can occur singly or together (Meindl et al. 1993). The gene responsible for ocular albinism is located more proximal (Zhang et al. 1993). Both contiguous and non contiguous deletions have been described.

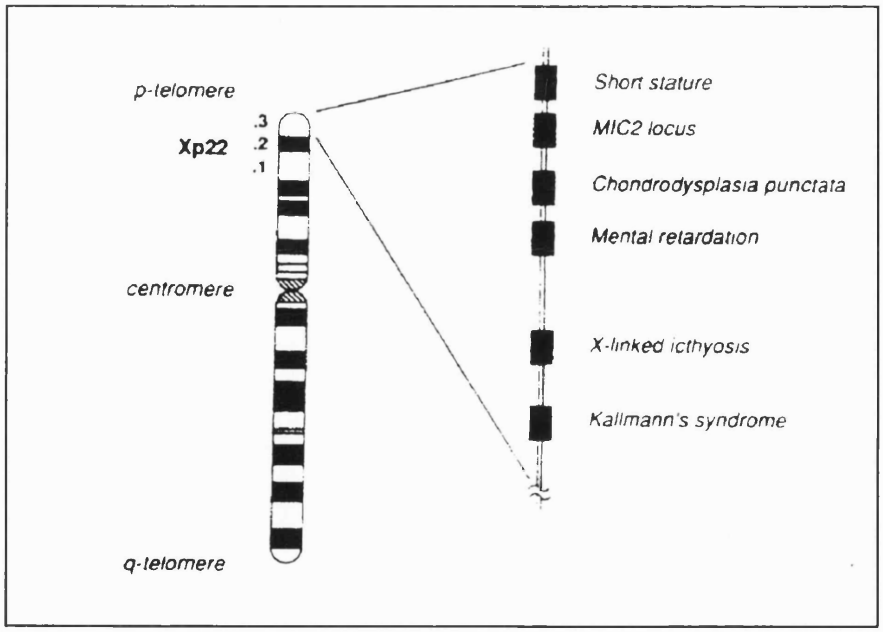


Fig.2.2 The X chromosome.

2.3 Homology of X and Y chromosomes.

The Xp22.3 region contains an area of strict homology with the Y chromosome since there is a single obligate X-Y crossover event during male meiosis, including the pseudoautosomal regions of the X and Y chromosomes. In Xp22.3, but outside of the pseudoautosomal region, a segment of the X chromosome shares extensive homology with Yq11. Two genes are now known to be involved in this sequence homology; the steroid sulphatase-*STS* and *KAL* genes. (Johnson et al. 1991) Both genes are functional on the X chromosome and escape X inactivation (Webster et al. 1978). Since mutations in *KAL* cannot be compensated for by the Y homologue, it can be hypothesised that the Y homologue is a non-functional pseudogene or insufficiently expressed.

During the search for the X chromosome locus, a YAC contig was constructed . These YACs contained Y homologous sequences and mapped to Yq11.2. The YACs were subcloned into lambda phage vector (del Castillo et al. 1992),and a library was screened with PCR amplified KAL cDNA segments. Each positive phage was subcloned and sequenced. By comparing with the KAL cDNA sequence, no phage containing Y sequences homologous to KAL exons 1,3,8 and 9 were found. Even under low stringency conditions no Y specific bands were detected for these exons when Southern blots were performed on genomic DNA from both normal males and females suggesting that they are absent in the Y homologue (Incerti et al. 1992).

When X and Y sequences were compared there was found to be a high degree of homology; 86.2%-98.3% for exons and 86.3%-99.1% for introns. It is noteworthy that five of the ten exons of the *STS* gene are also missing on the Y homologue. However, the similarity indices are high in both exon and intron sequences.

In order to investigate X and Y sequences divergence the chromosomal location of these genes was studied in other species (del Castillo et al. 1992); notably, the old world monkey (macaque), new world monkey (callimico) and prosimian. Genomic DNA was isolated from several pairs of each of these species and restricted with EcoR1 and blotted onto filters. The blots were then hybridised under low stringency to probes corresponding to exons 2, 4 and 10 of the KAL gene. In macaque and callimico, the comparison of the signal between male and female revealed a dosage difference corresponding to X-specific bands. Y specific bands

were detected in macaques but not in callimico. In the lemur there was no difference between male and female signals which would suggest that *KAL* could be either autosomal or pseudoautosomal. These results match the results previously found for *STS* in this taxonomic group. *STS* is pseudoautosomal in the mouse and other rodents and since *KAL* undergoes the same rearrangements as *STS* it has been hypothesised that *KAL* is pseudoautosomal in the mouse too but has yet to be identified.

2.4 Expression of *KAL* in the developing chick.

Evolutionary studies were performed on *KAL* in the form of "zoo blots" analysis using the human cDNA as a probe. Sequence conservation was found among several species including; monkey, cow, rabbit, sheep and chicken but not the mouse. Since the mouse could not be used to perform developmental studies, (as it does not have the gene) observations have been confined to chickens. Chicken has often been used to study development and perform functional studies. As the gestational period is short, their fast development makes them convenient experimentally. The human cDNA clone was used to screen a cDNA library of 10 day old chick embryos and a 666 bp clone was isolated. This clone was then used to screen further libraries of later stage chicks. The clones were combined to assemble a 4,604bp sequence which had a single open reading frame and a predicted protein product of 675 amino acids, highly homologous to the human protein, but five amino acids shorter (Legouis et al. 1993b).

In order to characterize the temporal expression of this gene during chick development, RT-PCR was performed together with RNase protection assays on chick embryos before olfactory bulbs develop ie stage 8 through to stage 35 when olfactory bulb development is complete. The RT-PCR showed the same result in all stages of development. The expected product was amplified in all cases. In situ hybridisation was then performed on olfactory placode, olfactory epithelium, olfactory nerves and olfactory bulbs using two non overlapping antisense ribo probes from the *KAL* gene (Legouis et al. 1993b). Hybridisation was detected in the neuroepithelium of the forebrain at the presumptive olfactory bulb region, at low levels which increased when the bulb differentiated. Expression was seen to be high in the outer layer of the neuroepithelium. This region contains the mitral cell layer in the chick. As the bulb continues to develop and reaches maturity, expression becomes concentrated in this layer. The mitral cell layer also showed expression in the adult chick. Expression of KAL was also seen in tissue types other than the olfactory bulb in the

developing chick notably in the brain in the ocular motor primordium. These are the cell bodies and neurons that will later innervate the muscles that control the eye. Expression was also seen in the cerebellum in Purkinje cells. Another area of expression is the ventrolateral part of the anterior forebrain, an area responsible for discrimination of colour intensity and pattern in birds. Other areas of the central nervous system that showed expression were the trigeminal motor nuclei and choroid plexus. Expression was also detected in the developing limb bud, but not in the tip of the bud .

These findings have been confirmed by a very similar study performed by Legouis et al (Legouis et al. 1993a), who screened a chicken genomic DNA library using probes from the human KAL gene. These were PCR probes corresponding to exons 3 to 5. Positive clones from this low stringency screening were cloned blunt ended into M13, and sequenced. There was found to be a high degree of sequence homology both for nucleotide and predicted amino acid sequence. In situ hybridisation was then performed and the main sites of expression found within the central nervous system. At stages 6 and 8 the expression was low and diffuse, and only detected in the germinal cells of the developing neural tubes. In the olfactory bulbs the expression was first detected at stage 8 and was stronger in the periphery than in the ependymal layer of cells. At stage 9 and 10, expression was detected at the mitral cell layer, the first neuronal layer to form. As the other layers of the bulb formed during development, the expression remained only in the mitral cell layer. The olfactory nerve and glomerular nerve layers did not show any expression.

Expression was seen in the neuroretina from stage 6. The pattern began as a weak diffuse one becoming far more specific by stage 8. In the cerebellum, expression was seen at stage 9 in the immature Purkinje cells. There was also some labelling in the spinal cord, essentially in the medial part of the grey matter.

Once this study was performed in the chick, homology of this gene with other birds was sought. Chick cDNA was used to screen a genomic DNA library from the total quail embryo and from the quail embryonic retina. The complete coding sequence for the quail was obtained by overlapping several clones. Chick and quail sequences are 95% homologous and are 73% and 72% respectively homologous with the human sequence (Lobie et al. 1993).

2.5 The predicted Kallmann protein.

As yet all that is known about the protein produced from the X-linked Kallmann gene is by prediction and deduction from the gene sequence. The protein has yet to be isolated by conventional tissue extraction. However analysis of the sequence data suggests that the protein has three domains (A, B and C) which are similar to other known human proteins. Domain A has a sequence characteristic of the four disulphide core domain shared by several protease inhibitors and neurophysins. Domains B and C represent part of the previously identified fibronectin type III domain found in most neural cell adhesion molecules (NCAM). This motif is often found to be repeated several times in such proteins. The predicted sequence begins with an uncharged hydrophobic region, characteristic of a signal peptide. The molecular weight of the predicted protein is 74 Kd and it has six potential glycosylation sites. The rest of the amino acids are hydrophilic with no evidence of a transmembrane domain for phosphatidyl inositol anchorage. The KAL protein is probably secreted, and homologies with other proteins suggest a role as a signal peptide ie. the N terminus enables the protein to be transported out of a cell. The protein may be involved in neuronal pathfinding and migration both by cell-cell interaction and cell-matrix interactions.

In the context of the clinical features of KS, the protein is probably involved in the migration of both neurons in the central nervous system and non-neuronal tissues. It has also been suggested that the protein is necessary for the maintenance of the olfactory bulb. Alternatively the KAL protein could be a growth factor responsible for the growth and maintenance of the olfactory bulb. KAL is likely to be one of a group of proteins involved in cell migration and maintenance of the olfactory bulb and there is a probable association with other molecules such as NCAM rather than independently. However, this is largely speculative at present.

Chapter 3

THE NORMAL DEVELOPMENT OF THE HUMAN GONADOTROPHIN RELEASING HORMONE AND OLFACTORY SYSTEMS.

The two main defects of Kallmann's syndrome are anosmia and hypogonadotrophic hypogonadism. The underlying pathogenesis of Kallmann's syndrome is best understood by reference to the developmental interrelationship between the olfactory and GnRH systems.

3.1 Gonadotrophin releasing hormone.

The neuropeptide gonadotrophin releasing hormone (GnRH) serves both as a hormone and a neurotransmitter. It has multiple roles in reproduction as well as behaviour. This hormone is also known as LHRH (luteinizing hormone releasing hormone)(Linda E.Muske, 1993). Reproduction involves many complex interactions between neural and endocrine mechanisms at different times and developmental stages. GnRH plays a very important role in initiating long term maturational events as well as short term endocrine and behavioural responses. The endocrine function of GnRH peptide regulates gonadotrophin release from the pituitary. It also acts directly on targets in the brain (Schwanzel-Fukuda and Pfaff, 1989; Sherwood et al. 1993).

3.1.1 Identification of GnRH

GnRH was isolated, characterized and synthesized in 1971 using porcine, ovine and bovine hypothalamic extracts (Cheung et al. 1991). The molecule was originally thought to exist in one form but has since been found to exist in seven different forms. Six of the forms of GnRH are distinct from mammalian GnRH and make up a family of decapeptides with at least 50% homology to one another. GnRH shows evolutionary conservation. Five of the 10 amino acids (residues 1,2,4,9 and 10) are conserved in all the forms identified so far.

3.1.2 Distribution of GnRH cells and their ontogeny

Distribution of GnRH has been studied in many species using immunohistochemistry. There is remarkable similarity across species in the distribution of these neurons, perhaps not surprising in view of other essential biological functions. GnRH occurs in the brain and other locations acting as a neurotransmitter, neuromodulator and local hormone. The first GnRH neurons were found in the caudal telencephalon and anterior diencephalon. Septo-preoptic neurons are the principal regulators of gonadotropin release in most vertebrates. The majority of GnRH axons within this region project to an area in or near the medium eminence suggesting that the main role is in the regulation of LH and FSH release.

GnRH immunoreactive neurons have been found in the peripheral part of the nervus terminalis before they are found in the brain (Schwanzel-Fukuda and Pfaff, 1989). The nervus terminalis extends centrally from the olfactory pit to the septal-preoptic region and GnRH neurons occur along its central and peripheral segments during embryogenesis. GnRH neurons differentiate from medial olfactory placode cells of the developing nose and migrate across the nasal septum and enter the forebrain with the nervus terminalis arching into the septal-preoptic area of the hypothalamus (El Amraoui and Dubois, 1993).

In mice, GnRH immunoreactive neurons are first detected in the epithelium of the medial olfactory pit at 11 post coitum. Using in situ hybridisation (S^{35} riboprobes) a GnRH transcript was localised initially in the olfactory pit, with no GnRH cells detected in the brain at this time. Cells hybridizing with a GnRH probe showed remarkably similar patterns of hybridization to the patterns previously seen using anti-GnRH antibodies (Wray et al. 1989). At days 12 and 13 post coitum, the neurons were seen migrating across the nasal septum towards the forebrain. Cells were detected adjacent to the olfactory pit and seen to be located on "tracks"extending from the olfactory pit to the junction of the nasal and telencephalic region. From days 16 to 20, an increase in the number of neurons was seen in the pre-optic area (Wray et al. 1989). H^3 thymidine autoradiography confirmed that the GnRH cells originate in the olfactory pit and not in the brain. A similar pattern was observed using in situ hybridisation and immunocytochemistry in the monkey (Ronnekleiv and Resko, 1990). In the developing monkey foetus, GnRH immunoreactive cells were found at day 36 of gestation within the nasal epithelium. By day 38 of gestation these cells have migrated into the brain along the dorsal surface of the olfactory bulb. By day 40 a greater number of GnRH neurons was seen in the brain and in the septal preoptic areas. A few cells were also found in the hypothalamus, showing a similar pattern of migration to that in developing mouse. It would appear that the GnRH neurons are the only neuroendocrine gene products to migrate from the olfactory pit.

3.1.3 NCAM and GnRH neuron migration

It is likely that both mechanical and chemical signals are included in the migration of GnRH cells. Cell adhesion molecules are also likely to participate. Studies using anti NCAM antisera, cytotactin, fibronectin, laminin, CTB proteoglycan as well as anti-GnRH antisera have been performed in order to determine the relationship between migration of GnRH and these different cell adhesion molecules (Schwanzel-Fukuda and Pfaff, 1991). NCAM is a large molecular weight molecule, found in the membranes of all nerve cells. It mediates cell-cell adhesion and is present on the cell body , the neurites, and on the tips of growing axons (Schwanzel-Fukuda et al. 1994). These experiments showed that NCAM was the only molecule of those tested to be reproducibly present in this migration, forming a scaffolding on either side of the midline, anchoring at the tip of the rostral forebrain to the epithelium of the olfactory placode. The GnRH neurons migrated out of the olfactory placode across the nasal septum and entered the forebrain alone and within NCAM immunoreactive fascicles. GnRH neuronal migration was never seen to be independent of NCAM. The NCAM immunoreactive fascicle of the vomeronasal and terminalis nerves did not grow directly into contact with the developing forebrain but aggregated into NCAM immunoreactive pioneer cells which then merged with the rostral tip of the forebrain. No NCAM immunoreactive neurons were observed in the forebrain before the bridge of NCAM immunoreactive cells and fibres were in place between the olfactory pit and the forebrain. Mesenchyme cells appear to be the source of NCAM which is not secreted by GnRH cells (Schwanzel-Fukuda et al. 1992).

The involvement of NCAM in this migration was proven by disrupting the action of NCAM in the developing mouse by injecting antiserum to NCAM onto the olfactory pit, in utero. At 11 days gestation fewer GnRH immunoreactive cell were found in the olfactory pit than in control mice. No migration of GnRH cells out of the epithelium of the olfactory pit occurred at this stage in the antibody treated mice, while a number of GnRH immunoreactive cells were seen in this region in the untreated control animals . However, the NCAM bridge seemed to be unaffected by the anti-NCAM injections. Fewer GnRH immunoreactive cells were detected in the brain at 12 days gestation than the control animals. In addition, the number of GnRH immunoreactive cells seen on the nasal septum or in the transverse crossing from the nasal cavity into the forebrain, or in any part of the forebrain, were greatly reduced compared to the numbers of these cells seen along the migrationary route in all categories of the control animals (Schwanzel-Fukuda et al. 1994).

Thus injecting anti-NCAM antibodies into the olfactory pit of 10 day old mice embryos causes the inhibition of GnRH neuronal migration into the nasal mesenchyme or forebrain.

Since there is no way of proving whether the antiserum was taken up outside the olfactory epithelium, the disruption of the migration of the GnRH cells into the brain may be secondary to the reduced number of GnRH cells originating in the epithelium of the olfactory pit. The results of this study suggest that cell adhesion molecules are essential for GnRH cell migration, and that a defect of GnRH migration may be the result of an interference with some property of adhesion.

3.1.4 GnRH neuronal migration in KS

Is the KAL protein also involved in the migration of olfactory axons and GnRH neurons? Evidence for this was sought from a 19 week old foetus which had a deletion of the terminal portion of the X chromosome and in which the migration of GnRH cells was totally arrested in the nasal area (Bick et al. 1992; Schwanzel-Fukuda et al. 1989). The brain and nasal region of this and three age and sex matched controls were examined using immunocytochemical methods in order to study GnRH tissue expression. The tissues were fixed in Bouin's solution, decalcified dehydrated and paraffin fixed. Sections were cut and unlabelled anti-GnRH antibodies were used followed by biotinilated secondary antibodies and an avidin-biotin horseradish peroxidase complex. The sections were then immersed in diaminobenzidine tetrahydrochloride (DBT).

In the normal brain, GnRH expressing cells were seen in, the medial septal nucleus, the medial preoptic area, the retrochiasmatic area, the anterior hypothalamus, above the anterior commissure, in the medial basal hypothalamus and in the premammillary and retromammillary areas. These cells were found singly or in pairs or in small clusters of three or four cells at most. A few GnRH cells were seen among the ganglion cells of the terminalis nerve in the nasal cavity and on the dorsal surface of the cribriform plate. Some GnRH immunoreactive fibres accompanied the branches of nerves of the nasal septum and throughout its course to the olfactory bulb.

In the Kallmann foetus, no GnRH expressing cells were found in the brain, including the preoptic area and the median eminence. Unlike the single cells observed in the normal tissue, dense clusters of immunoreactive cells were observed in the nose on either side of the midline and medial to the olfactory nerves. On the rostral part of the nasal cavity small clusters of immunoreactive cells were identified among bundles of nerve fibres, as well as on the nasal septum oriented perpendicular to the cribriform plate. The largest and most prominent GnRH nerve fascicle coursed upward through holes in the cribriform plate just caudal and lateral to the crista galli and ended in a dense plexus of GnRH immunoreactive cells and fibres on the dorsal surface of the cribriform plate below the forebrain. In this

foetus the olfactory bulbs and tracts were absent on both sides, and the olfactory, vomeronasal and terminal nerves never reached the brain.

This study of GnRH releasing neuronal migration in the Kallmann and normal foetus showed that there were no GnRH expressing cells in the brain of the KS foetus, in contrast to the control brains and that these neurons accumulated in the nose and dural layer of the meninges beneath the forebrain in KS. Further, axonal processes of the olfactory nerves and the vomeronasal and terminal nerves extended across the nasal septum but ended on the dorsal surface of the ethmoid bone never reaching the brain. The absence of GnRH neurons in the brain may be due in part to the absence of the projections of the terminal nerves in the brain. In this foetus the olfactory, terminal and vomeronasal nerves were all present as were GnRH immunoreactive cells. However, they did not reach their final destination, their migration being arrested before they reached the brain (Schwanzel-Fukuda et al. 1989).

Observations such as the above together with previous studies of GnRH migration in association with NCAM, imply that the terminal nerve together with NCAM provide a scaffolding over which the GnRH neurons migrate from the olfactory pit to the forebrain via the olfactory bulb during development. NCAM is important in providing mechanical support as well as messages for this migration to occur. KAL protein may complement NCAM in this migrationry pathway. Evidence for this can be obtained from the KS foetus which had all the necessary neurons present for migration but in whom the whole process came to a premature halt presumably due to absent Kallmann protein.

3.2 The Olfactory bulb.

Most mammals have a main and accessory olfactory system whose receptive elements, the primary sensory centres (the nose) and their connections to the brain, form separate pathways. The olfactory process takes place in four distinct stages, each stage involves a different cell type.

i) The receptor organ -the nose. ii) Glomerular layer iii) Plexiform layer iv) Primary olfactory cortex.

The olfactory receptor cells in the nasal mucosa receive signals from odorants and transmit that information to the main olfactory bulb in the brain which sends output fibres directly to the secondary centre in the primary olfactory cortex. Although the olfactory bulb serves as the first relay of the olfactory information pathway, it is not only a ganglion in which the olfactory pathway synaptically interchanges but a highly complex structure (Meng Inn Chuah and Farbman, 1995) The olfactory bulbs are paired oval evagination of the frontal wall of the telencephalon. In humans they are small and are located on the ventral surface of the frontal lobes.

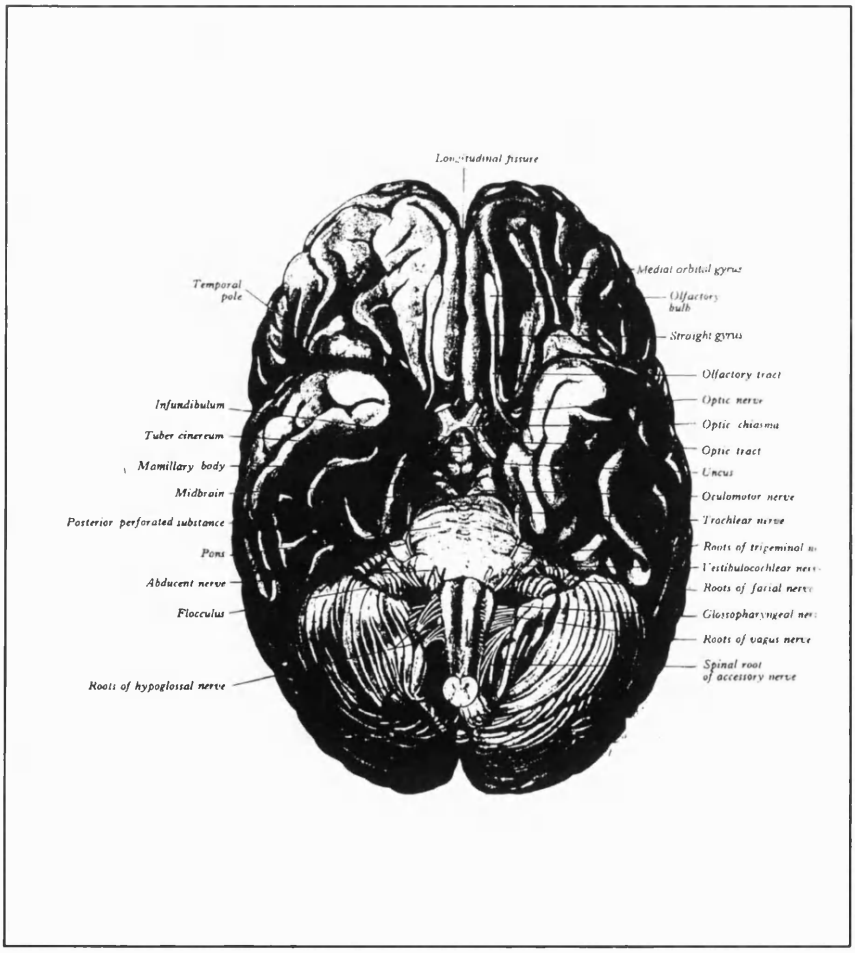


Fig.3.1 Basal aspects of the human brain

The bulbs are made up of six layers of cells: i) Olfactory nerve layer, ii) Glomerular layer, iii) External plexiform layer, iv) Mitral cell layer, v) Internal plexiform layer, vi)Granular cell layer.

These layers become less well organised and distinct with age. However, in the foetus they can be very clearly distinguished.

In the olfactory bulb, in the same way as any other brain centre, there is an input fibre (axons of olfactory receptor cells and axons from brain structures), an output neuron (mitral cell neurons and tufted cells) and an interneuron (periglomerular cells, short axon cells and axonless granule cells) which form a triad of neuronal elements.

Axons of human olfactory receptor cells form bundles with as many as 200 axons per bundle and they become packed into a single Schwann cell. The glomeruli are the most distinctive part of the olfactory bulb, making up the glomerular layer of the bulb. In this layer, receptor cell axons connect to dendrites of the output neurons.

The human olfactory receptor cells are constantly regenerated by new neurons. However, this does not disrupt the connection with glomeruli. As many as 2000 olfactory axons will connect to one glomerular cell. The glomerular layer is rich in neuronal cells. A dendrite of a periglomerular cell terminates within one or two glomerular cells in which terminals of olfactory axons and dendrites of mitral and tufted cells are mixed.

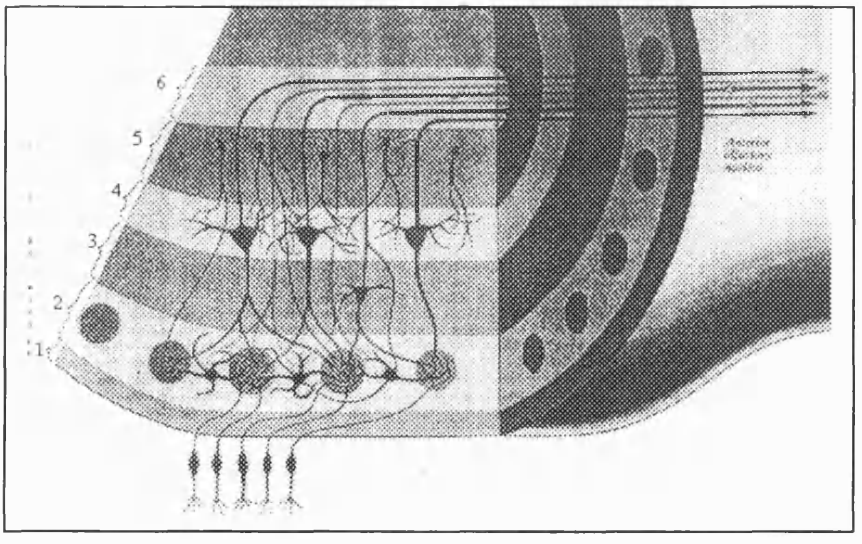


Fig 3.2 Scheme of the human olfactory bulb. Note the layers of cells: 1. Olfactory nerve layer, 2. Glomerular layer, 3. External plexiform layer, 4. Mitral cell layer, 5. Internal plexiform layer, 6.Granular cell layer.

Each olfactory bulb sends ipsilateral projections to the primary olfactory cortex. The mitral cell axons terminate throughout the whole olfactory cortex whereas the tufted cell projections are restricted to the more rostral part of the cortex.

In the olfactory system, unlike other systems in the central nervous system, the output signals from the primary system centre reach the specific cortical area directly without any processing from or through the thalamus.

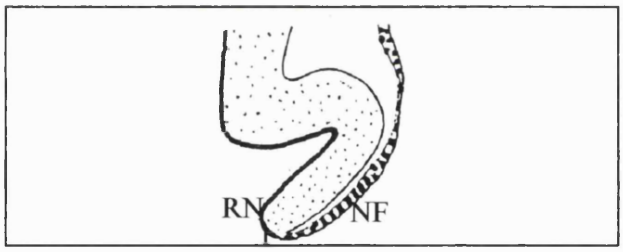
3.2.1 Developmental anatomy of the olfactory bulb.

There have been relatively few observations in the development of the olfactory bulb in humans. However, Humphrey in 1940 studied a series of 13 human foetuses between the age of 6.5 and 18.5 weeks of gestation (Humphrey, 1940). In 1973 O'Rahilly undertook a study on staged embryos from stage 11 (24 days) to stage 23 (57 days) and made the following observations (Meng Inn Chuah and Farbman, 1995).

Day 24-3.5 weeks

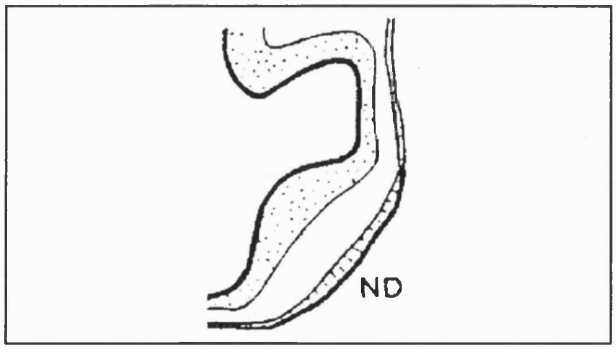
A small epiblastic thickening of cells is seen on each side of the rostral of the neuropore(RN). This primordium is a little less advanced than the lens.

NF-Nasal field



Day 26-3.7 weeks

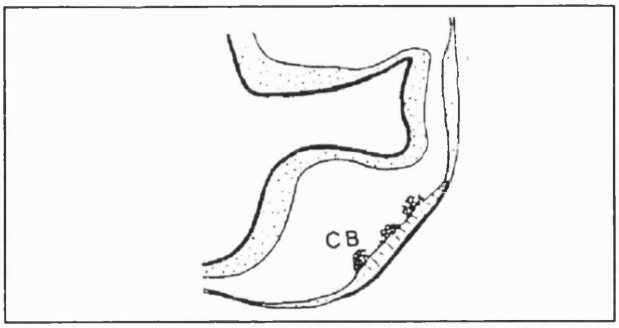
The nasal field and disc (ND) can be clearly seen at this stage.



1

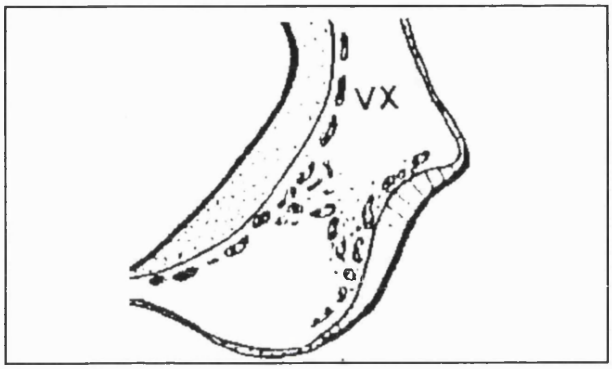
Day 28-4 weeks

An important vascular layer appears between the nasal and olfactory fields. The shape of the nasal field is still convex. Cellular buds (CB)can be seen on the basement membrane.



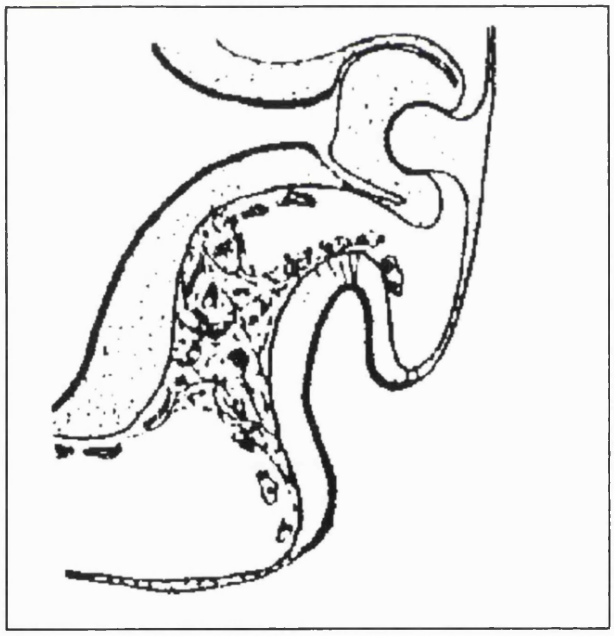
Day 32-4.5 weeks

The vascular layer spreads out giving rise to a strand between the nasal placode and the olfactory field. The number of cells in the placode increases and its shape may change from flat to concave.(VX vessels)



Day 37-5.2 weeks

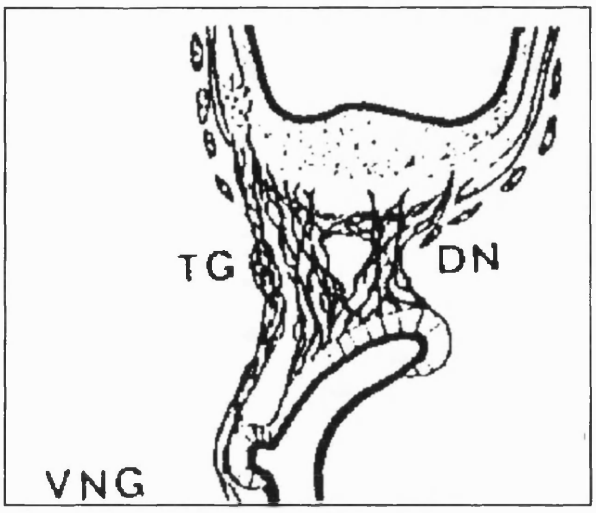
The nasal field shows a pronounced groove, the beginning of the vomeronasal groove. The olfactory nerve found between the olfactory field and the nasal groove increases in size. The presumptive bulb at this stage has a highly cellular inner ventricular layer and a very acellular outer layer. As time passes olfactory fibres penetrate into the ventricular layer of the bulb and the bulb increases in size considerably. The olfactory sulci can now be seen.



Day 44-6.2 weeks

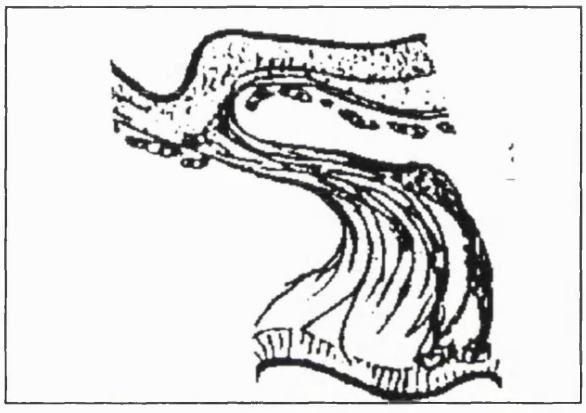
The olfactory nerve and bulb can now be seen in the same plane. The olfactory field is well outlined by two olfactory sulci. Terminal and vomeronasal structures are taking shape.

TG terminal ganglion VNG vomeronasal groove DN nasal disc



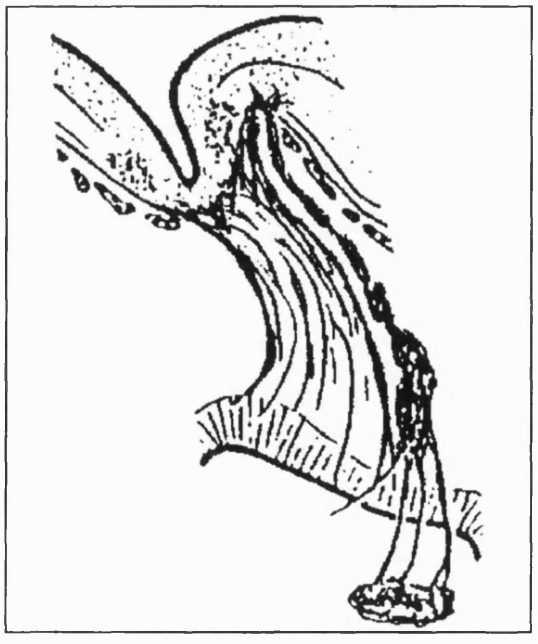
Day 48-7weeks

The origins of the olfactory nerve and terminal-vomeronasal complex are well differentiated. The terminal ganglion can also be seen.



Day 51-7.2 weeks

The olfactory nerve at this stage ends in the posteromedial, superior and apical areas of the olfactory bulb. When the olfactory nerve first appears and becomes attached to the brain there is no evidence of the bulb, only once the olfactory nerve has started to grow into the brain does the bulb begin to appear. The terminal nerve fibres endings are now isolated but the vomeronasal nerve fibres are still intermingled with the fibres of the olfactory fibres.



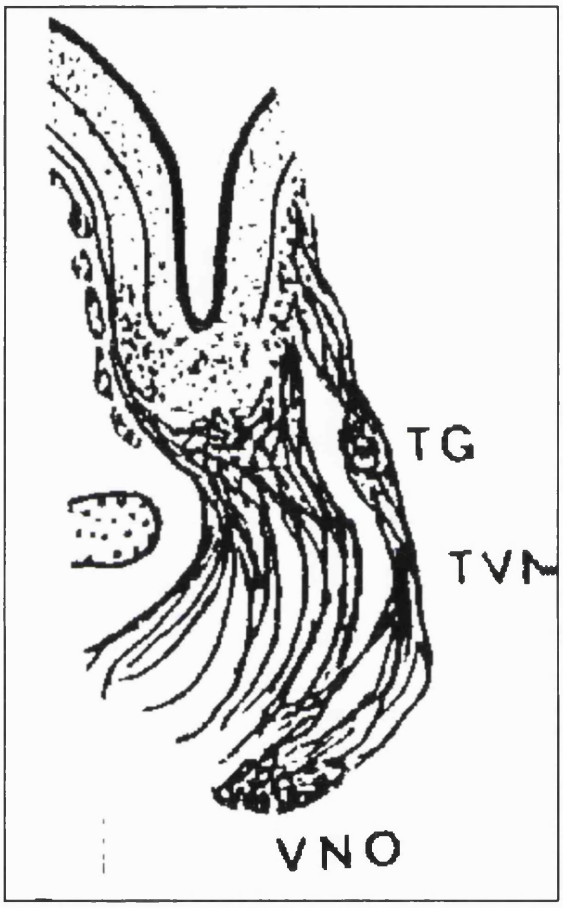
Day 52-7.3 weeks

The olfactory nerve is arranged in small threads in the area which will become the cribriform plate. The olfactory bulb can now be seen as a distinct protrusion of the wall of the telencephalon. At this stage the bulb is directed downwards and a little backwards. The terminal nerve can be distinctly seen, the right and left nerves approach one another and almost touch the median plane suggesting decussation is occurring.

Day 54-7.7 weeks

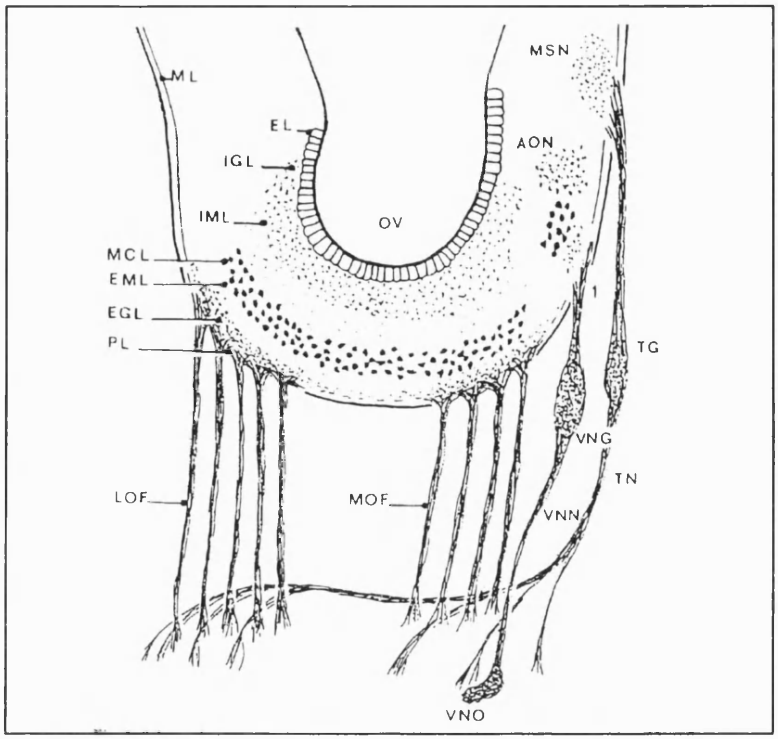
The bulb grows longer and larger and now looks more bulbar in shape.

(VNO-vomeronasal organ TVN-terminal vomeronasal nerve TG terminal ganglion).



Day 57-8 weeks

Within the olfactory and accessory olfactory formations, there are indications of layering occurring and a difference between these two structures can be demonstrated. In the olfactory bulb, numerous cells at the surface form a layer in intimate relation with the entering olfactory fila, which do not extend very deeply. Deeper in the developing bulb there is another cellular layer. These cells are separated from neuroblasts migrating out from the ependyma. The glomerular and granular layers differentiate in this cellular region and will become the granular (day 54) and mitral cell layers (day 52). The accessory olfactory bulb at this same stage shows some differences. It has fewer nerve fibres entering it and fewer cells.



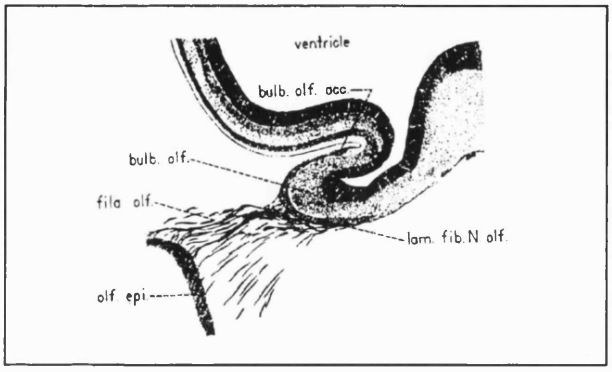
Day 57: (AON-anterior olfactory nucleus, EL-ependymal layer, EGL-external granular layer, EML-external molecular layer, IGL-internal granular layer, IML-internal molecular layer, LOF-lateral olfactory fila, MCL-mitral cell layer, ML-marginal layer, MOF-medial olfactory fila, MSN-medial septal nucleus, OV-olfactory ventricle, PL-plexiform layer, TG-terminal ganglion, TN-terminal nerve, VNG-vomeronasal ganglion, VNN-vomeronasal nerve, VNO-vomeronasal organ)

Day 66-9.5 weeks

The developing olfactory bulb now extends medially as well as ventrally. The accessory olfactory bulb now occupies its dorsomedial position and forms a slight superficial eminence. The internal granular, the external and internal molecular layers remain a similar size. The mitral cells become wider and more distinct. The mitral cells can be seen to form a distinct layer by day 60.

Day 77-11 weeks

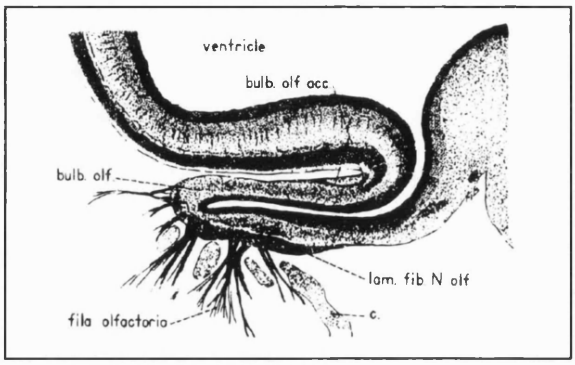
The olfactory bulb is directed posteriorly as well as downwards but will later extend caudo-medially. The most striking evidence of bulb formation at this stage is the



appearance of the mitral cell layer. The size of the cells is much the same but there is now less space between the layers of mitral cells and internal granular cells of the bulb. In the accessory bulb, the mitral cells are also more compact and clearly delimited. The accessory bulb does not have an external granular layer, unlike the olfactory bulb.

Day 91-13 weeks

The bulb turns forward and will now continue to grow in length and not in thickness.



Day 98-14 weeks

The granular cells of the bulb begin to clump in a very limited portion of the peripheral part of the internal granular layer and the onset of closure of the olfactory ventricle by the growth of cells into it from the ependyma.

By week 15 the fila olfactoria enter the bulb on all surfaces at its most frontal tip. Glomeruli are also beginning to form in the region were the fibres enter the bulb. Mitral cell dendrites can be traced into these developing glomeruli. Further regression of the accessory bulb has occurred. Finally at 18.5 weeks gestation, the bulb is as differentiated as the cells seen in the adult bulb. As in the adult the olfactory ventricle is no longer patent in the bulb. The olfactory fila surround the tip and enter it on all sides. There is marked infolding of the mitral cell layer in the ventral, lateral, and medial sectors of the bulb and the mitral cell layer on the dorsal side is more clearly defined. There are now fewer outward reaching mitral cells in the external molecular layer. The number of cells in the granular layer is now greatly increased. The accessory olfactory bulb is present. Although it is absent in adult man, it is present in the embryo and then disappears. The vomeronasal nerve however, is present in the embryo and remains in the adult.

By 19 weeks gestation the laminar pattern seen in the adult can already be seen in the foetus and the bulb is said to be fully formed (J.Bossy, 1980; Pearson, 1941b; Pearson, 1941a).

3.2.2 Structure, function and development, and of the vomeronasal organ.

The vomeronasal system is important in human reproduction and behaviour (David T.Moran et al. 1995). It is also called the accessory olfactory bulb. It is made up of three major components; i) the chemosensory organ in the nose, called the vomeronasal organ or organ of Jacobsen, ii) the vomeronasal nerve which connects the vomeronasal organ to the brain, iii) the region in the brain to which the accessory olfactory bulb connects. Until recently, the adult human vomeronasal organ was merely thought to be a vestigial structure. However, of late this theory has been challenged (Moran et al. 1991). The vomeronasal organ is a tube that is situated bilaterally on the anterior of the nasal septum beneath the surface of the nasal mucosa. The opening of the tube is the vomeronasal pit. The vomeronasal organ gives rise to a bundle of nerve fibres collectively called the vomeronasal nerve. This nerve connects to the accessory olfactory bulb in the brain. It is important at this stage to note that the vomeronasal nerve is separate from the olfactory nerve in the same way that the accessory olfactory bulb is separate from the olfactory bulb.

At 6.5 weeks gestation the vomeronasal organ (Jacobsen's nerve) is represented by a deep groove extending into the wall of the nasal septum. From the epithelium of this wall the fibres of the vomeronasal nerve extend forward to the forebrain vesicle. At this time the olfactory and accessory bulbs have three regions; ependymal, mantle and marginal. There is no sign of lamination of the olfactory and accessory bulb (Nakashima et al. 1985).

At 7 weeks gestation the groove forming the vomeronasal organ is less definite. Nerve fibres still pass from the medial wall of the developing organ to the forebrain. By 8 weeks the vomeronasal organ has deepened and elongated fully and appears as a closed off pocket, both the medial and lateral walls having a lining epithelium. Fascicles of the vomeronasal nerve pass to the rhinencephalon from both surfaces of the organ. Vomeronasal fibres enter the olfactory bulb just medial and slightly dorsal to the fila olfactoria.

At 8.5 weeks gestation the vomeronasal organ has elongated and deepened to appear as a saccular structure having a tube like connection to the nasal fossa. In each region, the wide ependyma and the adjacent area of neuroblasts proliferating from it are still present about the ventricle but towards the surface, changes have occurred.

By 9.5 weeks gestation, the vomeronasal organ no longer opens into the nasal fossa but is connected with it only by a solid cord of epithelial cells .According to original articles by week 10 of gestation, the "vomeronasal organ varies considerably and complete degeneration in early foetal life may ensue!" However the development of the vomeronasal organ is not in fact complete until week 20 of gestation. There have not been any recent studies on the development of the vomeronasal organ in human embryogenesis.

3.2.3 The Nervus Terminalis

The nervus terminalis or "terminalis nerve", as it is also called, is a cranial nerve of unknown function (Marlene Schwanzel-Fukuda and and Donald W.Pfaff, 1995; Pearson, 1941b; Pearson, 1941a). It is located rostral to the 12 cranial nerves. It shares a common developmental path with the vomeronasal nerve or organ, from the epithelium of the medial olfactory pit. The central process of the vomeronasal and terminal nerves ascend the nasal septum, course through the cribriform plate of the ethmoid bone medial to the olfactory nerves and enter the ventromedial surface of the forebrain just caudal to the olfactory bulb. The vomeronasal nerve ends at the accessory olfactory bulb and the terminalis ends at the brain (Schwanzel-Fukuda and Pfaff, 1991). The nervus terminalis has ganglia at nodal points. The intracranial part of the nerve lies along the ventromedial surface of the olfactory bulb. The nervus terminalis remains constant in its anatomy across species. It is even present in animals that do not have an olfactory and vomeronasal system (chickens and bats). The fact that it is present in the human adult implies a physiological role; it is not just a transient embryonic structure.

Since it has such a close association with the olfactory, vomeronasal and trigeminal nerves, it is difficult to isolate the function of the nervus terminalis with any degree of certainty. In addition, the fact that this nerve is seen in animals that do not have a vomeronasal or olfactory nerve suggests that the role is other than an olfactory one.

With the use of immunohistochemical techniques, it has been discovered that GnRH cells are contained in the nervus terminalis. These cells are necessary for the reproductive behaviour of humans. In fact, these GnRH producing and releasing cells originate in the nose- in the olfactory pit, in the same place as the terminal and vomeronasal nerves and they migrate to the brain along a pathway laid down by these nerves. This suggests that the nervus terminalis may be acting as a scaffold.

In man, the nervus terminalis is located lateral to the midline and medial to the vomeronasal and olfactory nerves and deeper in the mucosa covering the nasal septum. The nervus terminalis emerges through the cribriform plate of the ethmoid bone medial to the olfactory and vomeronasal nerves. It forms a plexus along the medial surface of the olfactory bulb in mammals and in humans it forms a plexus on either side of the crista galli. At this point the terminalis joins the vomeronasal nerve and runs to its terminus in the olfactory bulb.

In the developing human, the terminal nerve cannot be easily distinguished from the vomeronasal nerve. By the time the embryo is 17 mm in length, the terminal nerve is seen

as a broad band of cells stretching from the olfactory placode to the brain. By the time the foetus is 24mm long the fibres were seen in the forebrain.
In conclusion the terminal nerve is a superhighway for the migration of GnRH neurons from the nose to the brain. Any other role it may have has yet to be discovered.

3.3 GnRH and the olfactory systems in KS.

In KS, irrespective of the mode of transmission, both olfactory and GnRH systems are disrupted. The reason why two seemingly unrelated systems should be seen to break down together to give rise to one syndrome has become clear by studying the normal development of the two systems involved. The origins of the GnRH and olfactory neurons and the nature of their migrationry routes during development being so closely linked; this allows us to understand how these two features come together to give rise to KS.

What causes KS? It is now know that the anatomical correlations of KS are aplasia or hypoplasia of the olfactory bulbs and hypothalamic deficiency of GnRH. However, what causes these anomalies? Since the cloning of the gene, it has been possible to hypothesise that the protein produced by *KAL* is a cell adhesion molecule. It is thought that the KAL protein is necessary for the maintenance of the olfactory bulb and possibly necessary for its formation. This is likely since bulbs are seen in some KS patients but the fact that these bulbs are hypoplastic supports the role of the protein in the maintenance of the bulb. We know that the protein is not entirely responsible for the migration of the olfactory and GnRH neurones, since some migration occurs in the Kallmann foetus, but this is arrested. This suggests that the KAL protein is necessary only for later events possibly in the migration rostral to the cribriform plate of the olfactory and terminalis neuron, which later synapse with the forebrain. Failure of the olfactory neurones to synapse with the brain results in failure of bulb formation. Studies of the olfactory mucosa of patients with KS has shown a very fast turn over of the olfactory neurones, and these neurons fail to reach maturity. KAL protein may therefore be involved in maturation of the neurons as well as migration. Full characterization of XKS, must await bio-assay systems in which the intact functioning protein can be investigated in-vitro.

AIMS OF THIS THESIS

This thesis addresses three main areas of Kallmann's Syndrome.

1) The role of KAL protein in development: Monoclonal antibodies have been raised in mice to a short sequence of the KAL putative protein sequence and then used to screen human foetal tissues for protein expression.

2) Spatio temporal expression of *KAL* in the developing human: This has been investigated by performing *in situ* hybridisation using *KAL* as a ribo probe on normal human foetal tissue at different stages of gestation. Reverse transcriptase PCR was also performed on first trimester samples to identify areas of *KAL* expression.

3) The molecular genetics of KS: Patients with X-linked and sporadic KS have been screened for mutation of *KAL* using PCR, single stranded conformational polymorphism analysis and direct DNA sequencing in order to identify:

a) mutations in KAL

b) carriers

c) a mutation hot spot.

Chapter 4

A STUDY OF KAL PROTEIN EXPRESSION IN THE DEVELOPING HUMAN USING MONOCLONAL ANTIBODIES.

4.1 Introduction

Many genes have been isolated, their sequences elucidated and their expression mapped, generating information crucial to unravelling the molecular basis of disease. Understanding the role of a gene requires a knowledge of behaviour and function of its protein product in normal humans, either in the adult or during development.

Since the cloning of KAL in 1991, little research has focused on the investigation of the function of the encoded protein. Knowledge of the Kallmann protein has been derived from sequence data and computer models. As yet neither the whole protein nor fragments have been generated to enable functional studies to be undertaken. Nor has the protein been purified from human brain or olfactory bulb. In the absence of any purified KAL protein, an investigation of Kallmann protein expression in human tissues was undertaken in both foetal and adult using a protein sequence apparently unique to KAL based on computer derived sequence data. This oligopeptide sequence was synthesied and subsequently used for generation of antibodies. These antibodies were then be used to screen a range of foetal tissue for areas of protein expression.

4.1.2 Antibodies, structure and function.

Antibodies are a group of glycoproteins present in the serum and tissue fluids of all mammals. Their production is induced when the host's lymphoid system comes into contact with immunogenic foreign molecules-antigens. They bind specifically to the antigen which induced their formation. They are therefore an element of the adaptive immune system.

There are five distinct classes of immunoglobulin molecules recognized in higher mammals- IgG, IgA, IgM, IgD and IgE. These differ from each other in size, charge, amino acid and carbohydrates composition. The immunoglobulin molecules show heterogeneity even within the same class but have the same basic four chain structure. Each immunoglobulin molecule is bifunctional; one region of the molecule is concerned with binding to the antigen while the other mediates binding of the immunoglobulin to host tissues.

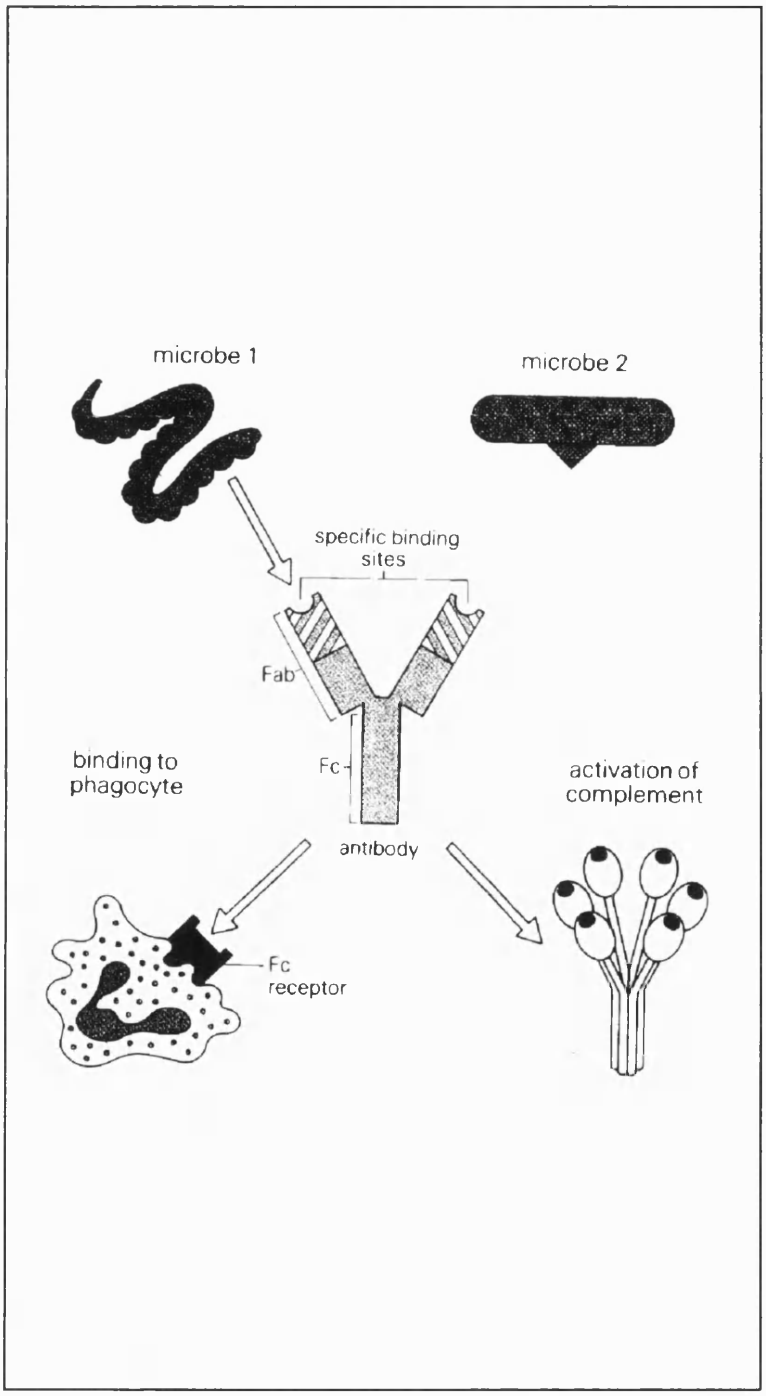


Fig. 4.1 An antibody. Note the antigen binding portion-Fab region to which the microorganism becomes attached and the Fc end which becomes attached to the host's tissue cells, particularly phagocytes.

4.1.3 Generation of Monoclonal Antibodies.

A monoclonal antibody is a single pure antibody produced in quantity by a cultured clone of a special cell type called B lymphocyte. Each normal B lymphocyte is capable of producing a single antibody. Animals, usually mice or rabbits are immunized with antigen. B lymphocyte producing antibodies recognize the antigen and are thereby stimulated. Once antibody response initiated, the spleen is removed and a cell suspension prepared. These cells are fused with myeloma cell lines by the addition of polyethylene glycol, promoting membrane fusion. Only a small number of the cells fuse successfully. The fusion mixture is then set up in culture medium containing hypoxyanthine aminopterin and thymidine (HAT). Spleen cells can grow in HAT whereas myeloma cells fail to grow and die. The spleen cells die within 1 to 2 weeks, so the only cells remaining are the fused immortal cells. Some of these fused cells will also have the antibody producing capacity of the spleen cells. Any wells containing growing cells are tested for antibody production using the enzyme linked immunoabsorbant assay-ELISA test and if positive the cultures cloned. This involves plating them out so that only one cell is in each well. This produces a clone of cells derived from a single progenitor, which is both immortal and produces a monoclonal antibody.
The advantage of using monoclonal antibodies over polyclonal antibodies is the increased specificity of the antibodies. The antibody will bind far more selectively to antigens in the tissue than polyclonal antibodies.

This initial approach was chosen in preference to isolation of KAL protein using *In vitro* expression systems; these are varied and have been shown to have different degrees of success. The difficulty of producing large enough amounts of peptide and then isolating, purifying and characterising them could have been an entire project in itself. Monoclonal antibodies to a part of the KAL protein were therefore generated and used to screen both adult and foetal tissues. Mice were chosen to be the source of antibodies for convenience, being cheap to keep and feed.

4.2 Methods and Materials

4.2.1 Selection of the antigen.

In order to stimulate *in vivo* production of an antibody, an antigen that will produce a immune response in the mouse is needed. The putative protein sequence of KAL was examined to find regions that had no homology to any other known proteins, in order to avoid potential cross reactivity. The protein sequence selected avoided sequences that were

too hydrophobic as these are known not to be immunogenic.

In general, small synthetic peptides used to generate antibodies in mice need to be conjugated to a carrier protein such as keyhole limpet haemocyanin in order to render them immunogenic when injected into the mouse. The peptide antigen usually represents a very small part of the total molecular weight of the antigen-carrier complex. Antibodies of interest generated will therefore only be a minor percentage of the total amount of antibodies produced. The process of conjugating peptide and carrier can be difficult and result in loss of significant amounts of the peptide. This can be expensive. Therefore an alternative to conjugating peptide to carrier is preferable. Conjugating peptide to itself is problematic in that the shape and structure of such a conjugate is difficult to predict, and it is also difficult to predict its immunogenicity. For all the above reason a multiple antigenic peptide (MAP) was chosen and used as the antigen (Sette et al. 1989; Tam, 1988; Posnett et al. 1988).

The KAL specific peptide antigen was synthesised on an automated peptide synthesizer RaininPS3 (Protein Technologies, Inc.) by a step wise solid phase procedure (Posnett D.N. et al. 1988). The polypeptide was cleaved from the resin and purified by reverse phase HPLC. An octameric version of the sequence was synthesised. This involved two steps:

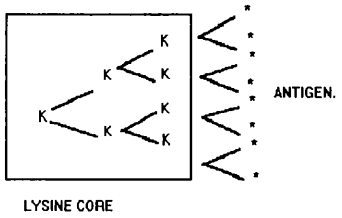
1) an octa branched core matrix composed of seven lysines and

2) eight copies of the KAL sequence were attached to the α amino groups of the terminal lysines.

The small lysine backbone represented a minor portion of the total octameric structure and was non-immunogenic. The exact amount of carrier/peptide was known as well as its structure.

The KAL sequence chosen corresponded to amino acid 389 to 404.(see page 31-37)

K V S L H F T S T H A T N N K E



Antigen Seq: V H A A H A K V S L H F T S T H A T N N K E *K K K*

Fig. 4.2 KAL Multiple antigenic peptide

It was decided to include a six amino acid sequence derived from ovalbumin (VHAAH, underlined) as this sequence has been found to increase immune responses to MAPs and other processed antigens in the mouse (Sette et al. 1989).

4.2.2 Inoculation of mice.

Experimental Protocol

1. 10µg of protein was required for each of the 10 mice inoculated (∴ 100mg of protein in 1 ml of saline.) Lyophilised protein was at a concentration of 100mg.

The antigen was prepared as follows:

To prepare 0.1mg/ml stock; 1mg of dry protein was added to 1ml of saline.

100µl of this stock was added to 900µl of saline to achieve a final concentration of 0.1mg/ml.

2. 1ml of complete Freunds adjuvant (CFA) (Sigma) (containing 1mg of tuberculosis, dried paraffin oil and mannide monoleate) was mixed with 1 ml of protein solution to form a thick emulsion. Failure of a drop of emulsion to dissolve in water indicated that the emulsion was sufficiently viscous.

3. The mice inoculated were female, B albino/C mice, 10-12 weeks of age, (white with pink eyes). 100µl of CFA/ protein mixture were injected subcutaneously twice ie. a total of 200µl was injected into each mouse.

4. After four weeks, the mice received booster injections. The protein was emulsified this

time with incomplete Freunds adjuvant (paraffin oil and mannide monoleate).

5. 1ml of protein (0.1mg/ml) was mixed with 1ml of incomplete Freunds adjuvant to form an emulsion as previously described and 0.3ml of the emulsion was injected into each mouse subcutaneously.

6. After a further two weeks, the mice were anaesthetized and a blood sample taken from the heart.

7. The blood sample was left at 37°C for 10 mins in order to allow clot formation.

8. Samples were then centrifuged in order to obtain the serum.

4.2.3 Serum dilutions for use in ELISA

1. 100µl of serum would be needed for each well of the microtitre plates in which the ELISA were to be performed. Each dilution was performed in duplicate.

A: 1 in 100 dilution ie. 2µl of serum in 200µl PBS

B: 1 in 1,000 dilution ie. 0.2µl of serum in 200µl PBS

C: 1 in 10,000 dilution ie.2µl of serum in 200µl⇨ 2µl of this was then added to 200µl of PBS ie. 1 in 100 dilution followed by a second 1 in 100 dilution.

2. Normal mouse serum was also prepared at the same time taken from a non- immunised mouse.

4.2.4 Enzyme linked immuno absorbent assay-ELISA

1. 4.8mg of dry protein (the antigen) was suspended in 2ml of PBS (2.4mg/ml). This stock was stored in 100µl aliquots (2.4µg/µl) at -20°C.

2. 100µl of stock was added to 12ml of PBS. 100µl of antigen was added to each well of a 96 well microtitre plate. This was left at RT for 1 hour. A control plate was prepared using PBS instead of antigen to coat the plate.

3. The contents of the plate was poured away and the plate vigorously shaken in order to ensure that the wells were empty.

4. The wells were washed twice with PBS.

5. Bovine Serum Albumin was prepared in PBS (1.5g BSA powder in 50ml PBS) to achieve a 3% solution. 150µl was added to each well plate and left at 4°C O/N. The BSA acts as a blocking agent preventing the antibody in the serum from binding non-specifically to the plastic, ie. once the antibody containing serum is added there is competition for binding to the antigen between the antibody and the BSA.

6. The following day the plates were washed twice with PBS.

7. 100µl of diluted serum was added to each well of the microtitre plate as follows.

	1	2	3	4	5	6
A	1A	2A	3A	4A	5A	6A
B	1A	2A	3A	4A	5A	6A
C	1B	2B	3B	4B	5B	6B
D	1B	2B	3B	4B	5B	6B
E	1C	2C	3C	4C	X	6C
F	1C	2C	3C	4C	X	6C
G	NMA	NMB	NMC	PBS	X	X
H	NMA	NMB	NMC	PBS	X	X

Table 4.1 ELISA to check for antibody production in mice

A=1 in 100 dilution B=1 in 1,000 dilution C=1 in 10,000 dilution. NM=normal mouse.

8. The plate was left at R/T for 1 hour and then was washed three times with PBS tween (a detergent-250µl of Tween BDH in 500ml of PBS) and a further three times with distilled water.

9. Anti- mouse peroxidase conjugate (Sigma) was then added. This is the secondary antibody which binds to mouse antibodies in the serum. Peroxidase conjugation enables visualization of positive reaction. The second antibody was diluted 1 in 500 ie. 20µl in 10ml of PBS. 100µl was added to each well and the plate was left for 30 mins.

10. The plate was washed three times with PBS tween and then three times with distilled water.

11. One tablet of OPD-O-phenylerediamine dihydrochloride (Sigma) was added to 4ml of 0.1M citrate phosphate buffer (pH5) and the volume made up to 40ml with distilled water.

12. When the OPD had dissolved, 8µl of 30% H_2O_2 was added. 100µl of this mixture was added to the plate and the wells assessed for colour changes. A minute after the wells were seen to go yellow, the reaction was stopped using 100µl of 4N HCl.

13. The quantity of antibody present in the serum was then measured by passing the micro titre plate through a plate reader at 490 nm.

4.2.5 Modified ELISA

Since BSA was included in the growth media, blocking the plates with BSA may have caused some cross reaction. Therefore plates were blocked with both BSA and FCS separately and a comparison between the two blocking agents made.

Experimental protocal

1. One vertical track of the microtitre plate was coated with BSA (ie. blocked) and one track was coated with foetal calf serum FCS and left O/N at 4°C.

2. BSA was included in the preparation of the secondary antibody in order to eliminate non-specific antibody interaction.

3. The tracks that had been blocked O/N were washed with PBS twice.

4. 100µl of each serum dilution for samples 6 and normal mouse serum were added to the wells and the plate was left to stand for one hour at RT.

5. The tracks were washed three times with PBS tween and then with water.

6. A 1 in 1000 dilution of the antimouse peroxidase conjugate (Sigma) was prepared. This was more dilute than the first experiment where the dilution was 1 in 500.

For track 1(blocked in FCS): 1µl of antimouse peroxidase conjugate in 1ml of 20% FCS

For track 2(blocked in BSA): 1µl of antimouse peroxidase conjugate in 1ml of 3%BSA

100µl of the appropriate antimouse peroxidase conjugate was loaded into wells. (Two wells on each track did not have any serum added to them and acted as blanks. They only had the blocking agent added and no antimouse peroxidase conjugate.)

7. The secondary antibody was left for 30 mins prior to washing three times in PBS tween and then water.

8. 100µl of the visualising mixture (containing 4ml of 0.1M citrate phosphate made up to 40 ml with water, 1 tablet of OPD and 8µl of 30% H_2O_2,) was then added. The reaction was allowed to proceed for a minute after a colour change to yellow was seen. It was then stopped with 100µl of 4N HCl.

9. The optical density-OD at 490nm was then measured.

4.2.6 Secondary immunisation of mice

15µg of antigen was injected intraperitoneally into two mice; nos 2 and 6.

These two mice were chosen as they showed the best response to the antigen by producing the highest antibody titre in the ELISA test.

4.2.7 Cell Fusions in order to generate hybridomas

1. Mouse no 6 was anaesthetized as previously described. 1ml of blood was taken from the heart as before (this is about 50% of the mouse's blood and leads to the demise of the animal).

2. The fur was swabbed down with alcohol, removed and the skin pulled back.

3. The peritoneal cavity was then snipped open. The spleen was removed in one piece. Henceforth all work was performed in a class II hood.

4. The spleen was placed in a mini petri dish with 2ml of sterile PBS. Using L shaped needles, the spleen cells were teased out from the thick elastic walled spleen in preference to macerating the tissue, as the wall is too fibrous.

5. Using a 21G needle, cells were drawn into a syringe and transferred to a universal container.

6. The petri dish was washed with 1ml of fresh PBS and added to the universal container. This was then filled with PBS (total volume 24ml) and centrifuged at 1500rpm for 5 min to pellet the cells.

7. The myeloma cells SP2/0 were removed from the CO_2 incubator and the flasks gently shaken in order to remove the cells from the sides of the flasks. The media containing cells were then transferred to universal containers and the volume made up to 24ml with fresh PBS.

8. These cells were centrifuged at 1500rpm for 5 mins to pellet the cells. Centrifuging the cells allows the removal of protein and albumin.

9. Both sets of cells were washed with PBS three times, and then re-suspended in 10ml of PBS.

10. A drop of the cell suspension was added to a haemocytometer for cell counting.

0.1mm deep x 1mm x 1mm x 10^4= cells per mm

splenocytes 10^8 in 10ml $\therefore$ 10^7 cells in 1ml

myeloma cells 4 x 10^7 in 10ml $\therefore$ 4 x 10^6 in 1ml.

The optimal ratio of cells is 1 myeloma cell to 10 spleen cells. In this case there are 2.5 splenocytes to 1 myeloma cells.

ie. 10^8 splenocytes: 1 x 10^7 myeloma cells would give the 1 to 10 ratio needed. Therefore only a quarter of the volume of the myeloma cell stock was used.

11. The two cell types were mixed and then decanted into a fresh universal tube in order to remove the fibrous tissue. The final volume was 15ml.

12. The cells were centrifuged at 2000 rpm for 5 mins in order to pellet the cells.

13. 50% polyethyleneglycol PEG was prepared with 10% DMSO in PBS. The PEG was equilibrated at 37°C and then 0.5ml was poured over the pellet of cells very slowly over a period of 1 min. This allows the cell membranes to coalesce instead of bouncing off one another. PEG assists fusion of the cells; this must be done very carefully to avoid cell rupture.

14. The universal flask containing the spleen and myeloma cell pellet was filled with media and the cell pellet carefully disrupted. Half the media was removed so that the pellet could be further disrupted and then the media replaced.

15. The cell containing media was pipetted out onto a 25 well plate (this is a plastic plate with 25 x 5ml wells used for growing small cell cultures), 0.5ml into each well. A total of 240 wells was prepared (ie. 10 plates) The plates were then left at 37°C in a CO_2 incubator O/N (this type of incubator maintains the pH of the growth media and prevents it from becoming alkaline).

NB. The growth media used was DMEM: Dulbeco's modified Eagles medium (Sigma), which contains glucose (100mg/L), Na pyruvate (100mg/L), Na Bicarbonate (100mg/L) and amino acids. Foetal calf serum -FCS was added to the medium at a concentration of 20%. It contains growth factors which will encourage growth of cells. Glutamine (2M) was also added to the DMEM (5mls of 200mM stock) as well as penicillin and streptomycin (50µg/ml).

16. Twenty four hours after cell fusion, hypoxanthine aminopterin thymidine-(HAT) was

added to 125mls of the DMEM at a concentration of 2%. This will kill any unfused myeloma cells. Splenocytes will die within 1 to 2 weeks after removal from the spleen if they have not fused. HAT acts to select the hybridomas and kill any other cells, the aminopterin inhibiting the main purine and pyrimidine pathways. 0.5mls HAT containing medium was added to each well in plates 1 to 5. Only cells in plates 6 to 10 received the HAT later. Once the HAT was added, the plates were returned to the incubator.

4.2.8 Screening of cell fusions

1. The modified ELISA designed above(4.2.5) was used to screen the cell fusions. The plates were coated with 2.4µg/µl KAL antigen solution for 1 hour and then blocked O/N with DMEM containing FCS, penicillin, glutamine and streptomycin.
2. The 48 well dishes were removed from the incubator where they had been left for two and a half weeks after the cell fusion was performed in order to look for cell growth. The orange colouration of the DMEM in the wells indicated the wells in which cell growth had occurred.
3. 0.1 ml of media from each positive well (ie. where cell growth had occurred, change of colour of DMEM from pink to orange) was removed and placed in a sterile bijou.
4. The wells, from which growth medium was removed for testing, were carefully marked on the plates, and 100µl of growth medium added to the microtitre plate. The ELISA was performed as above (4.2.5).
5. 1 in 1000 dilution of the original polyclonal serum form mouse 6 was included as a control.

4.2.9 Cloning of 2A1, 5C2 and 8C1.

Cloning is performed in order to obtain one antibody secreting cell per micro titre well, ie. a clone.

Cloning was performed in 20% FCS and HT. The aminopterin was omitted so that the hybridomas would revert to their own purine and pyrimidine pathways, encouraging better growth.

Experimental protocol

1. The cells in well (5C2) which gave the highest antibody titre in the ELISA, were agitated by drawing up and down a 1ml pipette repeatedly.
2. 0.1ml of the cell suspension was removed and placed in a bijou.
3. A drop of the cell suspension was placed on the haemocytometer:

20 cells were counted ie. there were 20 x 10^4 cells in 1ml

20 x 10^3 in 0.1ml, 500 cells were needed; 20 x 10^3=20,000 cells

20 fold dilution =1000, after a further 2 fold dilution = 500 ∴ 20 x 2 = 40 fold dilution required.

∴ 3.9 ml of media was added to the 0.1ml of cell suspension.

4. 0.1ml of the diluted cell suspension was taken and transferred to a fresh bijou.

5. 2.3mls of fresh media was added to this. 1.2mls of this was drawn up into the pipette and 0.1ml dispensed into each well of row 1 of a 96 well micro titre plate (there are 12 wells in each row of these plates).

6. 1.2mls of fresh media was added to the bijou containing the diluted cell containing media, in order to dilute the 500 cells by 50%.

7. 1.2 mls from this dilution was pipetted into the second row of the plate. This dilution and plating out was repeated until the plate was filled.

8. The above cloning was also performed on 8C1 and 2A1, as they also had high antibody titres when the ELISA was performed. Two sets of cloning plates were prepared for each sample.

9. All six samples found to be positive in this screening had back ups prepared by adding 0.1ml of cell containing media to 10ml of fresh media. (5C2, 2A1, 8C1, 2D2, 3D1 and 6D3)

10. All the wells not screened or found to be positive in this screen were fed 1ml of fresh media.

4.2.10 Ovalbumin control

Given that an ovalbumin hexapeptide sequence was included in the original antigen and that cell fusion had generated five antibody secreting hybridomas, it was necessary to ensure that the immune response was not to the ovalbumin.

Experimental protocol

1. Ovalbumin (Sigma) was prepared in the identical manner as the original KAL antigen ie., 2.4mgs/ml.

2. 100µl of the ovalbumin solution was used to coat a microtitre plate for one hour.

3. The plates were washed in PBS and then blocked with growth media for 90 mins.

4. The following controls were used, all of which had previously been positive in ELISA except for 8D4

	1	2
A	5C2	9A2
B	2A1	7C4
C	8C1	5B6
D	2D2	4B1
E	3D1	3D5
F	6D3	X
G	X	X
H	8D4	X

Table 4.2 ELISA using ovalbumin to block plate.

The samples included in this ELISA, had all been screened previously.

4.2.11 Cell fusion screening

The original fusion plated were inspected on a weekly basis for cell growth and antibody secretion (a change in colour of the growth medium). Wells in which a change was noted were screened using the ELISA (4.2.5) and the wells topped up with 1ml of fresh growth medium. Wells that gave a high antibody titre at ELISA (higher than 1.5) were cloned as 4.2.9. All screenings were performed in duplicate, one on a plate with KAL bound to it, the other with ovalbumin. 10ml back up cultures were prepared for each sample cloned (0.1ml of cell culture was added to 10ml of fresh growth media).

4.2.12 Expansion of plated clones.

The plates in which the cloned cells had been aliquoted were expanded. Wells where one cell was seen under the microscope, were expanded by removing the media in the well and transferring it to a fresh 24 well plate. 1ml of fresh DMEM containing HT and 20% FCS was then added to each new well. These expanded out clones were screened using the modified ELISA in order to ensure that they were still secreting antibodies. These cells were called clone +1, since they have been cloned and then expanded once. Many of these samples did not give a positive result in ELISA after expansion. This is not unexpected since even though there was one cell in the well, continued antibody secretion is not guaranteed. Continuous screening is therefore imperative. The positive clones were expanded on a further two occasions and after each expansion they were screened using the ELISA to ensure continued antibody secretion. Cells producing the highest antibody titre were chosen and grown in large cultures. The 10ml cultures were expanded into 20ml cultures and then further into 50ml cultures. Six cultures were grown up for each clone and once the cells had grown well they were centrifuged and cells frozen.

4.2.13 Storage of antibody producing cells.

The antibody secreting cells which were sub cloned through three stages, and on each ELISA screen found to be producing antibody, were grown in several 10ml, 20ml and 50ml cultures and cells observed for continued growth and growth medium added at regular intervals. After a few days, the flasks were gently shaken in order to lift the cells off the sides of the flasks and the media was transferred to sterile tubes and centrifuged at 1500rpm for 5 mins. The supernatant was transferred into a fresh container and sodium azide added (this prevents growth of any bacteria in the supernatant). The growth media containing antibody was then stored at 4°C.

The pelleted cells were resuspended in 0.5ml of 10% DMSO in DMEM medium. The

resuspended cells were transferred to cryo tubes and frozen at -70°C.

4.2.14 Classification of the antibody generated.

It was necessary to identify the antibody produced in order to choose the correct method of antibody purification. This was performed using a kit (Serotec).

Experimental protocol

1. A 1 : 50 dilution of the antibody supernatant was prepared ie. 20µl in 980µl PBS.
2. 30µl of diluted supernatant was transferred into each of eight wells of a 96 well microtitre dish.
3. The reagent bottles in the kit were each shaken in turn and 30µl was loaded on the microtitre plate as follows:
4. The plate was shaken on a plate shaker for 6 secs and then left at RT one hour.
5. Aggregation of the antibody with the reagent was used for classification purposes.

	7C4 A	7C4 B	5C2 A	5C2 B	2A1 A	2A1 B
A	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1
B	IgG2a	IgG2a	IgG2a	IgG2a	IgG2a	IgG2a
C	IgG2b	IgG2b	IgG2b	IgG2b	IgG2b	IgG2b
D	IgG3	IgG3	IgG3	IgG3	IgG3	IgG3
E	IgA	IgA	IgA	IgA	IgA	IgA
F	IgM	IgM	IgM	IgM	IgM	IgM
G	+ control	+ control	+ control	+ control	+ control	+ control
H	- control	- control	- control	- control	- control	- control

Table 4.3 Classification of antibodies generated to KAL using Serotec kit.

4.2.15 Purification of antibody using Protein A.

The glass column was packed with Sepharose 4A beads coated with protein A. This is a bacterial protein which binds to the IgFc portion of IgG antibodies.

Experimental protocol

1. 1.5g of Protein A Sepharose Cl 4B was rehydrated in PBS pH 7.45 in a flask. The Sepharose was allowed to swell for a few minutes.

2. The protein A was then transferred to a sintered glass filter. The filter chamber was filled with PBS and a vacuum was used to aspirate the PBS. The Protein A remained in the filter chamber. This was repeated several times until a litre of buffer had been used to wash the protein A. The aim was to wash any preservative off the Sepharose which would probably denature the protein that would run down the column.

3. The Protein A was then transferred to a 50ml glass tube and large volumes of PBS were washed through the tube in order to settle the Sepharose beads. The column was allowed to settle for a couple of hours and then stored at 4°C.

4. A UV metre was rinsed with 10ml of ethanol followed by 10ml of PBS before being attached to the column in such a way that the elute from the column would pass through the meter and into a collecting flask.

5.Running buffer (0.05M phosphate buffer with Na azide at pH 8.15) was washed through the column in order to rinse it out before it was used. The UV meter was zeroed.

6. A column volume of ammonium thiocyanate (3M NH_4SCN) was then passed through the column, removing any protein from the column.

7. Three column volumes of phosphate buffer were then run through the column to rinse off the thiocyanate.

8. A few crystals of Tris HCl (Sigma) were added to the supernatant of culture 7C4 in order to make it more alkaline. (pH 8.75)

9. The antibody supernatant was loaded onto the column and allowed to run through at the rate of one drop per second.

10. The OD at 280nm was measured as the supernatant ran through the column. Once all the supernatant had run through the column, one column volume of PBS was run through and the UV meter was zeroed again.

11. Once the supernatant had run through the column, 0.1 M citrate buffer (pH 3) was used to elute off the protein. As soon as the reading on the meter began to rise, indicating when the protein was being eluted off the column, the elute was collected and stored with Na azide at 4°C.

12. Phosphate buffer was then run through the column to rinse it out before it was clamped

off and stored at 4°C for future use.

13. The antibody eluted off the column was then dialysed against PBS using dialysis tubing that had been preboiled in water for 5 minutes.

14. The dialysis tube containing the sample was placed in a large flask containing two litres of PBS and placed on a magnetic stirrer.

15. Two hours later the buffer was changed and the flask was left at 4°C stirring O/N.

16. The following morning the tubes were removed and the contents transferred to concentrating (centriprep) columns in order to concentrate the antibody containing solution.

17. The tubes were centrifuged at 4200 rpm for 20 minutes at 4°C.

18. The final antibody solution was removed and the concentration measured at 280nm

4.2.16 Use of ELISA to check purified antibody

The Protein A purified antibody and unpurified antibodies were tested in an ELISA (4.2.5) to determine any difference in the concentration of the antibodies and their ability to bind to the antigen.

4.3 RESULTS

4.3.1 Enzyme linked immunoabsorbant assay- ELISA

The antigen (KAL MAP) was used to coat a 96 well microtitre plate. Small quantities of antigen become absorbed in the plastic and free antigen washed away. At the same time control plates were prepared with no antigen coating the plate, only PBS. The plate was then blocked with excess of the irrelevant protein BSA to prevent any subsequent non-specific binding of proteins. The test antibody in the diluted mouse serum was added and left to bind to antigen. Any unbound protein was washed away. The antibody antigen binding was detected by adding a second antibody conjugated with horseradish peroxidase. This was an anti mouse antibody which will only bind to the test antibody present in the mouse serum. Any free ligand was washed away and the bound ligand was visualised by the addition of a chromogen-a colourless substrate which in the presence of enzyme portion of the ligand, produces a coloured end product.

In this test, the amount of antibody in the mouse serum or growth media was measured by assessing the amount of coloured end product by optical density scanning of the microtitre plate in which the reaction was performed. All readings greater than 0.5 were suggestive of the presence of antibodies in the serum.

	1	2	3	4	5	6	7
A	0.725	0.559	0.253	0.379	0.267	0.560	-0.488B
B	0.303	0.265	0.226	0.235	0.115	0.656	-0.488B
C	0.500	0.336	0.290	0.338	0.192	0.841	-0.488B
D	0.314	-0.006	0.173	0.213	0.129	0.807	-0.488B
E	0.083	-0.076	0.293	-0.045	0.088B	0.453	-0.488B
F	0.129	-0.046	0.162	0.051	0.081B	0.342	-0.488B
G	0.068N	-0.109N	0.122N	0.037P	0.089B	0.020B	-0.488B
H	0.197N	0.066N	0.080N	0.068P	0.093B	0.020B	-0.488B

N= normal mouse P= PBS B=blank

Table 4.4 Results of ELISA to measure antibody response in mice to KAL MAP.

i) The duplicates performed in this experiment did not appear to be very similar.

ii) The normal mouse serums all had very low readings, suggesting that the immunised mice have produced an immune response to the KAL antigen.

iii) The PBS (G4 and H4) should have read 0 or at least lower than they were, since they only had antigen, BSA and second antibody in them. There could have been a cross reaction between the BSA and the antibody and therefore by using BSA when preparing the secondary antibody this cross reaction can be eliminated. A second method of eliminating the cross reaction with BSA would be to block the plate with FCS-foetal calf serum rather than BSA.

4.3.2 Modified ELISA

It was necessary to modify the ELISA for two reasons.

1) To eliminate the possible cross reaction with BSA.

2) To determine the origin of variation between the duplicates seen in the first test.

	FCS	BSA	BLANK
A	1.477	0.990	-0.143
B	1.552	1.140	-0.143
C	0.182	0.125	-0.143
D	0.176	0.132	-0.143
E	-0.099	-0.058	-0.143
F	-0.048	-0.066	-0.143
G	-0.009	-0.096	-0.143
H	-0.099	-0.099	-0.143

Table 4.5: Results of modified ELISA

i)These results show the anticipated very close readings between the duplicates.

ii)The normal mouse serums gave very low readings again suggesting no KAL antibodies in the normal mouse.

iii)The two sets of controls, the PBS and the PBS with no secondary antibody are excellent ie. they are zero.

iv) These results suggested that the mice had produced an immune response to the antigen injected and that booster injections of antigen could be administered.

4.3.3 Screening of cell fusions

There were three samples that gave optical density readings greater than 1.0. This was suggestive of a high antibody titre, were therefore cloned.

In each ELISA screen of fused cells, and then the later cloned cells, both pre-immune serum and PBS blanks were included as controls.

4.3.4 Ovalbumin control

Since a six amino acid sequence from ovalbumin was included in the sequence of the MAP it was necessary to ensure that the antibodies produced were in fact to KAL and not to ovalbumin. This was tested by coating a 96 well microtitre plate with ovalbumin instead of KAL antigen and then performing the same ELISA tests previously performed with the KAL antigen and antibody secreting hybridoma growth media.

All the optical density reading were zero, confirming that the antibody response seen in the mice was in fact due to KAL and not to the ovalbumin.

4.3.5 Expansions of clone plates.

Each of the samples that tested positive from the original fusion was cloned. After two weeks, the cloned cells were observed under the microscope to identify wells where only one cell could be seen. These were expanded out ie. transferred to new microtitre plates with fresh growth media, to ensure that only one antibody secreting cell was present in each well. This expanding out of the clones was performed three times for each clone. After each expansion, the clones were screened using the ELISA (4.2.5) to ensure that the cell was continuing to secret antibody. The final number of antibody secreting clones was three. These were given code numbers which corresponded to their original location in the fusion wells.

4.3.6 Classification of the antibody generated

Using a kit (Serotec), the antibodies could be classified relying on a system whereby the antibody would react and form an aggregate or a cloudy clump with the correct reagent. Both the antibody clones tested were found to be IgG2a.

4.3.7 Purification of antibody using Protein A column.

The antibody in the cell culture supernatant was purified in a protein A column in order to isolate the antibody from the other proteins in the supernatant ie. foetal calf serum.

1. The concentration of the eluted 7C4 when OD was measured at 280nm was calculated as follows:

0.018(OD @ 280nm) x 0.7 (constant) = 0.0126mg/ml

25ml was eluted ∴ 0.0126 x 25 = 0.315mg

yield = 0.315 ÷ 45ml (total supernatant) = 7µg/ml of antibody.

2. The concentration of the eluted 2A1 antibody when eluted from column and optical density was measured at 280nm:

0.03 x 0.7 (constant) = 0.021mg/ml

45ml was eluted ∴ 0.021 x 45 = 0.945mg

yield = 0.945 ÷ 45ml (total supernatant volume) = 2µg/ml of antibody.

3. After purification of antibody through the column and dialysing it the concentration was measured after concentrating the antibody solution.

OD x 0.7 = concentration.

Sample	OD (280nm)	conc (mg/ml)
2A1a	0.159	0.1
2A1b	0.149	0.1
2A1c	0.344	0.24
2A1d	0.294	0.21
7C4a	0.133	0.08
7C4b	0.107	0.07
7C4c	0.260	0.1

4.3.8 Use of ELISA to check purified antibody

These results (OD readings of 1.149 for purified antibody and 1.179 for the same clone unpurified) suggest that there is no difference between the purified and unpurified anti body in their reaction with the antigen in the ELISA test. The only way of identifying a potential functional difference is to investigate the two sets of antibody out on tissue sections (see Chapter 5). Further confirmation that there is no cross reactivity with ovalbumin was also achieved.

4.4 Conclusion

The results showed that IGg2a class monoclonal antibodies to a small region of the KAL putative protein have been successfully generated in mice. The next step was to test these antibodies against different panels of tissue(Chp 5) in order to:

a) determine their ability to bind with KAL protein expressed in various tissues ie. in vivo

b) to map areas of KAL protein expression by binding of the KAL antibodies to KAL protein in tissues.

It was evident from all the ELISA results that the antigen had caused an immune response in the mouse, and that antibodies had been produced. It was possible to confirm that the antibodies did not crossreact with the ovalbumin included in the MAP.

Production of high affinity antibodies against a particular synthetic peptide epitope does not guarantee their absolute specificity against that epitope. It is but one of a series of steps needed in fully validate the antibodies produced. Full validation of an antibody depends upon verification of its binding to the KAL peptide. When the above studies were conducted, it had not been possible to extract and purify X-linked Kallmann peptide from either foetal or adult tissue. At the time of writing this thesis, there have been no published reports of any generation of recombinant intact X KAL protein and therefore it has not been possible to establish *in vivo* specificity of the antibodies generated.

Chapter 5

IMMUNOHISTOCHEMICAL ANALYSIS OF HUMAN FOETAL MATERIAL USING KAL MONOCLONAL ANTIBODY.

5.1 Introduction

The antibodies generated to a portion of the putative KAL sequence were generated in order to perform immunohistochemistry on human foetal tissue, thereby identifying areas of KAL protein expression in developing human tissues.

The antibodies generated were monoclonal, highly specific antibodies and the following studies were performed on fresh human tissues.

5.2 Methods and Materials

5.2.1 Tissue preparation-frozen sections

1. Ethical approval was obtained from the Royal Free Hospital Ethics Committee to collect human foetal material for this study.

2. The tissue obtained from first trimester termination of pregnancies was frozen in liquid nitrogen and stored at -70°C until needed.

3. The tissue was mounted on a chuck using O.C.T. mounting gel (this supports the tissue and seals it onto the chuck). Sections were cut on a cryostat, with a chamber temperature of -25°C.

4.The thickness of the sections were 10μm. The tissue sections were layered onto frosted glass slides, air dried and then stored at -20°C.

5.2.2 Tissue preparation-paraffin fixed sections

1. A comparison was performed between frozen tissue and paraffin fixed tissue sections in order to ascertain the type of fixation binding the antibody better. Tissue was fixed in formalin for 24 hours.

2. The tissue was dehydrated by washing in increasing concentrations of alcohol (70%, 90%, 95%, 100% ethanol.) Tissue was then subjected to three washes of 100% IMS, two washes in xylene and two soaks in paraffin wax at 58°C. The block of tissue coated in wax was then ready for sectioning.

3. Sections were cut on a microtome. A block of ice was placed on the tissue for a few

minutes before the tissue was cut since the best tissue sections are achieved when the tissue is cold.

4. The ribbons of tissue were allowed to float in a heated water bath. Sections were separated and tissue layered onto slides by placing the slide under the floating tissue in the water bath.

5.2.3 Slide preparation prior to staining.

1. Paraffin mounted tissue had to be de-waxed prior to staining and then rehydrated. Slides were soaked in Histoclear (Fissons) for 10 mins, twice, and then transferred to 100% ethanol for a few seconds.

2. Cryostat cut sections were removed from the freezer and left to air dry for 10 mins before use. Slides were then soaked in acetone for 15 mins in order to fix the tissue before staining.

3. All slides were then soaked in methanol and 0.3% H_2O_2 for 15 minutes (3ml of 30% H_2O_2 in 250ml of methanol). This step reduced the amount of endogenous peroxidase in the tissue, particularly in the kidney.

4. Slides were washed once in water before the staining.

5.2.4 Immunostaining.

This was performed using the vector stain kit. (Vector)

1. Slides were blocked with normal horse serum (20µg/ml-6 drops in 10ml PBS) for 20 mins.

2. The primary antibody (either 7C4 or 2A1) was layered onto the slide so that the whole tissue section was covered. Anti carcinoembryonic antigen (CEA) (Atta M Abbasi et al. 1992) was used as a positive control. The negative control slides were not exposed to the primary antibody and were instead washed and soaked in PBS. Slides were allowed to incubate for 45 mins.

3. Slides were washed in PBS three times over a 10 min period.

4. The secondary antibody was layered on the slides (all slides) and left for 1 hour. This second antibody was a biotinylated anti mouse antibody (BDH).

5. Slides were washed in PBS three times over a 10 min period.

6. The avidin DH (2 drops) and biotinylated horseradish peroxidase H (2 drops) were added as a mixture to 10ml of PBS and left on the slides for 1 hour.

7. The slides were washed again in PBS before visualising under a microscope.

8. Diaminobenzadine tetrahydrochloride (DAB) (Vector) was weighed out in the fume cupboard and dissolved in PBS and filtered before use (final concentration 1mg/ml). 0.3%

H_2O_2 was added just prior to use (1ml of peroxide for 1ml of DAB). The DAB mixture was layered onto the slides and left for exactly 5 minutes before washing with copious amounts of tap water.

9. The slides were then counterstained using haematoxylin for 1 min only. Slides were then washed in running tap water for a few minutes before being dehydrated and mounted.

5.3 RESULTS

5.3.1 First screen using strongest pre-clone ELISA positive.

The growth media in which the fused hybridomas were growing were tested for the presence of antibody using an ELISA (4.2.5) two weeks after the cell fusion was performed. The samples that gave highest antibody titres were used to screen a set of different tissues.

7C4: COLON: weak positive on laminal and glandular surface ie. internal surface but not on the external surface.
PITUITARY: negative
OLFACTORY BULB: negative
FOETAL HAND: positive in neural tube cells, small nuclei and large cytoplasm. This was a very specific reaction. Bone and connective tissue were all negative.

9A2: COLON: strong binding on internal surface and weak binding to stroma and connective tissue.
PITUITARY: positive staining
OLFACTORY BULB: weak binding
FOETAL HAND: some binding in connective tissue next to the bone, however not in bone.

6A4: NEGATIVE in all tissues. Even though this was positive in the ELISA, it was not positive now because ELISA is 100 times more sensitive.

2A1: COLON: internal surface like 7C4, small amount of staining in connective tissue.
OLFACTORY BULB: positive
FOETAL HAND: like 7C4, more intense.

8A1: COLON: weak staining
FOETAL HAND: weakly positive

5C2: COLON: strongest antibody binding
FOETAL HAND: very weak signal

5.3.2 Screen using both cryostat and paraffin fixed sections.

Paraffin fixed sections when stained with antibody showed some staining. However, when the cryostat sections were stained, better staining was observed and the tissue also appeared well preserved. It was decided therefore to use only frozen tissue sections. Using frozen tissue sections proved easier than having to collect fresh paraffin fixed tissue.

5.3.3 Screen using both purified and unpurified monoclonal antibodies

Using the purified antibodies did not result in any staining of tissue sections, probably due to the low concentration of antibodies. Alternatively part of the purification process may have changed or damaged the antibody eg. use of citrate buffer.

The unpurified antibodies, however, gave very strong signal, consistent with previous results. Staining was seen in the intercostal muscle cells (Figs 5.2 and 5.3). Staining was also seen in the neuroretina of the eye (Figs 5.4 to 5.6).

5.3.4 Screen on human adult tissue using unpurified 7C4

Antibody staining was seen in the following tissue types.

Uterus-smooth muscle cells, blood vessels and connective tissue.

Cervix-muscle, basal and squamous epithelium and cervical glands.

Breast-ductal glands

Colon -connective tissue, stroma, muscle cells and lumina glands.

Although interesting, the widespread distribution of antibody binding suggested lack of antibody specificity. Antibody was observed to bind to a large variety of tissue types in adult, particularly to muscle cells and connective tissue. However, this pattern was unlike the antibody binding seen in foetus which was confined to muscle cells.

5.3.5 Screen of ten week old human foetal material using unpurified 7C4 and 2A1

Striated muscle cells in the muscles of the fingers rib cage and muscle cells of the spine (Figs.5.1, 5.2 & 5.3) showed very strong antibody binding. Strong antibody binding was also seen in the foetal eye, in particular in the neuroretina (Figs. 5.4 to 5.6).

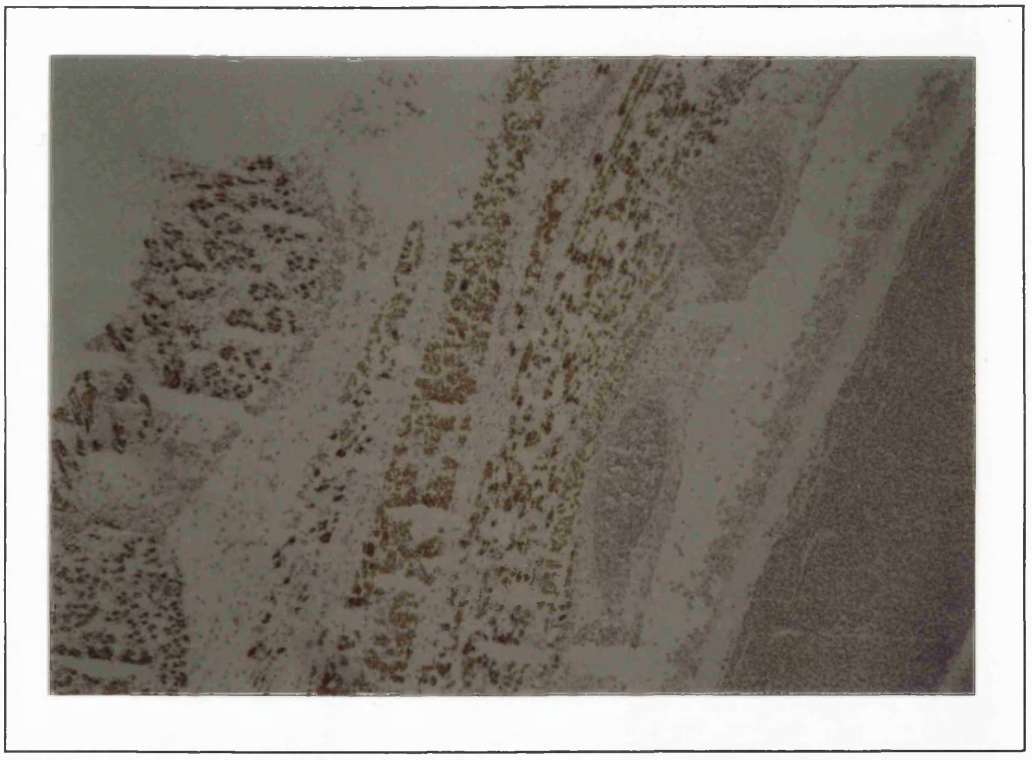


Fig. 5.1 Spine and ribcage of 10 week old human foetus. (x100)(Positive antibody staining is seen as brown.)

Fig. 5.2 Spine and ribcage of 10 week old human foetus. (x100)(Positive antibody staining is seen as brown.)

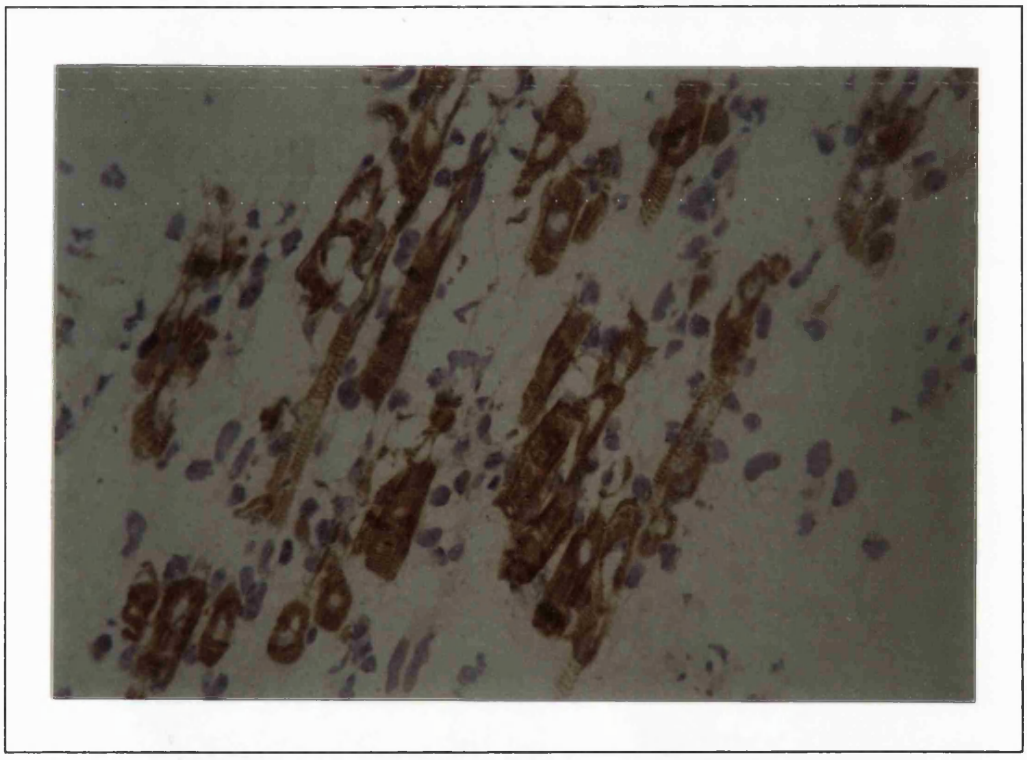


Fig 5.3 Positive antibody staining in primitive myoblasts surrounding the spine and ribcage of 10 week old human foetus. (x400)

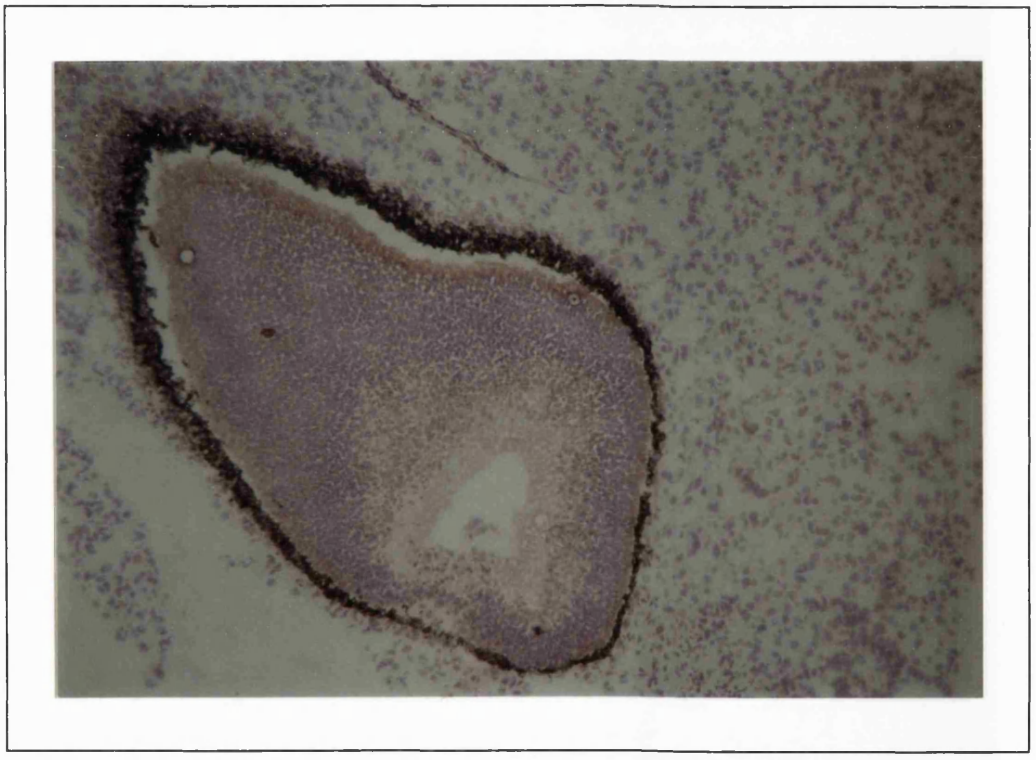


Fig. 5.4 Positive antibody staining in human foetal eye (10weeks). P-pigmented retina (x200)

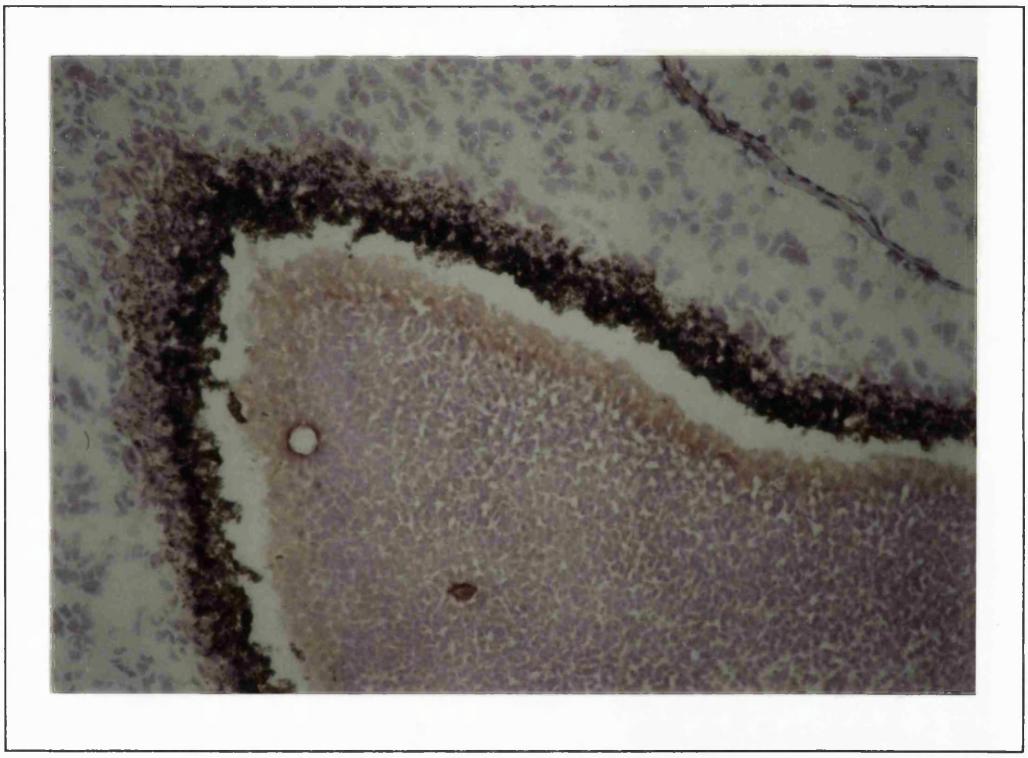


Fig. 5.5 Positive antibody staining in human foetal eye (10weeks). nr-neuroretina (x400)

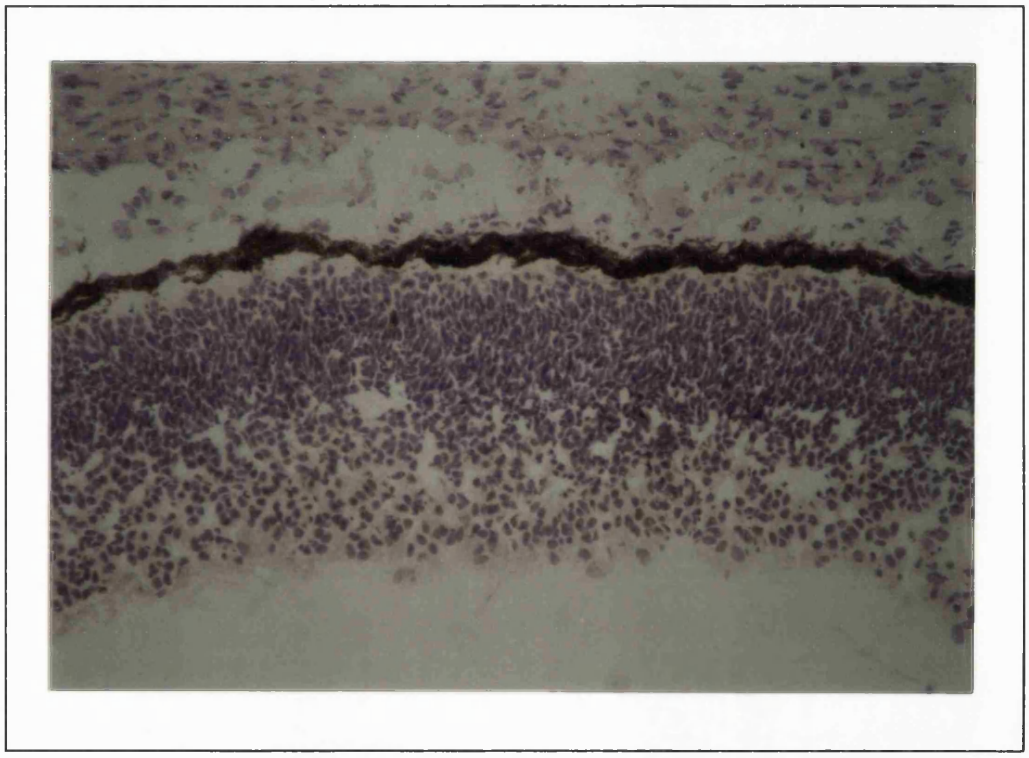


Fig. 5.6 Negative control ie. no antibody (x400)

5.3.6 Screen using 2A1 and 7C4 on Kallmann foetus

Tissue sections from the head of a 19 week old foetus with XKS and three age and sex matched controls were examined with the two monoclonal antibodies generated. This experiment was kindly performed by Prof Marlene Schwanzel Fukuda at the Rockerfeller Institute, New York. Staining was seen in brain cells in the forebrain and diencephalon. Some staining was seen in the retina and connective tissue near the eye. Strong staining was seen in the trigeminal ganglia cells and muscle. Unfortunately, the same staining patterns were observed in the normal controls suggesting and confirming lack of specificity of KAL. In some slides, staining was seen in areas of the brain of the Kallmann foetus which were not observed in the normal foetuses, again suggesting lack of specificity of the antibody.

5.4 Discussion

Using the monoclonal antibodies generated against a short sequence of KAL has given some unexpected results and at the same time has highlighted the difficulties involved in generating anti-protein antibodies.

The area in which the anti-KAL antibody bound (ie. muscle cells) were unexpected because there is no association of any muscle abnormalities with the other clinical features of KS. Moreover, the expression patterns seen in adult tissues varied from the patterns seen in developing foetuses in that areas of expression where more wide spread in the adult. None of the areas of expression in the adult correlated to the clinical features in KS either. There were areas in which KAL protein expression were seen in the foetus correlated with areas of *in situ* expression of the KAL gene ie. in the neuroretina of the eye.

These results suggest that the antibody generated binds strongly to a muscle protein but the protein is not the KAL protein. The only way to ascertain whether the antibody is sensitive to KAL protein would be to bind the protein, then run the antibody down the column and see how much, if any, bound to the immobilised protein. This experiment has yet to be performed since the KAL protein is still unavailable.

This experiment has highlighted the difficulties of generating anti-protein antibodies which are highly specific. However carefully the protein sequence to be used as the antigen is chosen, there is always a risk that either the animal will not generate antibodies to it ie. it is not immunogenic or as is suspected in this case, there will be some cross reactivity with another protein sequence. However, these results are evidence that it is possible to generate highly sensitive monoclonal antibodies in mice.

SDS-POLYACRYLAMIDE GELS AND WESTERN BLOTTING.

5.5 Introduction

The monoclonal antibody raised to a short part of the putative KAL sequence, when used to perform immunohistochemistry, gave strong signal in the muscle cells of both adults and foetus, indication of the antibody recognition of a specific muscle antigen.

It was therefore necessary to investigate the antibodies generated further. Western blotting was performed in order to achieve this.

5.5.1 Western Blot Analysis

Western blotting uses a highly cross linked gel. The proteins to be separated in this gel are not just in an aqueous solution, but in a solution that includes a powerful negatively charged detergent (sodium dodecyl sulfate-SDS). The detergent binds the hydrophobic regions of the protein, causing the proteins to unfold into extended polypeptide chains. The protein-detergent mixture is then electrophoresed through a polyacrylamide gel towards the positive electrode. The mixture of proteins is fractionated into a series of protein bands arranged in order of molecular weight. A specific protein can then be identified on such a gel by exposing all the proteins to an antibody that has been coupled to either a radioactive isotope, an enzyme or florescent dye. This is usually performed once the proteins on the gel have been transferred onto nitrocellulose paper.

The method of SDS polyacrylamide gel electrophoresis is a more powerful procedure for protein analysis because, it can be used to separate all types of protein including those insoluble in water. Since this method relies on separation on the basis of the size of the protein, it is possible to use this method to determine whether the monoclonal antibodies generated could detect a tissue protein of the predicted size of KAL (72Kd).

5.6 Methods and materials

5.6.1 Sample preparation

1. Tissue to be analysed was weighed. Human foetal tissue, MDX mouse (tissue from the dystrophin knock out mouse), and normal mouse tissues were obtained.

2. Tissue was homogenised in 20x weight to volume sample buffer, using a polytron homogenizer.

3. Tissue was further sheared using different size needles..

4. Samples were divided into 1ml aliquots and frozen at -70°C.

5.6.2 Gel preparation

See appendix B

5.6.3 Loading samples

1. The comb was removed from gel, and wells flushed gently with reservoir buffer three times.

2. The gel was removed from casting stand and clipped into holders. The holder was slowly filled with reservoir buffer.

3. The samples were boiled for at least 5 mins, microfuged for 5 mins and 40µl loaded per well.

4. 30ul of pre-stained molecular weight marker (Sigma) was loaded onto a specific gel lane.

5. Gels were lowered into the buffer tank and the tank filled with reservoir buffer making sure the gel was completely covered.

6. The gel was electrophoresed at 20m Amps for at least 1 hour. The marker was carefully watched to ensure the gel was electrophoresed sufficiently and that the proteins had been separated adequately.

5.6.4 Semi- dry western blotting

1.The power was disconnected and the gel removed from the buffer tank. The glass plates were slid out and layed flat on the bench, separated and using a scalpel, the stacking gel was cut off the top of the separating gel and discarded.

2. The size of the separating gel was measured and the gel then lowered into a tray containing 100ml of transfer buffer. The gel was then left on a shaker for at least 10 mins.

3. 6 pieces of 3MM Whatman filter paper were cut exactly to size for each gel . At the same

time, a piece of nitrocellulose membrane was cut.

4. The pieces of membrane were allowed to soak in transfer buffer for a few minutes and then three membranes were layered onto the blotting platform, one on top of the other.

5. The nitrocellulose was layered onto the filter paper. Care was taken to ensure that it was flat and that no air bubbles were trapped underneath it.

6. The gel was layered on top of the membrane ensuring it remained moist and that no air was caught underneath it. The sandwich was completed by layering on the remaining filter papers.

7. Transfer of the protein in the gel onto the nitrocellulose was carried out using a constant current of 1.6mA/cm^2 for 1 to 2 hours.

8. After transfer was complete the filter papers were discarded. The position of the lanes in the gel were marked on the membrane with a pencil and then left between two sheets of filter paper to dry O/N.

9. Gel was placed in stain solution O/N to test efficiency of transfer of protein out of the gel.

5.6.5 Immunostaining-enhanced chemiluminescence

1. The marker was cut off the nitrocellulose membrane, ensuring the orientation of the protein on the membrane had been carefully marked.

2. The membrane was placed in a 50ml plastic tube, and 30ml of TBS added to wash the membrane on a shaker for 10mins.

3. The solution was poured away and replaced with 30ml of blocking solution. The tube was left on a shaker for 2 hours.

4. The blocking solution was poured away and the membranes washed twice for 5mins each wash with 30ml of TTBS.

5. 10ml of antibody buffer together with 200µl of antibody supernatant (not purified) was added to the membranes which were then left on the shaker for 2 hours.

6. Membranes were washed three times for 10 mins with 30ml of TTBS.

7. 10ml of antibody buffer with 3µl of secondary antibody-anti mouse IgG Horse Radish Peroxidase linked antibody was added and membranes left shaking for 1 hour.

8. The membrane was washed three times for 10mins each wash with 30ml of TTBS.

5.6.6 Detection

1.This was performed using RPN 2106 ECL kit. The membrane required a final volume of detection solution of 0.125ml/cm^2 . The volume of solution needed was calculated and then divided by two. This was the volume of each solution in the kit used.

2.The following was prepared and taken into the dark room: scissors, timer, cling film wrap, cassette for X-ray film, sticky tape, Amersham Kit, pipette and clean tips, X-ray film, plastic tray.

3.In the dark room with safe light on, the membrane was placed in a plastic tray and the appropriate amount of detection fluid added (solution 2 first, as solution 1 would start the reaction off). The tray was agitated for 1 minute.

4. The membrane was removed and wrapped in plastic and firmly stuck down in the X Ray cassette.

5. X-ray film was placed over the membrane for 10sec, 30sec and 1 minute. Care was taken to mark each film so that it could be orientated at the end.

6.The film was processed. In order to get a result the marker was realighened with the membrane.

5.7 Results

5.7.1. Screen using human foetal tissue together with mouse tissue and cell lysate from muscle cell cultures together with 7C4 antibody.

Human material was included in order to see if the antibody reacted differently than to the mouse tissue. The experimental layout was as follows:

Lane 1: Blank 50µl of sample buffer

Lane 2: Marker 10µl + 10µl of sample buffer

Lane 3: Blank 50µl of sample buffer

Lane 4: Blank 50µl of sample buffer

Lane 5: 50µl of human foetal homogenate

Lane 6: 40µl of human foetal homogenate + 10µl of sample buffer

Lane 7: 30µl of human foetal homogenate + 20µl of sample buffer

Lane 8: cell lysate-normal muscle cells

Lane 9: cell lysate-DA 70P14-dystrophin cell line

Lane 10: cell lysate-DA 98P17-dystrophin cell line

Lane 11: 50µl MDX mouse homogenate

Lane 12: 50µl BIO normal mouse

Lane 13: 30µl MDX mouse homogenate + 20µl of sample buffer

Lane 14: 30µl BIO normal mouse + 20µl of sample buffer

Lane 15: Blank 50µl of sample buffer

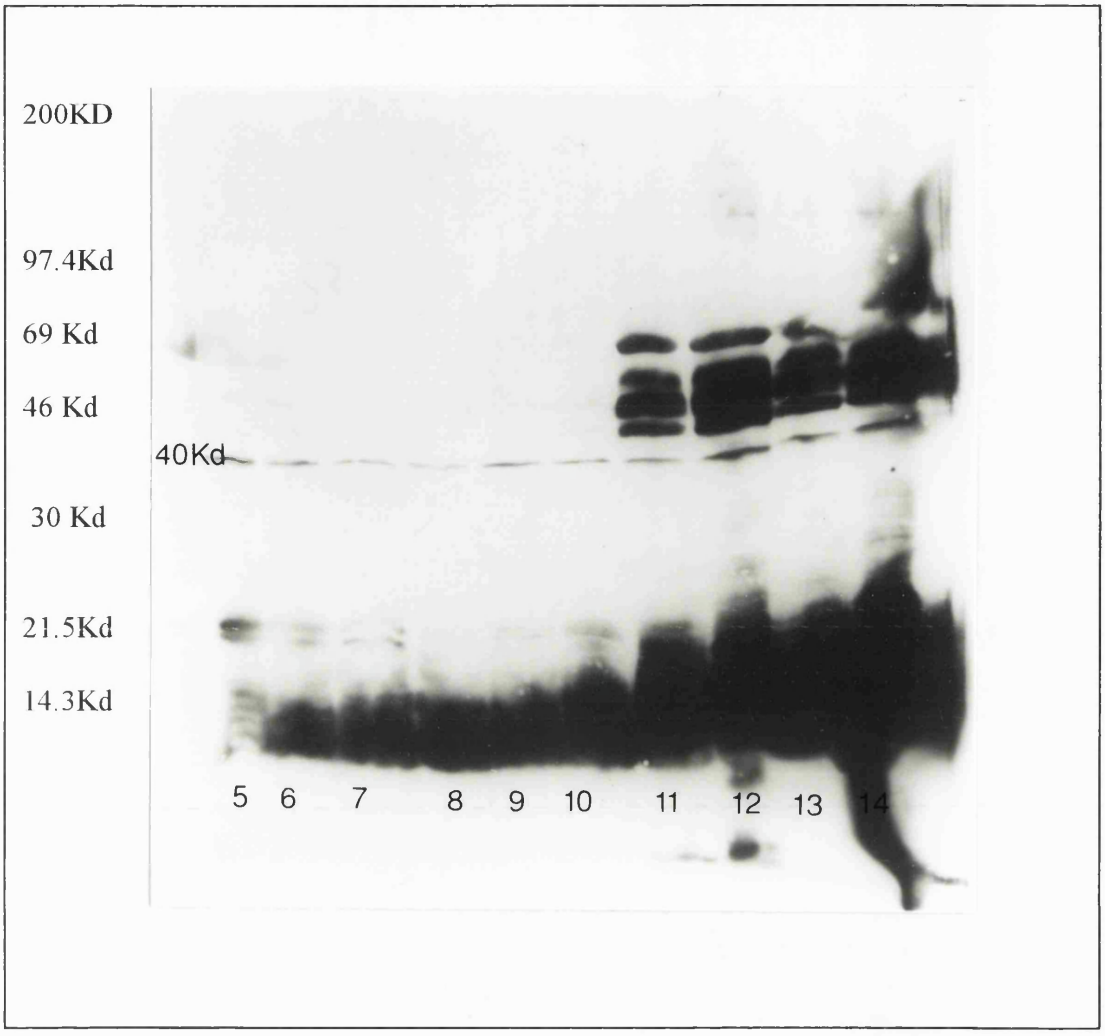


Fig 5.7 Western blot protein extract from human foetal and mouse tissue using antibodies raised in mice to a short sequence of KAL putative protein sequence.

The mouse samples show several bands. These are cross reacting bands due to the origin of the antibody. There is a band of about 40 Kd in all the samples, probably a non-specific band. There are also bands smaller than the 40 Kd band about 28Kd but there are no distinctive bands in any one sample suggesting that although the antibody binds successfully to different tissue types, it does so in a non specific manner.

5.7.2. Screen of human foetal brain using both 7C4 and 2A1.

There was a large amount of binding of antibody to the membrane when 7C4 was used(Fig 5.8A) and a small amount in the cerebellum when 2A1 was used (Fig 5.8B). However this antibody binding was in a non specific manner. The gel was probably overloaded with protein. This probably lead to reduced resolution of the protein on the gel, as these lanes in which the protein had been loaded could not be seen after blotting.

Lane 1: cerebellum

Lane 2: cerebellum

Lane 3: temperol lobe

Lane 4: temporal lobe

Lane 5: membrane from outer surface of the brain

Lane 6: membrane from outer surface of the brain

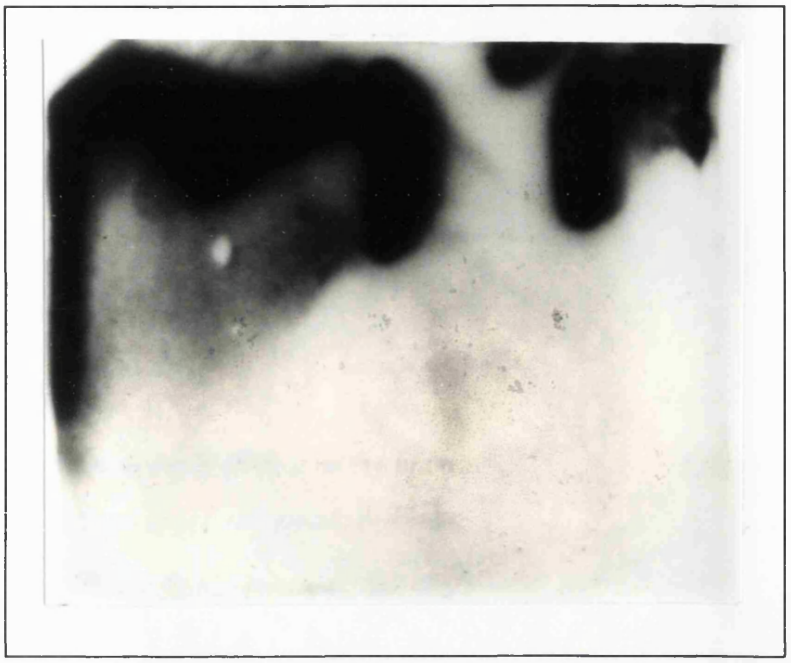
Lane 7: olfactory bulb

Lane 8: olfactory bulb

Lane 9: Marker

20µl of sample was loaded in each lane.

A) 7C4



B) 2A1

Fig. 5.8 Western blot of protein extract from different areas of human foetal brain with 7C4 and 2A1 anti KAL antibodies.

5.7.3. Screen using fresh samples of human foetal brain tissue and 7C4 antibody.

Since the results of the previous Western blots have failed to identify a specific protein site to which the antibody was binding, antibody was then run on the gel to ensure that the antibody could actually bind correctly. Fresh tissue samples were obtained for this experiment.

Lane 1: Marker

Lane 2: 7C4 antibody.

Lane 3: Blank

Lane 4: olfactory bulb

Lane 5: Blank

Lane 6: temporal lobe

Lane 7: Blank

Lane 8: cerebellum

30µl of sample was loaded into each lane.

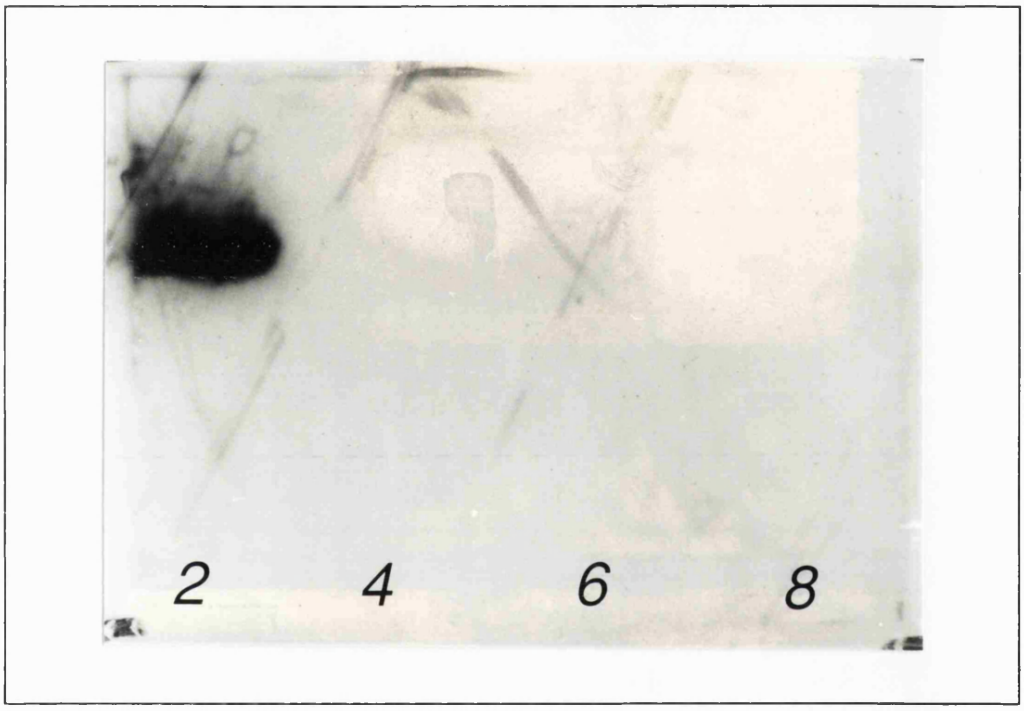


Fig 5.9 Western blot of protein extract from human foetal brain as well as 7C4 antibody

The only lanes in which antibody was seen to bind to the membrane were the lanes in which the antibody had been run ie. it only bound to itself no other protein.

5.8 Discussion

From the above experiments there is little that can be concluded with any degree of certainty. It can be suggested that the monoclonal antibodies raised in mice to a short sequence of the putative KAL protein sequence (the KAL MAP), binds to protein in the brain including the olfactory bulb and the eye as well as muscle cells in various parts of the body but binding is also occurring within these and other tissues in a non-specific manner.

Due possibly to short falls in the Western blotting methods used, it has not been possible to estimate precisely the size of protein to which the antibodies are binding. It has also not been possible to obtain any consistent results. Taken together, these Western blot results and the immunohistochemistry data suggest that the antibody generated cross reacts with a muscle protein, although this is not dystrophin. As purified mature KAL is not available, the generated antibody cannot be validated more extensively.

Although the sequence used to generate the antibody was carefully chosen using computer data bases of known protein sequences avoiding a sequence with homology to any other known protein, an obvious weakness of this approach is that the antibody raised can react with as yet unidentified proteins.

This is an unavoidable weakness of using reverse genetics to generate a putative protein sequence when the protein has not been isolated and purified from tissues. Generating the putative peptide sequence by using transcription, translation systems was technically outside the scope of this project. It was therefore decided to generate antibodies to an oligo peptide instead. Given the difficulties encountered in interpreting immunohistochemical and western blot analysis, it was decided to look at mRNA expression instead, as a guide to the anatomical locations of expression of the Kallmann gene.

Chapter 6

IN SITU HYBRIDISATION OF KAL cDNA TO HUMAN FOETAL TISSUE.

6.1 Introduction

The understanding of the pathogenesis KS requires the study of the role of *KAL* in normal human development. Given the difficulties encountered with the immunohistochemistry it was decided to perform *in situ* hybridisation on normal human foetal material in order to construct a map of the spatio temporal expression of *KAL*.

The process of *in situ* hybridisation relies on the hybridisation of a radio labelled RNA probe to a specific complementary mRNA sequence in the tissue. In this case the ribo probe used is complementary to *KAL* mRNA. The tissue sections must be treated with heat or acid before exposure to the probe to ensure that the RNA and DNA are denatured . The tissue is allowed to hybridise to the probe and after hybridisation, the unhybridised labelled probe is removed and the slides coated with photographic emulsion. This is followed by autoradiography to reveal areas of hybridisation and therefore the presence and location of KAL specific mRNA within the individual cells. A sense probe is used to detect non specific hybridisation.

6.2 Methods and materials

6.2.1 Tissue collection and pretreatment.

After consent from the individual patients and from the Hospital Ethical Committee, normal human foetuses were collected from five chemically induced terminations of pregnancy performed at 45 days- 6.5 weeks (n=2), 48 days- 6.9 weeks (n=1) and 11 weeks (n=3) after fertilisation. The tissues were fixed in ice cold 4% paraformaldehyde in phosphate buffered saline(PBS) (pH7.0) overnight at 4°C, and then soaked in 10ml of saline for 30 minutes at 4°C twice and dehydrated through graded alcohols- 50%, 70%, 70%, 85%, 95%, 100%, 100%, toluene twice, toluene:wax (1:1) followed by three changes of wax before being transferred to a mould for embedding.

6.2.2 Slide preparation.

Care was taken to ensure that the slides were only handled with gloves at all times, to avoid the possibility of RNase destroying or damaging the tissue.

1.Glass slides were immersed (subbed) to prevent the tissue sections floating off the slides. The slides were dipped in acid/alcohol (10%HCl /70% ethanol).

2. Slides were then dipped in DEPC H_20.

3. Slides were then dipped in 95% ethanol and then dried at 80°C for a few minutes while the rest of the solutions were prepared.

4. Slides were then dipped in 2% TESPA (3-aminopropyltriethoxy-silane in acetone)

5. Slides were then dipped in acetone twice, and DEPC H_20 once.

6. Slides were then loosely wrapped in tin foil and placed in a 37°C incubator over night.

6.2.3 Tissue sectioning.

1. Tissue sections were cut 5μm thick using a microtome and floated on DEPC H_20.

2. The slides were then placed under the tissue so that tissue could settle on the slides.

3. The slides were then allowed to dry on a hot plate for a couple of hours.

6.2.4 Pretreatment of slides for *in situ* hybridisation.

1. Glass slides with tissue sections on them were dewaxed in 400ml of Histoclear (Fissons) for 10 minutes, twice.

2. They were soaked in 100% ethanol for 2 minutes to remove the Histoclear.

3. Slides were transferred through the following series of ethanols in order to dehydrate the tissue: 100%, 100%, 95%, 85%, 70%, 50% and 30%.

4. Slides were soaked in 400ml of 0.83% saline for 5 minutes followed by 400ml of PBS-DEPC also for 5 minutes

5.Slide were soaked in 400ml of 4% paraformaldehyde (PFA) for a minimum of 20 minutes. This further fixed the tissue (in the fume cupboard).

6. Slides were then soaked in 400ml of PBS-DEPC twice for 5 minutes each.

7. Slides were placed on foil on bench top. 1ml of Proteinase K solution was layered onto each slide and left for exactly 5 minutes. This digested protein in the tissues.

8. Slides were placed in a rack and soaked in PBS for 5 minutes.

9. Fixation in 4% PFA was repeated for 20 minutes, followed by washing in distilled water.

10.Slides were placed in 400ml of 0.1M Triethanolamine resting on a petri dish lid, with a

magnetic stirrer under the slides. 1ml of acetic anhydride was added to stirring solution (in the fume cupboard). This neutralises the charge of the tissue on the slides to optimise binding of the probe.

11. Slides were soaked in 400ml of PBS-DEPC for 5 minutes and then 400ml of saline solution for a further 5 minutes.

12. Slides were transferred through the following series of ethanols in order to dehydrate them: 30% 50%, 70% (for 5 minutes in order to remove any salt deposit on the slide) 85%, 95%, 100%,100%.

13. Slides were allowed to air dry for at least one hour before performing the hybridisation process.

6.2.5 Preparation of riboprobe for *in situ* hybridisation.

The KAL cDNA was kindly donated by Dr C.Petit (Pasteur Institute, Paris). The KAL cDNA was inserted into Bluescript vector at Bam H1 and Cla 1 sites and was 2.3Kb in size. The orientation of the insert was not known and therefore it was necessary to determine this before any probes could be prepared.

1. The vector was digested as follows:

10µg of DNA was used in each restriction digest; stock = 2.4µg/µl thus 4µl

10µl of bovine serum albumin (BSA) was also included in each reaction.

The reaction was left at 37°C for 1 hour and 10µl was electrophoresed on a 1% agarose gel.

2. These digests were then further restricted with each of the following enzymes in turn: EcoR1, Hind 111, Pst 1, Xho 1 and Kpn1. and electrophoresed on agarose gels and the fragments sized by comparing the bands seen on the gel to those of molecular weight markers.(see appendix B)

	A	B	C
Enzyme 5µl	Bam H1	Cla 1	Bam H1 & Cla 1
Buffer 10µl	react 3	H	H
Water	71µl	71µl	66µl

Table 6.1: Restriction experiment to determine orientation of KAL in Bluescript vector.

These results together with a map of the restriction sites in KAL allowed the orientation of KAL in the Bluescript vector to be determined.

EcoR1 revealed a restriction site at 1380bp of KAL.

Hind 111 cut once at 420bp.

Xho 1 restriction site in the polylinker was cleaved as well as at 960 bp of KAL.

Xba 1 a second fragment was only observed in the double digest and not in the linearised vector suggesting that the only restriction site was in the polylinker. Although there is a Xba 1 site in KAL at 5000bp, it has not been included in this insert.

Kpn 1 restricts in the polylinker and at 900bp.

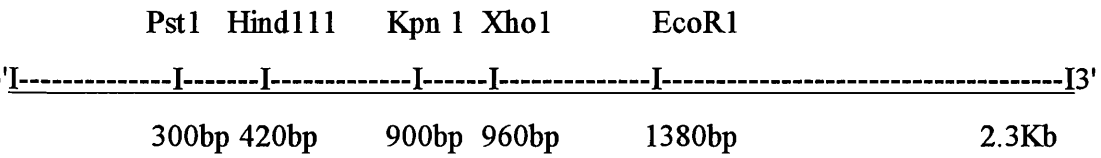


Fig 6.1 Map of restriction sites in KAL cDNA inserted in Bluescript vector.

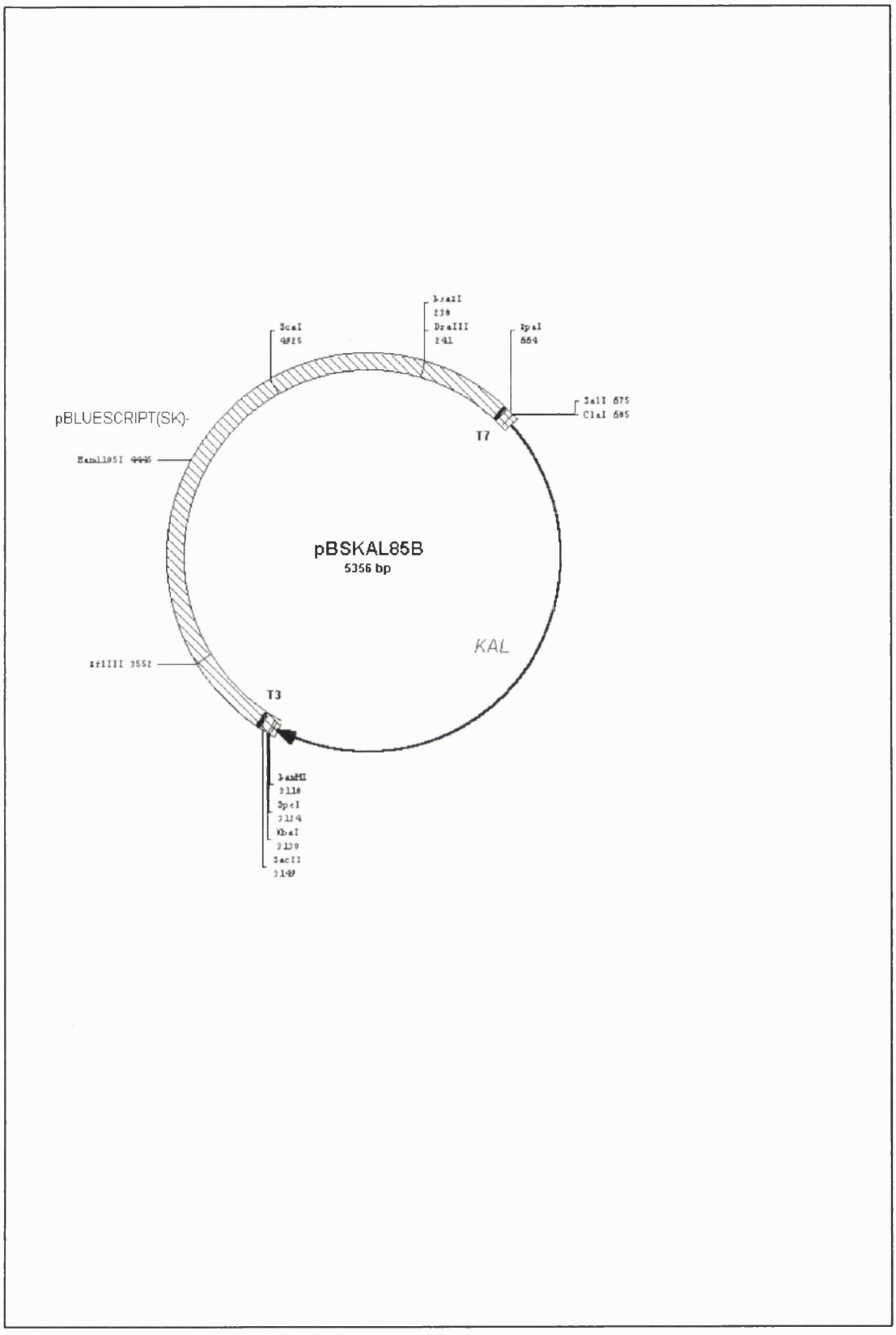


Fig 6.2 KAL inserted in Bluescript vector

3. KAL cDNA was restricted with BamH1 enzyme. The T7 promoter was then used in order to generate antisense probe. The T7 promoter was used as the start site of synthesis of DNA, generating the complement of the native KAL DNA/RNA sequence and so when denatured, the antisense probe will hybridise to the KAL sequence in the tissue.

The plasmid was digested with Cla1 in order to generate sense probe by using the T3 promoter. The sense probe is the same sequence as KAL and will therefore not hybridise to KAL sequence. Rather it is used as a negative control to ensure that areas where KAL antisense is seen to hybridise are in fact KAL expression and not non specific hybridisation. This reaction was performed as follows in a total reaction volume of 30ul made up as follows:

7.5ul plasmid DNA (2.4µg/µl KAL maxi prep)

3ul 10 X restriction buffer

1ul enzyme BamH1/Cla 1

18.5ul water

The reaction was left in water bath at 37°C for 1 hour.

Lectin p1.13a was restriction digested with Bam H1 was used as a positive control. The T3 promoter was used in order to generate an antisense riboprobe which would bind ubiquitously, as lectin is expressed at low levels in large number of tissue types during human development.

4. The transcription reaction was prepared as follows:

4ul restricted plasmid DNA

4ul 5X transcription reaction buffer

2ul 0.1M DTT

2ul nucleotide mix GCA (2.5M 5ul of each 10mM stock plus 5ul of water DEPC)

10ul ^{35}S UTP

1ul ribonuclease inhibitor

0.5ul polymerase enzyme T7 or T3

The reaction was left in water bath at 37°C for 2 hours.

5. Columns through which unincorporated ^{35}S and nucleotides could be separated from the probe were prepared:

Using autoclaved glass pipettes the bottom was plugged with sterile wool from a disposable pipette and the column filled with sephadex G50. Column buffer (0.1%SDS in TE) was run

through the column.

6.Sample were loaded onto column (24ul) and then 3X 150ul of column buffer was loaded onto the column and eluted into Eppendorf tube 1.

7. 150µl of column buffer was loaded onto column and eluted into Eppendorf tube 2. This was performed thrice and then the column was transferred into a final tube were it remained.

8. The radioactivity of the elutes was measured by adding 1µl of sample from tubes 1 to 5 to 2ml of scintillation fluid. The tubes with the two highest counts were kept, the others discarded.

9. 50% volume of 6M ammonium acetate was added (150ul) followed by 200% volume of absolute ethanol (900µl).

10. Tubes were left at -20°C for at least 2 hours or O/N.

11. Tubes were centrifuged for 20 minutes and the position of the pellet marked on the tube.

12. Supernatant was removed and the pellet was washed first with 200µl of 80% ethanol and then 100% ethanol. The pellet was then left to air dry.

13. The number of counts per minute of probe were calculated (ie. count is the count for 1ul multiply this value by 150ul ie reaction volume). This figure was divided by 1 x 10^6 to give the volume of DTT needed. This was multiplied by nine to give the volume of hybridisation buffer needed.

14. The necessary amount of DTT and hybridisation buffer was added to the pellet of probe and then aliquoted out into 125µl tubes and frozen.

15.The probe was heated to 95°C for 5 minutes on a heating block in order to denature it and then pipetted onto pre-treated slides. 25µl of probe was layered onto each slide and cover slips were placed on the slides.

16. Slides were placed in a designated rack and put in a plastic box. 50% formamide solution was poured over a paper towel in the lid of the box, in order to keep the slides moist.

17.The box was wrapped in cling film. Again this keeps the slides moist during hybridisation.

18. The box was sealed and placed in an oven at 60°C O/N.

6.2.6 Post hybridisation treatment of slides.

DEPC treated solutions and baked or autoclaved glass wear were no longer necessary. However, care was taken as the slides were radioactive.

1. The following day the slides were transferred to 400ml of prewarmed (60°C) 5X SSC solution with 10% DTT added. The slides were left for 30mins at 60°C. At the same time a fresh solution of 5X SSC was prepared and warmed (5X SSC was prepared using 100ml of 20X SSC and 300ml of water).

2.The slides were washed in the second 5X SSC wash for a further 30 mins at 60°C.

3.Slides were transferred to slide boxes containing 30ml of pre-warmed 50% formamide and left gently shaking for 30 mins in 65°C water bath.

4.Slides were returned to the slide rack and soaked in 400ml of pre-warmed NTE solution. The slides were soaked in three washes of NTE for 10mins at a time in the 37°C water bath.

5.Slides were treated with Ribonuclease A (20µg/ml) in 400ml of NTE in the 37°C waterbath. (Ribonuclease A needed, 20µg in 400ml, therefore 8000µg needed or 8mg. Stock was 10mg/ml therefore 0.8ml used). The slides were left to soak for 30 mins.

6. Slides were left to soak in fresh NTE at 37°C for 15 mins.

7. Slides were washed again in 50% formamide for 30 mins at 65°C.

8. Slides were soaked in 2X SSC at room temperature for 15 mins.

9. Slides were soaked in 0.1X SSC at room temperature for 15 mins.

10. Slides were dehydrated in a series of ethanols containing 0.3M ammonium acetate. 2.5ml of NH_4Ac was added to the 30% 60% 80% and 95% ethanol washes and finally two 100% ethanols.

11. Slides were then left to air dry before dipping in photographic emulsion.

6.2.7 Autoradiography

1. 20 ml of photographic emulsion was measured into a 50ml plastic tube which was wrapped in foil and placed in the water bath (50°C).

2. 6ml of 2% glycerol was measured out in the slide mailer and then transferred to a fresh tube which was also placed in the water bath for 30 mins.

3. The 6ml of pre-warmed glycerol and emulsion were mixed in the slide mailer. The mixture was allowed to settle before dipping the slides in the emulsion one at a time. The emulsion was then wiped off the back of the slide which were then lain flat and allowed to dry for at least two hours.

4.Once dry the slides were put in a rack and light tight black box. Desiccant was placed in the lid of the box which was then sealed and stored at 4°C for five days.

6.2.8 Developing slides.

1. Slide box was removed from the fridge and left at room temperature for 1 hour.
2. Developer was prepared by adding 8g of D19 developer to 50ml of water in a glass flask which was wrapped in tin foil and allowed to stir for 1 hour.
3. A stop solution of 30% Na thiosulphate was prepared and stored in dark room wrapped in foil at room temperature.
4. Under safe light the slides were unwrapped, removed from the box and transferred to the D19 developer for 2mins, fixative for 1 min, and then 30%Na thiosulphate stop solution for 2 mins.
5. The slides were then washed in tap water twice and counter stained in Toluene Blue, (0.05%) for 2.5 mins.
6. The slides were then dehydrated through 70% and then 100% ethanol.
7. Slides were allowed to air dry before they were mounted and examined by dark and light field microscopy.

6. 3 RESULTS- IN SITU HYBRIDISATION

6.3.1 OLFACTORY BULBS

The highest signal seen as silver grain under the dark field microscope was detected over the outer most layer of the bulbat 11 weeks. This layer is constituted by olfactory nerves. The inner layer of the bulb gave a weak signal. This area is made up of mitral and granular cells. In contrast the antisense probe for L14 lectin showed strong signal over all layers of the bulb. The KAL sense probe showed no significant hybridisation (Fig. 6.3).

6.3.2 EYE

The neuroretina expressed KAL at 11 weeks, although the low intensity and diffuse nature of the signal made it difficult to conclude the specific cell type in which the expression was being seen. Transcripts were also detected in the intrinsic ocular muscles. The intensity of KAL was less than that of lectin. However it was greater than the signal seen using the KAL sense probe (Fig. 6.4).

6.3.3 KIDNEY

Transcripts were detected using the KAL anti sense probe in the outer cortex of the metanephric kidney where undifferentiated cells and nephron precursors are located. Mature nephrons and interstitial tissue located in the centre of the organ showed no signal above that of the sense probe (Fig. 6.5).

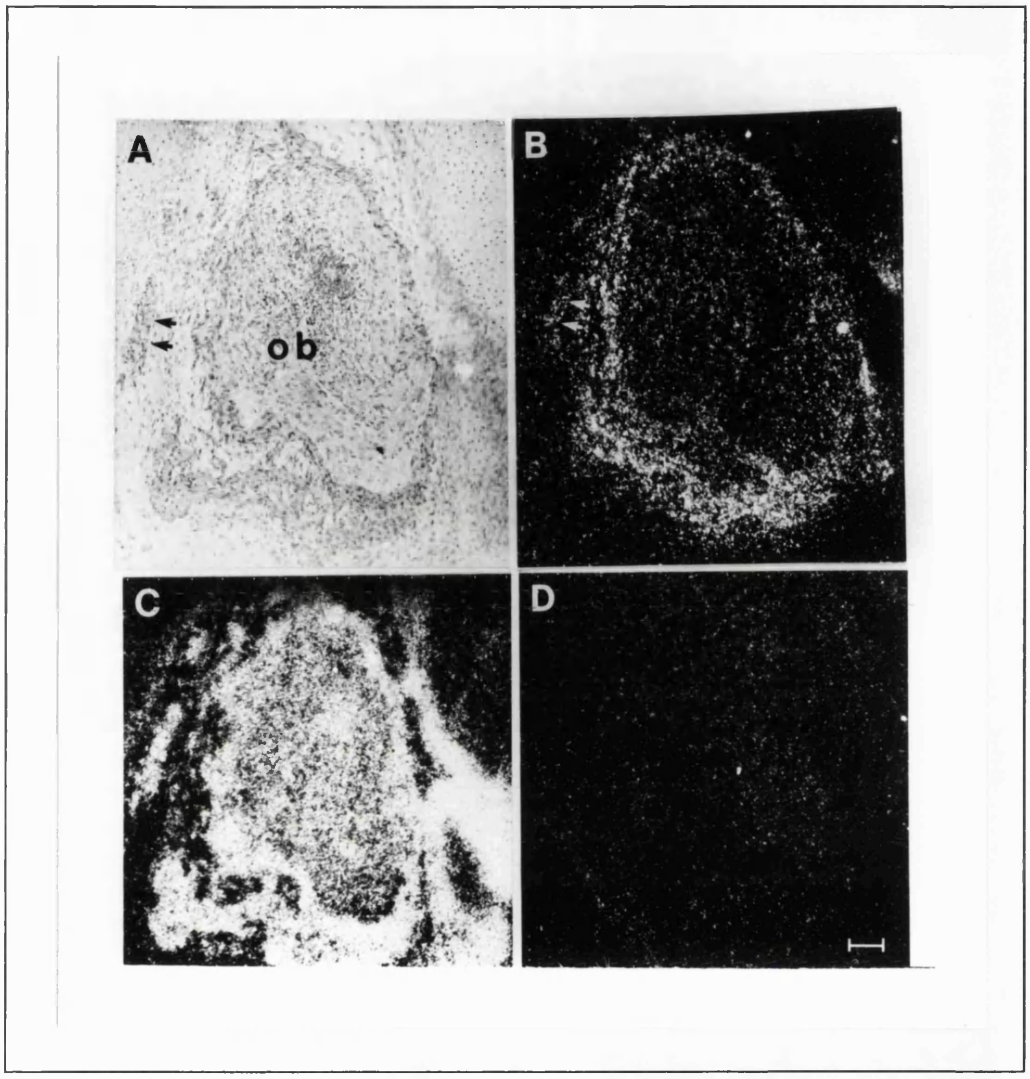


FIG 6.3 KAL expression in the developing human olfactory bulb. (A) Bright field coronal section at 11 weeks gestation counterstained with haematoxylin and eosin. Olfactory neurons migrating into the olfactory bulb (OB) are arrowed. (B)-(D) In situ hybridisation with dark field illumination. (B) The strongest signal from the S^{35} labelled KAL antisense riboprobe was detected in the olfactory neurons situated in the olfactory layer of the bulb. Inner layers of the bulb showed weak signal. (C) Hybridisation with L14 lectin antisense riboprobe shows high levels of expression throughout the tissue as expected for a ubiquitously expressed gene. This confirms the integrity of the mRNA after embryo processing. (D) KAL sense probe of the same area showed no significant signal. Bar was 100μm.

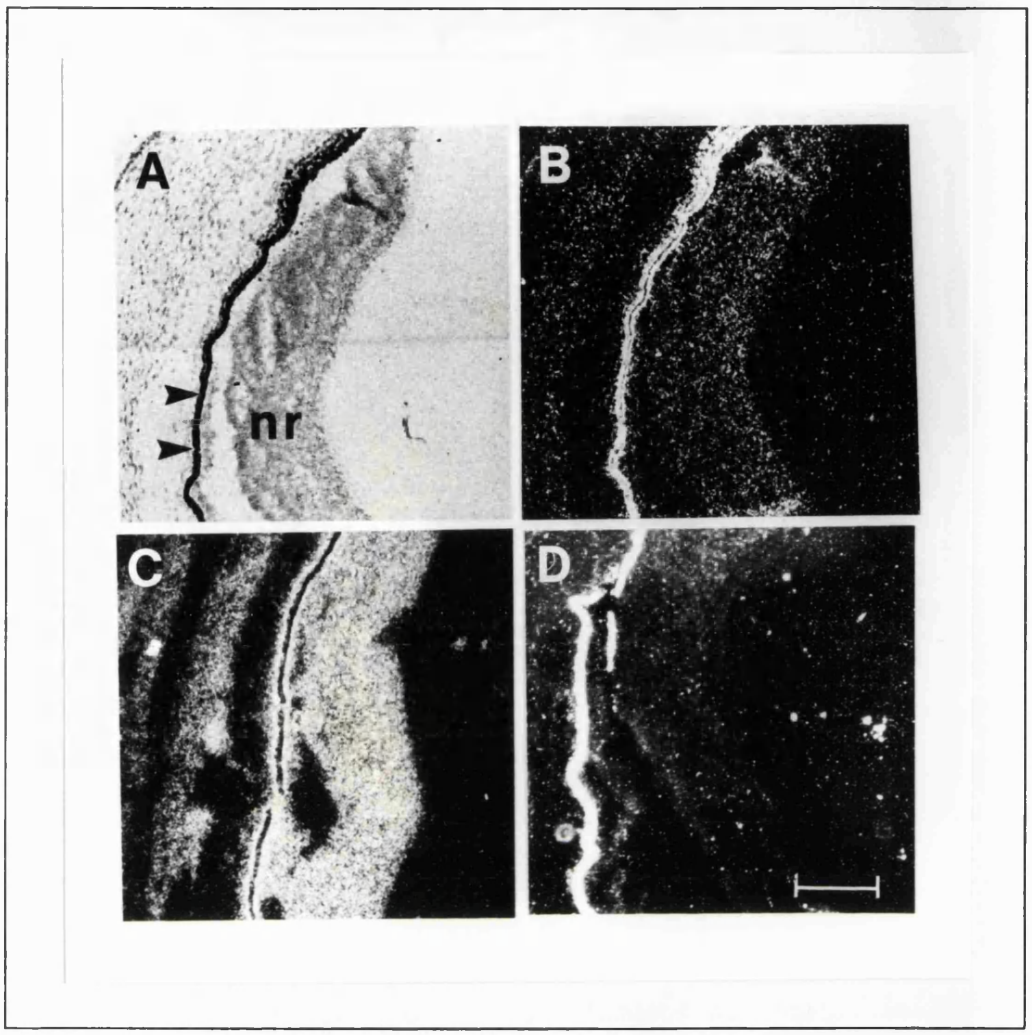


FIG 6.4 KAL expression in the developing human retina. (A) Coronal section at 11 weeks gestation shows the developing retina (nr). (B)-(D) In situ hybridisation and dark field illuminations (B). The KAL antisense riboprobe hybridised with the mRNA in the neuroretina. (C). L14 lectin antisense riboprobe showed wide spread expression, confirming the integrity of the RNA. (D). Minimal signal with the KAL sense riboprobe in the eye. Note that the apparent signal in the pigmented retina (arrowheads in A) arises from the melanin granules and is not due to binding of the riboprobe. Bar is 100μm.

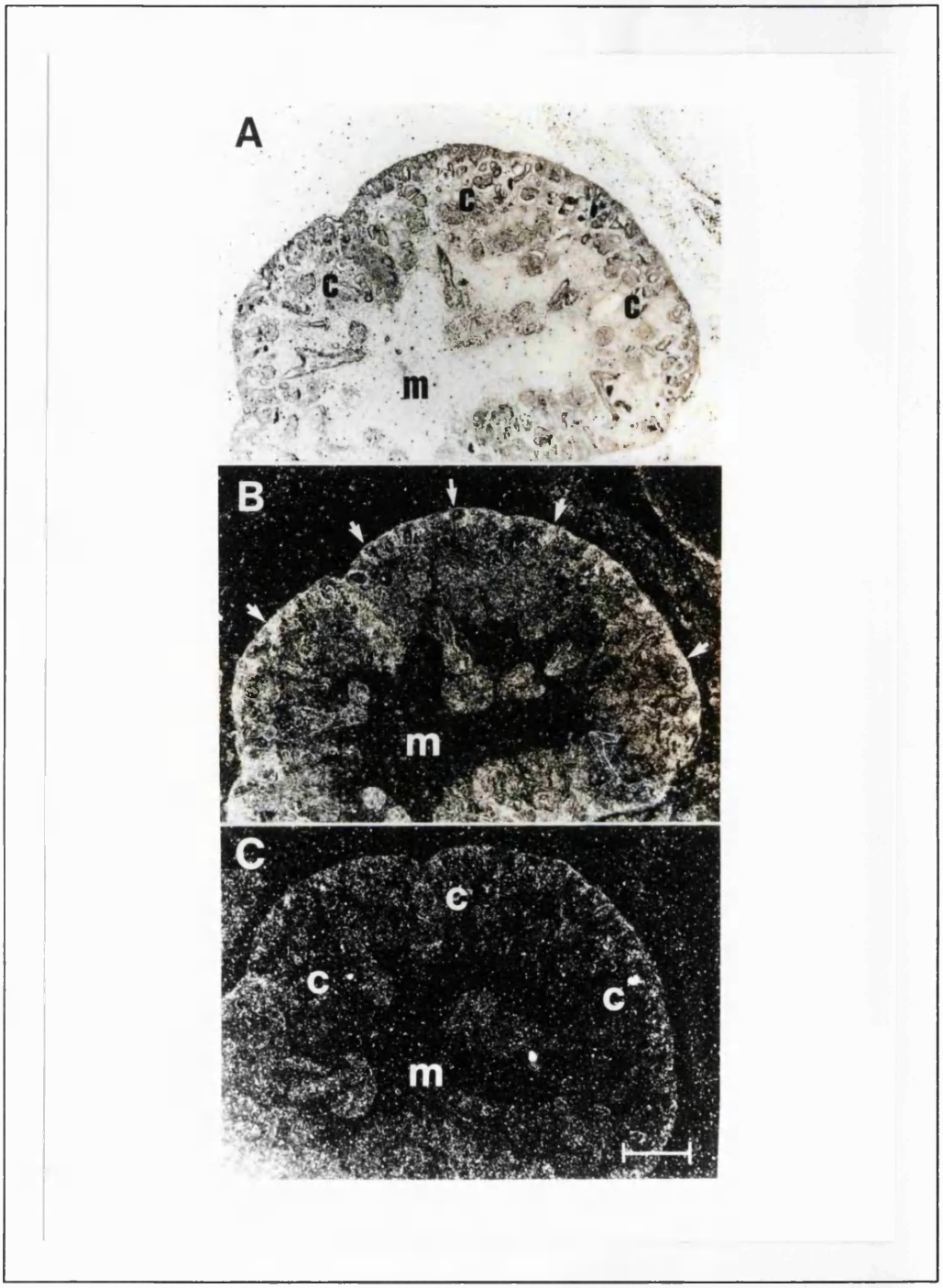


FIG 6.5 KAL expression in the developing human kidney. (A) Bright filed of sections at 11 weeks gestation counterstained with haematoxylin and eosin showing the cortex (c) and medulla (m). (B,C) In situ hybridisation and dark field illumination. (B) The KAL antisense riboprobe hybridises with the mRNA in the outer cortex of the kidney (arrows). (C) KAL sense riboprobe of the same area shows no significant signal. Bar 40μm.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION. (RT-PCR)

6.4 Introduction

Since DNA is double stranded, it is far more stable than RNA, a single stranded molecule with a short half life. For these reasons, it is very useful to be able to isolate total RNA or mRNA from tissue and then use it as a template to generate more stable DNA. The process by which this is done is called reverse transcription using reverse transcriptase (RT) enzymes. This enzyme is produced by a virus that generates its DNA by copying its RNA ie. the reverse of the usual transcription reaction where DNA is copied to RNA. The enzyme generates single stranded cDNA molecules which are soon converted to double stranded molecules by DNA polymerases. These double stranded cDNA clones can then be used for various purposes including cloning. cDNA generated only contains coding sequence since it has been generated from spliced RNA. In contrast, genomic DNA contains both coding and non coding sequences. cDNA therefore reflects the underlying mRNA template.

The mRNA extracted from the various tissues is converted to DNA by reverse transcriptase enzyme together with a short primer which anneals to the 3' end of the RNA-random primer. This starts off the transcription reaction. Once the DNA has been generated, it is used in a PCR reaction together with primers that are specific to a region of KAL. Provided KAL sequence is present in any sample, it will be amplified. This RT-PCR method allows one to identify tissues in which KAL is expressed in the developing foetus. β Actin is used as a control to test the integrity of the RNA extracted. Actin is ubiquitously expressed, ie. is it is a housekeeping gene (a gene that is active in every cell type). House keeping genes are ubiquitously expressed in cells in all cells. Each sample is used in a PCR reaction together with primers specific for actin.

Nested PCR is a method used to increase the specificity of the result. It consists of using a second set of PCR primers that will amplify a sequence generated by the first set of PCR primers in the first round reaction. In this case the first round set of primers are specific for a KAL specific sequence. The second round primers are within this sequence and will amplify a region within the product generated from the first round reaction. If the first round amplified a product, then the second round PCR reaction will have a template to amplify. In this way nested PCR reactions improve the specificity because the second round relies on the success of the first round PCR reaction. Therefore the advantage of this method is that

it is both very sensitive and highly specific!

6.5 Methods and materials

6.5.1 Tissue collection

A 45 day old human embryo was collected and placed in ice-cold L15 medium, and then dissected within half an hour of delivery.

6.5.2 RNA extraction

RNA was extracted from the whole brain, eye, spinal cord, heart, liver, limbs, gonads, mesonephros and metanephros as follows:

1. The tissue was homogenised in 0.5ml of Solution D by passing tissue through a syringe and needle several times
2. 50µl of 2M Na acetate pH 4 was added to each tube which was then mixed by inversion.
3. 500µl of water saturated phenol was added to each tube and the samples mixed by inversion.
4. 100µl of 49:1 chlorofom:octanol was added ,mixed and the tubes left to incubate on ice for 15 minutes.
5. The tubes were spun at 4°C for 20 minutes and the supernatant (upper phase) transferred to fresh tubes.
6. 500µl of isopropanol was added and the tubes left to incubate at 4°C for 30 minutes after which they were centrifuged for 10 minutes at 4°C.
7. The supernatant was discarded and the pellet resuspended in 300µl of solution D.
8. The RNA was precipitated with 300µl of isopropanol for 30 minutes at 4°C followed by centrifuging for 10 minutes at 4°C. The supernatant was then discarded.
9. The pellet of RNA was washed in 500µl of 75% ethanol, centrifuged for 10 minutes at 4°C and the ethanol then discarded and the pellet allowed to air dry.
10. The RNA pellet was resuspended in 50µl of DEPC water and then stored at -70°C.

6.5.3 Reverse transcription.

1.5µl of RNA was mixed with 5µl of DEPC water and heated to 90°C (on hot block) for 10 minutes and then snap frozen on ice.

2. While the RNA was cooling down a master mix was prepared (per RNA sample) according to the following protocol:

1µl random primers

4µl first strand buffer (Gibco)

2µl dNTP's 2.5mM (Pharmacia)

1µl RNA guard (Pharmacia)

1µl reverse transcriptase enzyme

3µl 0.1M DTT

Total volume=12µl.

3.12µl of master mix was added to each sample and the tubes were incubated at 37°C for one hour.

4. 30µl of DEPC water was added to each tube which was then heated to 95°C and then snap cooled on ice.

5. 2.5µl of this reaction was then used in the following PCR reaction:

5µl 10X reaction buffer (Bioline)

3µl $MgCl_2$

1µl dNTP's 2.5mM

2.5µl primer 1

2.5µl primer 2

0.5µl Taq polymerase (Bioline)

33µl H_20

Total volume=50µl

The primers used were as follows:

primer 1: 5' GCT GGA CGA GTC GCT GTC TGC CGG 3' sense primer corresponding to nucleotides 100 to 123 of KAL cDNA.

primer 2: 5'CCC CCT GCT TCA CCA ACA GGC TGT 3' antisense primer corresponding to nucleotides 394 to 371 of KAL. (Legouis et al. 1991)

For each tissue sample RT-PCR for β actin, a ubiquitously expressed gene was also performed in order to confirm the integrity of the extracted RNA.

6. The amplification times were as follows: 94°C for 5 mins followed by 30 cycles of 94°C

for 1 min, 55°C for 1 min and 72°C for 1 min followed by 72°C for 10 mins.

7. The PCR products were electrophoresed through a 1.5% agarose gel and visualised after ethidium bromide staining (see appendix B).

8. Nested PCR was then performed using the PCR product together with the following primers:

primer 3: 5'CGT CCA GCG CGC TCC TGC GCC TCC 3' sense primer corresponding to nucleotides 128 to 151 of KAL

primer 4: 5'TGA GGA ACT CAC AGC TGG TCA AGC 3' antisense corresponding to nucleotides 367 to 344 of KAL.(Legouis et al. 1991)

9. The products from the first round PCR were digested with *Hind III* and separately with *Pst 1* and the fragments electrophoresed on an agarose gel to ensure specificity of the PCR reaction.

6.6 RESULTS OF RT-PCR

KAL mRNA was detected in the spinal cord, mesonephros and metanephros of a 45 day-6.5 weeks old human embryo. No signal was detected in the whole brain, eye, liver, limbs and gonads at this stage in development. β actin mRNA was detected in all tissue confirming the integrity of the RNA.

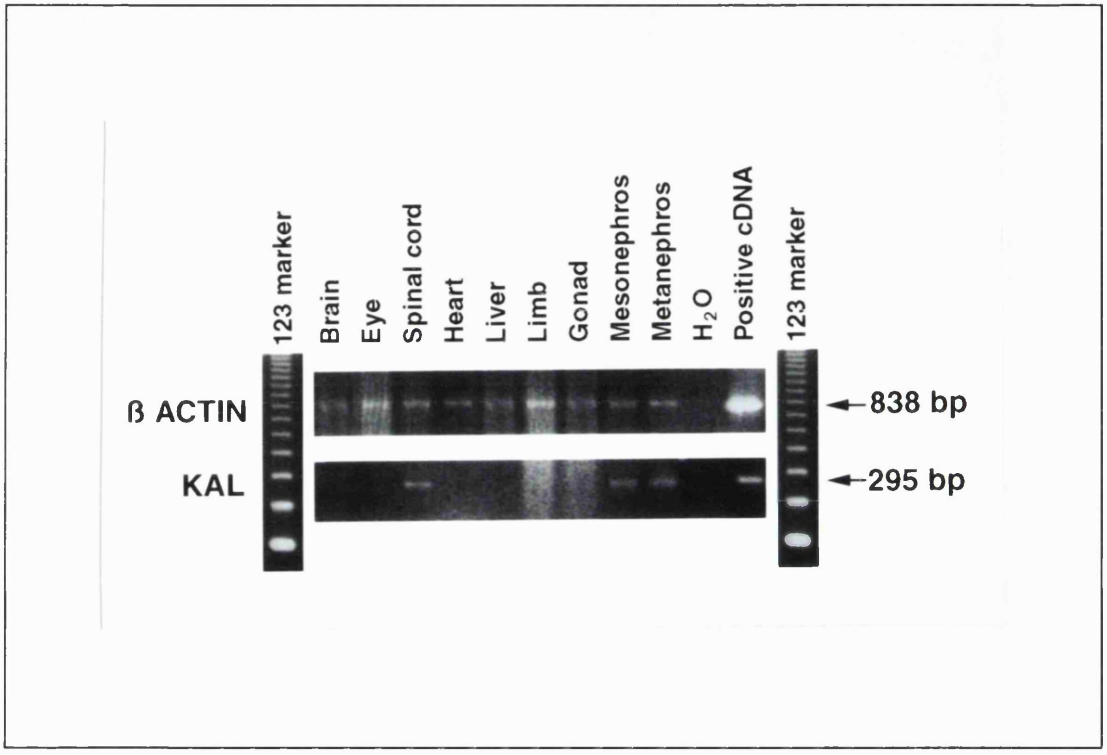


Fig 6.6 RT-PCR for KAL and β actin from 45 day old human foetus. Molecular weight markers indicate multiples of 123 base pairs. ie. 123bp, 246bp, 369bp etc. Lanes contain tissue as marked. H_20 is negative control with cDNA omitted, KAL and β Actin plasmid cDNA are positive controls. The KAL primers generate a 295bp product and the actin product is 838bp.

6.7 Discussion.

We reasoned that the key to reaching a better understanding of the pathogenesis of the clinical features of XKS would reside in establishing the normal expression of KAL during early development. Although recent studies have addressed KAL expression in avian development (Rugarli et al. 1993; Legouis et al. 1993a) and also in late human gestation-19 weeks (Lutz et al. 1994), this is the first report of KAL expression in early human gestation at a period when critical events of organogenesis are occurring. Using *in situ* hybridisation and RT-PCR KAL, mRNA was found to be expressed at low levels at 6.5 weeks (45 days) in the spinal cord and the excretory system but not in the brain. At 11 weeks after fertilisation, KAL was detected by *in situ* hybridisation in the central nervous system and kidney. Therefore the gene is expressed throughout the period of early nephrogenesis. We were however, unable to clearly define the time of onset of KAL expression within the olfactory bulb and neuroretina since no material was available between the seventh and eleventh weeks of gestation.

6.7.1 Expression in the central nervous system.

In this study it was not possible to detect KAL mRNA in the brain or the eye at 6.5 weeks gestation as investigated by the sensitive method of RT-PCR, although expression was present in the spinal cord (Fig 6.6). At this stage of development corticospinal axons are just about to reach their decussation point in the lower medulla oblongata (Humphrey, 1960). At the same time, olfactory nerve fibres are migrating from the epithelium of the olfactory ectoderm towards the forebrain where the olfactory bulbs will later develop. Using *in situ* hybridisation, KAL transcript was detected at 11 weeks gestation in the developing olfactory bulb (fig 6.3) where the highest expression was seen over the outer olfactory nerve layer. At the same stage of gestation, KAL was detected in the neuroretina. The study performed by Lutz et al. on a 19 week old foetus found expression by *in situ* hybridisation in the olfactory bulb with the highest level in the granule cell and olfactory neurone layers. The same study also reported expression in the cerebellum and thalamus at that stage of development but no other organs outside of the brain were examined.

More extensive studies have been performed in the chick (Rugarli et al. 1993; Legouis et al. 1993a). Chick KAL has 77% homology to the human gene with over 90% homology at protein level. At stage 30 (corresponding to 38-45days human gestation) KAL transcripts were detected by *in situ* hybridisation in the region of the presumptive olfactory bulb in the

developing telencephalon. By stage 36 (52-57 days human gestation) expression was found in the mitral neurones, the first layer to differentiate within the bulb. KAL expression is maintained in this layer during gestation but falls after birth. It is noteworthy that the olfactory and granular layers show the highest levels of expression in the humans but the mitral layer shows the highest expression in the developing chick olfactory bulb. These conclusions should be interpreted with two caveats in mind: 1) the limits of resolution of the radioactive *in situ* technique does not easily permit localisation of a signal over any one cell. 2) at the earlier stages of development the cell layers of the olfactory bulb are not well defined. Studies in chicks have also revealed transcript in the neuroretina and spinal cord in concordance with our findings (Fig 6.3 and 6.6).

Assuming that the mRNA is translated to functional protein it becomes possible to speculate on the role for KAL in the normal and aberrant development of the olfactory bulb. In a 19 week old human foetus with a Xpter deletion, initial migration of olfactory and GnRH neurons from the olfactory epithelium to the cribriform plate was not impaired but onward migration of these neurons to the hypothalamus failed to occur. From this study KAL would be expected to be expressed both at and before this stage. KAL expression therefore is not required for the initial migration of nerve fibres from the olfactory placode. Lutz made the important observation that KAL expression was seen in chick olfactory bulb area despite ipsilateral olfactory placode ablation (Lutz et al. 1994). In these animals, no olfactory neurones were present and therefore the initiation of KAL expression in the bulb was independent of this innervation. With these studies in mind it is possible to speculate that KAL expression in the olfactory bulb is necessary for later events, perhaps including the synapsing of olfactory neurons with the bulb and the subsequent migration of GnRH neurones along the pathway provided by the accessory olfactory nerves. In this respect it is of note that neural cell adhesion molecules (NCAM) which has homology to KAL putative protein, has been implicated in the migration of the GnRH neurons into the forebrain along the accessory olfactory nerve.

The function of KAL in other parts of the central nervous system including the eye and the spinal cord are as yet unclear. The neural retina begins to differentiate at the end of the sixth week of human gestation into the outer neuroblastic layer which gives rise to the rods and the cones, and the inner neuroblastic layer which develops into ganglion and supporting cells. Axons then sprout from the ganglion cells, growing towards the optic stalk and back into the brain. The retinal layer is not fully developed until the eighth month of gestation and

it is therefore possible that KAL may be involved in axonal migration or neural migration in the retina. Legouis also reported KAL expression in the developing chick retina at embryonic day 9.5 and 19 (equivalent to 52-57 days and eight months of human development) Transcripts were noted in the amacrine, horizontal and ganglionic layers of neurons.

Interestingly, the dystrophin gene is also expressed in the retina (Schmitz et al. 1993) and in the olfactory bulb (Goreki et al.) The dystrophin gene is mutated in patients with Duchenne Muscular Dystrophy (DMD).

The mechanism underlying bimanual synkinesis in XKS was thought to be due to defective inhibitory neuronal communication between the two cerebral hemispheres (Danek et al. 1992). However, more recent work has suggested that defective decussation of the corticospinal tracts is responsible for this clinical manifestation of XKS. The latter hypothesis is supported by the RT-PCR signal for KAL, which was found in the spinal cord.

6.7.2 Expression in the developing excretory system.

KAL expression was detected at 6.5 weeks gestation in the meso and metanephros using RT-PCR (Fig 6.6). At this stage, however mRNA levels were too low to be detected by the less sensitive *in situ* hybridisation. At this time of development, the paired mesonephric kidneys are fully differentiated. In the male, remnants of the mesonephric duct later in gestation gives rise to the vas deferens. The metanephros is the precursor of the adult kidney and these organs can first be detected in the human at 5 weeks after fertilisation. At 6.5 weeks, when KAL transcripts can be detected by RT-PCR (Fig 6.6). Critical inductive interactions which are known to be essential for normal kidney development are occurring between the renal mesenchyme and the ureteric bud epithelium (Hardman et al. 1994). At 11 weeks of gestation, low levels of KAL transcript were detected in the outer cortex of the developing kidneys (Fig 6.5). In this region nephrons continue to form until 34 weeks gestation. Up to 40% of XKS patients have unilateral absence of the kidney (Kirk et al. 1994) implicating KAL in nephrogenesis. Based on these clinical observations, and on the results of this study, it appears that KAL has a role in the formation or maintenance of the kidney. The homology of the KAL protein to cell-cell and cell-matrix adhesion molecules could explain this role because similar functions have been assigned to both uvomorulin and laminin A chain (Hardman et al. 1994; Klein G et al. 1988). These cell-cell and cell-matrix adhesion molecules are expressed in the transition of undifferentiated mesenchyme into

polarised nephron epithelia.

In conclusion, this study has shown that KAL gene is expressed in diverse tissues in the first trimester of normal human development. Some of these locations correlate directly with the clinical pathologies found in XKS patients who harbour mutations of this gene.

Chapter 7

MUTATION ANALYSIS OF PATIENTS WITH X-LINKED AND SPORADIC KALLMANN'S SYNDROME.

7.1 Introduction

Having studied the role of *KAL* in normal human development, it was necessary to study *KAL* in patients with XKS. By studying this gene in patients using established molecular biology methods two questions could be answered.

i) Is there a mutation hot spot ie. an area of *KAL* where most of the mutations lie? Identifying such an area in KAL may lead to a greater understanding of the functionally important parts of the KAL protein. For example does the mutation lie in a part of the protein involved in receptor binding is it folded on the inside of KAL protein?

ii) Is there a relation between patient phenotype and genotype? Since not all patient share the same clinical features (outside of the anosmia and hypogonadotrophic hypogonadism) eg. synkinesis and renal agenesis is not seen in all the patients. Do specific genotypes predispose to particular phenotypic variants?

7.2 Methods and Materials

The methods outlined in the first part of this chapter are general methods which have been widely used in various parts of this thesis. (7.2.1 to 7.2.7)

7.2.1 DNA extraction from whole blood.

Experimental protocol

1. 10ml of venous blood was taken from the patient in a K EDTA (red top) tube and stored at -20°C.
2. The blood sample was thawed while lysis buffer was prepared and chilled.
3. Blood was transferred to oakridge tubes and the tubes were filled with lysis buffer. The tubes were centrifuged at 15,000rpm for 20 minutes at 4°C.
4. The supernatant was decanted and discarded and the pellet resuspended immediately in

4.5ml of NaCl EDTA..

5. The solution was transferred to 10ml glass tubes and 250µl of 10% SDS added-this acts as a detergent. The tubes were then shaken.

6. 200µl of Proteinase K solution was added to each tube, which were then incubated in a 37°C water bath O/N. The Proteinase K digests proteins in the blood.

7. 5ml of Tris saturated phenol (RT) was added to each tube which was then vigorously shaken. The tubes were then centrifuged at 2000rpm for 10 minutes.

8. The top layer was transferred to a fresh tube, care being taken not to disrupt the interphase. 5ml of chloroform:octanol was added and the tubes vigorously shaken.

9. Tubes were centrifuged at 2000rpm for 10 minutes.

10. Step 8 was repeated.

11. The top phase was transferred to a fresh tube and 0.5ml of cold 3M sodium acetate added.

12. The tube was then filled almost to the top with cold absolute alcohol, and DNA was allowed to precipitate and float up to the top of the tube where it was hooked out using a glass hook.

13. The DNA was then rinsed in 70% ethanol and allowed to air dry.

14. The DNA was resuspended in 0.5ml of T.E. buffer, and then left on a wheel to go into solution for 48 hours. The sample was then stored at 4°C.

15. 25µl of DNA solution was taken and added to 3ml of T.E. buffer and the optical density at 280nm and 260 nm was determined.

The concentration of DNA was calculated as follows:

OD of 1 at 260nm = 50µg/ml of DNA

dilution factor =120 therefore concentration= OD at 260nm x 120 x 50 = µg/ml.

7.2.2 Endonuclease restriction enzyme digestion of DNA.

Restriction enzyme digestion of DNA was performed for several reasons, each of which will be discussed where relevant. Typical conditions were as follows:

Total reaction volume 50µl.

Restriction enzyme 2.5µl or 10units/µg of DNA (one unit of enzyme is the quantity necessary to digest 1µg of DNA in one hour. It is standard practice to use 10units/µg of DNA) The amount of enzyme used was always less than 10% of the total reaction volume.

Restriction buffer supplied by the manufacturer was included in each reaction (5.0µl-always

10% of total volume)

Bovine serum albumin 5.0µl-enhances the reaction but is optional and therefore not always included in the reaction. Approx 10µg of DNA was digested and the volume was made up to 50µl with water. The reaction was prepared in an Eppendorf tube, mixed and then microfuged before incubating in 37°C water bath for a minimum period of 1 hour and a maximum of over night. When more than one enzyme was being used in the same reaction, only one restriction buffer was necessary (see manufacturers notes).

7.2.3 Preparation of agarose gel for electrophoresis.

In order to visualise and estimate size, quantity and approximate concentration of DNA or RNA products, samples were run in agarose gels. The samples resolved on these gels were derived from a variety of sources (eg. DNA extracted from blood, DNA fragments resulting from restriction enzyme digests and PCR products). The % of the gel prepared depended on the size of the DNA fragments being separated. For high molecular weight nucleotide fragments, 0.5 to 0.8% gels were prepared and for low molecular weight nucleotide fragments 1.2 to 2% gels were prepared.

See appendix B.

7.2.4 Isolation of DNA from agarose gels.

DNA was isolated from a gel in order to prepare a probe for hybridisation with a southern or northern blot. The probe is digested out of the vector, and separated by agarose gel electrophoresis and the size ascertained by comparing with standard markers. There are several methods for isolating DNA from agarose gels, some of which rely on digesting the gel so that the DNA is left. Others rely on the DNA being precipitated out of the gel.

7.2.4.1 Isogene Purification Kit.

1. The target band of DNA was cut out of the gel while on the UV transilluminator and placed in a pre-weighed tube.
2. The tube containing the agarose was weighed and the weight multiplied by two to give the volume of NaI needed.
3. Once the NaI was added, the tube was incubated at 60°C for ten minutes.
4. The tube was vortexed and placed on ice until cool.
5. The DNA binder was resuspended by vortexing and then according to the volume of

agarose, the following was added.

Volume of agarose (µl)	Volume of DNA binder (µl)
25	10
50	20
100	20
200	30
300	40
400	45

6.Samples were mixed at room temperature on shaker for 10 minutes and then microfuged for 10 seconds.

7. The supernatant was discarded and the pellet resuspended in 400µl of cold wash buffer (0.1ml of stock buffer in 10ml of ethanol).

8. The pellet was vortexed, microfuged and the supernatant discarded twice.

9. 20µl of cold water was then added to the pellet, which was vortexed and then microfuged and the water containing DNA removed and placed in a fresh tube.

10. This was repeated giving DNA in a final volume of 40µl. 2µl of the DNA solution was then checked on an agarose gel.

7.2.4.2 β Agarase 1 Purification kit.

1. Gel was dissolved at 65°C, 10% buffer was added, and the gel incubated for a further 10 minutes.

2. The gel was left to cool before 1 unit of agarase enzyme was added for 200µl of gel solution. This was incubated for 1 hour at 40°C.

3. 10% volume of Na acetate was added to the tube, the tube mixed and placed on ice for 15 minutes.

4. The tube was microfuged for 15 minutes and the supernatant transferred to a fresh tube.

5. Two volumes of isopropanol was added to the supernatant, the tubes frozen at -70° for 15 minutes and then microfuged for 15 minutes.

6. The supernatant was discarded and the pellet washed in 70% ethanol before being left to air dry.

7. The pellet was then resuspended in 30µl of water. 2µl was checked on an agarose gel.

7.2.5 Transformation of KAL cDNA

A small aliquot of KAL cDNA was kindly provided by Dr.C.Petit of the Pasteur Institute. This was transformed into E Coli in order to generate large amounts of the DNA which would then be used for future experiments including *in situ* hybridisation.

1.10ng of KAL cDNA was aliquoted into a chilled Eppendorf.
Stock was 1.5µg/µl or 1500ng/µl
Stock was diluted 1µl in 600µl of sterile water to give a concentration of 2.5ng/µl .
4µl of the diluted stock was then taken.

2. The competent E Coli cells were removed from the freezer and allowed to thaw out on ice.

3. 100µl of cells were added to the 4µl of cDNA, the tube gently mixed and the cells left on ice for 30 minutes. The remaining cells were placed on an ice bath (ethanol and dry ice) to refreeze them before replacing them in the -70°C freezer.

4. The cells and DNA were heat shocked by placing them in a 42°C water bath for exactly 45 seconds and then placing tubes on ice for 2 minutes.

5. 900µl of SOC was added to the cells care taken to flame the mouth of the bottle. Future procedures were performed aseptically.

6.The cells and SOC were transferred to 10ml tubes and placed in a shaking incubator at 37°C for 1 hour.

7. Three previously prepared agar plates were used to spread the cell culture over using an ethanol dipped and flamed glass spreader as follows:
plate 1 - 100µl
plate 2 - 300µl
plate 3 - 500µl
The plates were left on the bench top for an hour to dry and then placed in a 37°C incubator over night.

8. The following morning, the plates were removed from the incubator and four 5ml minicultures were set up with colonies picked from these plates. The LB in which the minicultures were to grow had ampicillin added to it. Ampicillin was used at a concentration of 100µg/ml taken from a 50mg/ml stock solution.
The colonies were picked from the 500µl plate and added to the 5ml LB/ampicillin in 50ml tubes which were placed in the shaking incubator at 37°C O/N.

7.2.6 Small scale preparation of plasmid DNA-minipreps

This method was performed in order to ensure that the E Coli had been transformed.

1.1.5mls of the 5ml overnight culture was removed and placed in an Eppendorf tube which was microfuged for 30 seconds in the cold room.

2.The media was discarded and the bacterial pellet resuspended in 100µl of ice cold Solution 1 by vigorously vortexing the tubes to obtain a white cloudy solution.

3.200µl of freshly prepared solution 2 was then added, the tubes mixed by inversion and then placed on ice for a few minutes.

4. 150µl of ice cold solution 3 was added, the tubes gently vortexed and then left to stand on ice for 5 minutes.

5. The tubes were then microfuged in the cold room for 5 minutes.

6. The supernatant was then transferred to fresh tubes (450µl) and 500µl of phenol:chloroform added. The tubes were vortexed and then microfuged for 2 minutes in the cold room.

7. The supernatant was transferred to fresh tubes and DNA precipitated by adding 1ml of ethanol. The tubes were vortexed and then left to stand at room temperature for 2 minutes.

8. The tubes were then microfuged for 5 minutes in the cold room after which the ethanol was removed from the tubes and replaced with 200µl of ice cold 70% ethanol. Tubes were respun, the ethanol removed and the DNA pellet left to air dry.

9. The DNA pellet was resuspended in 20µl of TE buffer and 1µl of RNAase added.

10. 2µl of this DNA solution was then taken and digested as follows to ensure that KAL cDNA that had been grown up and isolated according to the following protocol:

2µl DNA

8µl water

1µl buffer

3µl Cla 1 enzyme

3µl Bam H1 enzyme

This was placed in 37°C water bath for 1 hour and then checked on a 1% agarose gel.

7.2.7. Large Scale preparation of plasmid DNA-Maxiprep

Once transformation had been confirmed to have been successful, the miniprep culture was taken (3.5ml) and added to 250ml of LB containing ampicillin (25mgs) and incubated overnight at 37° in a shaking incubator.

1. The 250ml overnight culture was poured into centrifuge flasks and centrifuged at 6000 rpm for 10 minutes at 4°C.
2. The medium was discarded and the cell pellet resuspended in 8.8ml of ice cold Solution 1 by drawing the solution and pellet up and down a pipette repeatedly.
3. 10mg of lysozyme was carefully weighed out and dissolved in 1ml of sterile water. This was added to the cell pellet which was left at room temperature for 10 minutes.
4. 17ml of freshly prepared Solution 2 was added and the flask was inverted a few times in order to mix the solution. The flask was then left on ice for 10 minutes.
5. 13ml of ice cold Solution 3 was added and the flask gently swirled around in order to mix contents. The flask was left on ice for 15 minutes.
6. The viscous solution was transferred to a sterile 50 ml tube, which was centrifuged for 20 minutes at 14,000rpm at 4°C. This spin would pellet out the protein in the solution.
7. The supernatant was transferred into two fresh sterile 50ml tubes. 10.5ml of isopropanol was added to each tube and the tubes mixed and left to stand at room temperature for 20 minutes.
8. The tubes were then centrifuged at 12,000 rpm for 20 minutes at 20°C.
9. The supernatant was poured away to leave the DNA. The pellet was washed in 70% ethanol, and centrifuged for a further 10 minutes at 12,000 rpm at 4°C to remove any remaining ethanol.
10. The pellet was dried in a vacuum dryer and then resuspended in 4ml of TE buffer. The separate DNA solutions were then pooled together.
11. 0.4ml of Tris and 0.4ml of Na EDTA was added to the DNA solution as well as 2.7ml of sterile water.
12. The 11.5ml of DNA solution was added to 12.7g of CsCl which had been weighed out in fresh tube. The tube was gently shaken to allow the CsCl to dissolve.
13. 1.2ml of EtBr was very carefully added to the tubes which were then spun in the bench top centrifuge at 2500 rpm for 10 minutes.
14. Using the barrel of a syringe as a funnel, the DNA CsCl mixture was transferred to an Ultra tube, great care taken to balance them.

15. The tubes were filled to the very top with liquid paraffin and loaded into the tube using a syringe.

16.The Ultra tubes were sealed with both plastic and metal caps and then centrifuged for 48 hours at 48,000rpm at 20°C.

17. At the end of the spin, the centrifuge was switched off and allowed time to slow down and stop fully. The Ultra tubes were very carefully removed and transferred to the dark room. Using long wave UV light, a large plasmid DNA band could be seen in the centre of the tube.

18.A needle was carefully inserted into the tip of the tube in order to allow air in and out of the tube. A needle and syringe was then used to draw the DNA band out of the tube. This was transferred to a fresh tube, and the rest of the Ultra tube carefully disposed of.

19. 5ml of CsCl saturated isopropanol was added to the DNA band. The tube was shaken and then allowed to settle, revealing two phases.

20. The top phase, pink in colour was removed and discarded. The bottom was kept and mixed with 5ml of CsCl saturated isopropanol twice more.

21. The DNA solution was transferred to a fresh Oakridge tube and the volume made up to 10 ml with water.

22. 1ml of Na acetate and 20ml of ice cold ethanol was added and the tubes were then frozen at -80 for 2 hours.

23. The tubes were centrifuged at 12,000rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet washed in ice cold 70% ethanol as above and then left to dry. Once dry, the pellet was resuspended in 400ul of TE buffer.

24. The optical density of the DNA solution at 260nm and 280nm was measured in order to calculate the concentration of the DNA.

25. A small aliquot of the DNA was digested using Bam H1 and Cla 1 to be sure that the correct plasmid DNA had been extracted.

7.2.8 Southern blotting of DNA samples

The transfer of DNA from an agarose gel to a membrane filter by capillary blotting was originally described by Southern (1975). The immobilisation of DNA on a membrane allows it to be hybridised with specific probes.

1.DNA from KS patients was digested with EcoR1 and HindII respectively. Digested DNA was loaded onto a 3% agarose gel (appendix B) and electrophoresed overnight at 30 Volt.

2.The gel was removed the following morning and photographed.

3. The gel was placed in a shallow plastic tray which was filled with 0.25M HCl and left on shaker gently shaking for 20 minutes.

4. The HCl was drained off and the gel rinsed in distilled water. The tray was filled with denaturing buffer and the gel replaced on the shaker for 1 hour.

5. The denaturing buffer was drained off and the gel rinsed in distilled water. The tray was refilled with neutralization buffer and the gel replaced on the shaker for 15 minutes.

6. Step 5 was repeated.

7. A capillary blot was set up as follows:

A tray was filled with 20 x SSC and a platform placed in the tray over which a wick was placed (made from 3MM filter paper saturated with 20 x SSC).

8. The gel was placed face down on the platform and a glass rod carefully rolled over the gel to ensure that no air bubbles were trapped under the gel.

9. A sheet of Hybond N+ (Amersham) was carefully placed on top of the gel, care taken not to trap air under the membrane, and at the same time ensuring that the gel was completely covered with the membrane.

10.Three sheets of 3MM filter paper were cut to exactly the same size as the membrane and gel, soaked in buffer, and carefully layed on top of the membrane.

11.A stack of absorbent paper towels were placed on top of the filter paper followed by a glass plate to ensure that the towels were level and covering the filter paper evenly. Finally a 1Kg weight was placed on top of the glass plate and the transfer allowed to proceed O/N.

12.After blotting the apparatus was carefully dismantled and the membrane marked with a pencil with the date and details of the blot for future identification.

13. The membrane was carefully and briefly rinsed with 2 X SSC and then wrapped in cling film before fixing the DNA onto the membrane by placing it on a UV transilluminator for 3 minutes. Care was taken to ensure that every part of the membrane was fixed in this way.

14. Once fixed, the membrane was stored wrapped in plastic in the -20°C freezer until needed for hybridisation.

7.2.9 Radioactive labelling of DNA probes.

In most cases the probe used to probe the Southern blots was one of the exons of the KAL gene or the KAL cDNA. A PCR would be performed in order to amplify the exon from a normal control, the products of the PCR reaction run on an agarose gel and isolated.

7.2.9.1Oligolabelling Kit-Pharmacia 27-9250-01

Using this kit the DNA was denatured and then mixed with hexadeoxyribonucleotides of random sequences. The random hexamers bind to random sites on the template DNA and act as primers for DNA synthesis by the Klenow fragments. With radiolabelled nucleotide present during this synthesis, highly labelled DNA could be generated. Since Klenow fragments do not have a 5' to 3' exonuclease activity, the labelled nucleotide will not be excised.

1. 10ng to 1µg of DNA in solution was denatured by heating to 100°C on a hot block and then snap cooled on ice.
2. 10µl of Reagent mix was added together with 5µl of αP^{32} dCTP, the volume made up to 49µl with distilled water.
3. 1µl of Klenow fragment was added, the tube gently mixed and microfuged.
4. The tube was incubated in 37°C water bath for 1 hour.
5. The reaction was terminated by adding 5µl of 0.2M EDTA. The volume was then increased by adding 45µl of water.
6. The probe was separated from unincorporated nucleotide by passing it down a separation column . A piece of glass wool was pushed to the end of a 1ml syringe which was filled with a solution of Sephadex G-50. The syringe was placed in a 10ml plastic tube and centrifuged at 1500rpm in order to pack the column.
7.The labelled probe was layered onto the column and the syringe was placed in a fresh tube and centrifuged at 1500rpm for 5 minutes.
8 The elute from the column was collected, comprising the labelled probe. It could be stored at -20°C for up to one week or used immediately.

7.2.9.2 T7 Quick Prime kit-Pharmacia 27-9252-01

The template DNA was denatured and then mixed with oligodeoxyribonucleotides of random sequence. These oligomers annealed to random sites on the template DNA and then served as primers for DNA synthesis by T7 DNA polymerase. With labelled nucleotide present during the synthesis it was possible to generate large amounts of labelled probe.

1. 25ng to 50ng of template DNA in solution was denatured.
2. 10µl of reagent mix was added to template together with 5µl of αP^{32} dCTP. The volume was made up to 49µl with distilled water.
3. 1µl of T7 DNA polymerase was added, the tube was mixed, microfuged and incubated

in 37°C water bath for 15 minutes.

4. The reaction was terminated and probe separated from unincorporated nucleotide as above.

For both kits the probe was either stored frozen for up to 1 week or used immediately. In the latter case, it was heated to 100°C for 5 minutes and then snap chilled on ice and added to prehybridised membrane.

7.2.9.3 *Redi*prime random primer DNA labelling system- Amersham RPN 1631

1.Template DNA was diluted to 2.5ng to 5.0ng concentration in 45µl of water.

2. The DNA was denatured by heating to 100°C for five minutes. The tube was then microfuged.

3.The template DNA was added to the labelling mix and the tube flicked in order to mix the contents and reconstitute the labelling mix (Colour changed to blue). The tube was then microfuged.

4. 5µl of Redivue αP^{32} dCTP was added and the tube mixed gently.

5. The tube was incubated for 10 minutes at 37°C.

6. The reaction was stopped by adding 5µl of 0.2M EDTA. The probe was then denatured by heating and then adding to the prehybridised membrane.

7.2.10 Hybridisation of DNA probe to Southern blot

1.Prehybridisation solution was prepared and added to the membrane, which had thawed out, and was placed in a plastic bag.

2.The bag was sealed and the membrane left in a shaking water bath for at least one hour at 65°C.

3. The radio labelled KAL cDNA probe was denatured by heating to 100°C and then immediately chilled on ice.

4. The probe was added to the prehybridisation solution and the bag resealed and replaced in the 65°C shaking water bath for over night incubation.

5.The following day the membrane was removed form the hybridisation fluid and washed in 2 X SSPE, 0.1% SDS at room temperature for 15 minutes, twice.

6. The membrane was then washed in 1 X SSPE, 0.1% SDS at 65°C for 15 minutes.

7. Finally the membrane was washed in 0.1 X SSPE, 0.1% SDS at 65°C for 15 minutes.

This was a high stringency wash ie. high salt concentration wash which is intended to reduce background hybridisation, and was not always included. It depended on how fresh the radioactivity was as to whether the warm washes were performed.

8.The membrane was finally wrapped in Saran wrap and autoradiographed over night.

7.2.11 Polymerase Chain Reaction-PCR.

This method is used to selectively amplify in vitro DNA or RNA sequences of interest from nanogram amounts of original template. The procedure generally depends on the presence and knowledge of the sequences that flank the region of interest. A pair of synthetic oligonucleotide primers complementary to the nucleotide compositions of the 5' ends of the sense and anti-sense flanking DNA segments respectively are synthesised and added to the DNA.

The three stages of the PCR reaction are, denaturation, primer annealing and extension. They are performed successively on the samples for 20 to 60 cycles and can achieve amplification of up to 10^8 to 10^{12} times. The double stranded DNA is denatured by heating to 94°C. On cooling to 55°C, the oligonucleotide primers anneal to complementary single DNA strands. Extension of the primers in the presence of DNA polymerase, the four dNTPs and magnesium ions occurs at an intermediate temperature of 72°C. The ability of the thermostable enzyme Taq polymerase to withstand repeated short exposures to high temperatures allows the PCR reaction to proceed without interruption and adding of new enzyme between each cycle.

PCR of individual exons were performed in X-linked and sporadic patients thus establishing whether the exon was present or not in each case. PCR can not reveal if the exon was harbouring a point mutation without sequencing it, although complete deletions could be identified using this method.

The primers and a protocol were kindly supplied by C.Petit (Pasteur Institute Paris). Hardelin Human Mol Gen 1993 & Hardelin PNAS 1992. However there were some modifications required as some of the PCR had not been optimised.

Oligonucleotides used for amplification of the KAL exons and splice sites.

Exon	PCR Primers 5' to 3'	Annealing temp (°C)	Amplified product (bp)
1	CCTCGCCCTCGCCCTCGACCCGCAG	63	329
	GAACTTTGCGAGCCCAGGCTGGGAG	(10% DMSO)	
2	TTGGAAGGGAAGGACAGCAGG	55	230
	GCACCATTCATACAGGTATAG		
3	TCTCAGCTTTGTTTGTTTCCA	55	174
	CGTAAGCATAGTCAGATTTGG		
4	ATGTCTTGGAAATCAGACTTC	55	334
	ATGTGACACTGCATGTGTCTT		
5	CAGATTGTTTTAATTGATACG	55	267
	GCAGACACTACCTCCAGGATG		
6	AGTGACATGTTCCCTGTGCTC	55	218
	CTGGTAGCAAGGATAGTATTC		
7	ATGATGTGTCTTTGTACTGGG	55	269
	TGGGAATAACAATCCTTCCTC		
8	GACGTGGAAGGTTTGTAACGC	55	237
	ATCATGTCACAATCATCTTGA		
9	TGCCCAGGAATCTATAATTAC	55	266
	ACTATCTCTATATTACTGTGC		
10	ACCTGGAATGTAACATCCAGC	55	293
	ACCATTCTGCTTTCCACTTCC	(10% DMSO)	
11	CATCCTTGTTGGATGGAATATG		278
	AATATGATTTCAATTCTTGCC	55	262
11C	GATGTAGAAGTCCTTCAGGTG		
12	TCTCCAGTCGCCTAATCCTGG	55	302
	CCAATGACACAGACATAGTAC		
13	GTGCATTGCATGTTGTCTCTG	55	241
	TGACAGGATGGCTTAATGCCC		
14	ATGTTACTGACATATTTTGTC	55	147
	GGCCGAAGTTCAACAAGCTTA		
14C	GCTTACACATCTGTGCAATTTCAC		131

Table 7.1 PCR primers used to amplify KAL

These primers were used at a concentration of 12.5µM in 50µl reaction therefore 0.125µM or 125nm each.

1µg of DNA was used in each reaction.

The volume of buffer was always 5µl this being 10% of the total reaction volume.

Exons 1 and 10 needed 10% DMSO to be added for the reaction to occur.

Although the annealing temperature had been given together with the other cycling conditions, the optimal amount of $MgCl_2$ and dNTPs was not known and therefore had to be determined. A stock of dNTPs was prepared; Stock of each nucleotide was 100mM. 10µl of each was taken, giving a concentration of 25mM. The volume was then made up to 100µl with sterile water, the final concentration of each nucleotide being 10mM.

In each and every PCR reaction the following controls were included. A normal control, a patient known to be deleted for KAL and a water control. This reaction contained the primers, Taq polymerase enzyme, dNTPs, $MgCl_2$, PCR buffer and water only and no DNA. This ruled out the presence of contaminants in the components of the reaction.

7.2.11.1 Exon 1:

The published data suggested that the annealing temperature should be higher for this primer than the temperature used in the other reactions and that 10% DMSO was needed. The following reactions were performed over a gradient of temperatures from 60°C each degree till 65°C in order to identify the optimal temperature for annealing of exon 1.

DNA (µg)	buffer (µl)	primers µl each	Taq (µl)	dNTPµ 10mM	water (µl)	$MgCl_2$ mM	DMSO (µl)
1	5	0.5	0.25	1	36.45	0.8	5
1	5	0.5	0.25	1	36.35	0.9	5
1	5	0.5	0.25	1	36.25	1.0	5
1	5	0.5	0.25	1	36.15	1.1	5
1	5	0.5	0.25	1	36.05	1.2	5
1	5	0.5	0.25	1	36.00	1.25	5
1	5	0.5	0.25	1	35.75	1.5	5
1	5	0.5	0.25	1	35.50	1.75	5
1	5	0.5	0.25	1	35.25	2.0	5

Table 7.2: Experimental conditions used to optimise PCR for Exon 1 of KAL

The identical experiment was then performed without the DMSO being added. ie. changing the temperature and the $MgCl_2$ concentration for each experiment.

7.2.11.2 Exon 2 to 14:

Although the conditions for the PCR reactions had been given the following experiment was performed in order to confirm that they were optimal. The concentration of $MgCl_2$ was maintained the same in each experiment ie. 1mM (1µl of 50mM stock in a 50µl reaction.) The concentration of dNTPs was varied.

	buffer (µl)	water (µl)	DNA (µg)	primers (µl each)	$MgCl_2$ µl of 50mM	Taq (µl)	dNTPs µl 10mM
A	5	40.75	1= 1.8µl	0.5	1	0.25	0.2
B	5	40.55	1=1.8µl	0.5	1	0.25	0.4
C	5	40.35	1=1.8µl	0.5	1	0.25	0.6
D	5	40.15	1=1.8µl	0.5	1	0.25	0.8
E	5	39.95	1=1.8µl	0.5	1	0.25	1.0

Table 7.3 Experiment to optimise PCR for Exons 2 to 14 of KAL

This optimising was performed for each exon so that the following conditions could be used to screen each exon.

exon	buffer µl	water µl	DMSO µl	primers µl each	dNTPs 10mM µl	$MgCl_2$ 50mM µl	Taq µl
1	5	35.5	5	0.5	1	1	0.5
2	5	40.5	-	0.5	1	1	0.5
3	5	40.5	-	0.5	1	1	0.5
4	5	40.5	-	0.5	1	1	0.5
5	5	40.5	-	0.5	1	1	0.5
6	5	40.5	-	0.5	1	1	0.5
7	5	38.5	-	0.5	1	3	0.5
8	5	40.5	-	0.5	1	1	0.5
9	5	39.5	-	0.5	1	2	0.5
10	5	40.5	-	0.5	1	1	0.5
11	5	38.5	-	0.5	1	3	0.5
12	5	40.5	-	0.5	1	1	0.5
13	5	40.5	-	0.5	1	1	0.5
14	5	40.5	-	0.5	1	1	0.5

Table 7.4 Optimised PCR conditions for amplification of KAL

7.2.11.3 Design of new PCR primers for exons 11 and 14 of KAL

When PCR reactions were performed using primers for exons 11 and 14 the negative controls amplified up bands of the correct sizes and then later when sequenced were found to be the correct sequence for the particular exon. This suggested that the PCR primers were not X specific as originally thought and that the primers were amplifying up sequence from the Y chromosome. Our collaborators in Paris were subsequently able to send the intronic sequence for both exons 11 and 14.

The original sequence of the primers for Exon 14 were:

ATGTGAACTGACATATTTTGTCA

GGCCGAAGTTCAACAAGCTTA

Inspection of X and Y sequences revealed that these sequences were present and identical in both chromosome sequences, thus explaining why every PCR reaction amplified the sequence even in the negative control. New primer were then designed.

For Exon 11 exactly the same was done by comparing X and Y sequences in order to design a new forward primer.

Exon 11X CATCCTT**G**TTGGATGGAATAT**G**A

InEX11Y CATCCTT**A**TTGGATGGAATATT**A**

The two differences in this sequence were sufficient to use the X sequence as a primer to amplify X specific sequence.

7.2.12 Single stranded conformation polymorphism analysis-SSCP.

This method allows the separation of DNA on the basis of size and sequence. A PCR reaction is performed in which P^{32} dCTP is included, so that the amplified products are radio-labelled and therefore easily detectable by autoradiography. The products are then diluted, and denatured both by heat and chemically. They are then run on a non-denaturing polyacrylamide gel. While running through this gel the single strands of DNA will re-anneal and according to the sequence of the sample the bands seen on the gel will vary accordingly. A change in sequence will cause a change in conformation of the DNA and therefore a shift in the bands seen on the autoradiograph (Olaf Hiort et al. 1993; A.Batch et al. 1992; Anu Suomalainen et al. 1992; R.Whittall et al. 1995).

The experiment was performed first using patients with a known mutation to ensure that a difference in conformation could in fact be seen before using patients whose genotype was unknown. Family A had a base substitution (G to A) causing a STOP codon in exon 5 (PNAS vol 89).

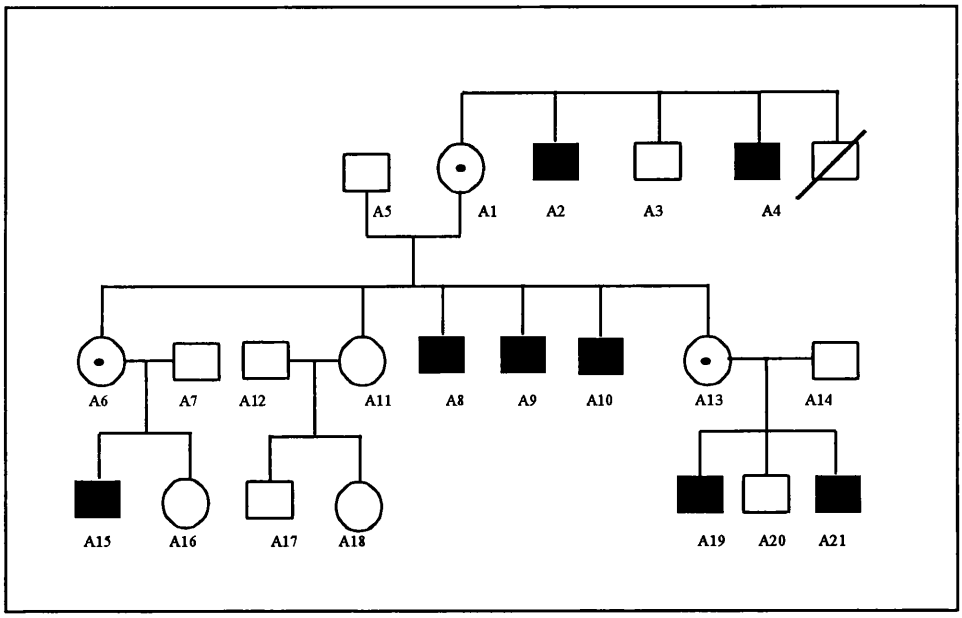


Fig 7.1 Family A-pedigree with XKS.

Family D have a base substitution in Exon 9 which caused a STOP codon (C to T).

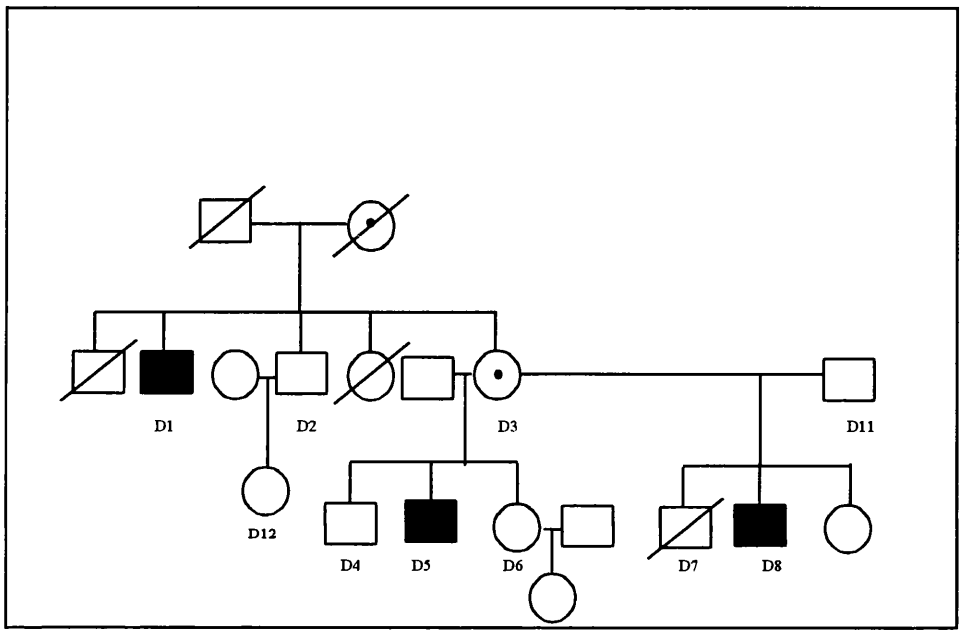


Fig 7.2 Family D pedigree with X

Family W also have a base substitution, (G to A) in Exon 6 also causing a premature STOP codon.

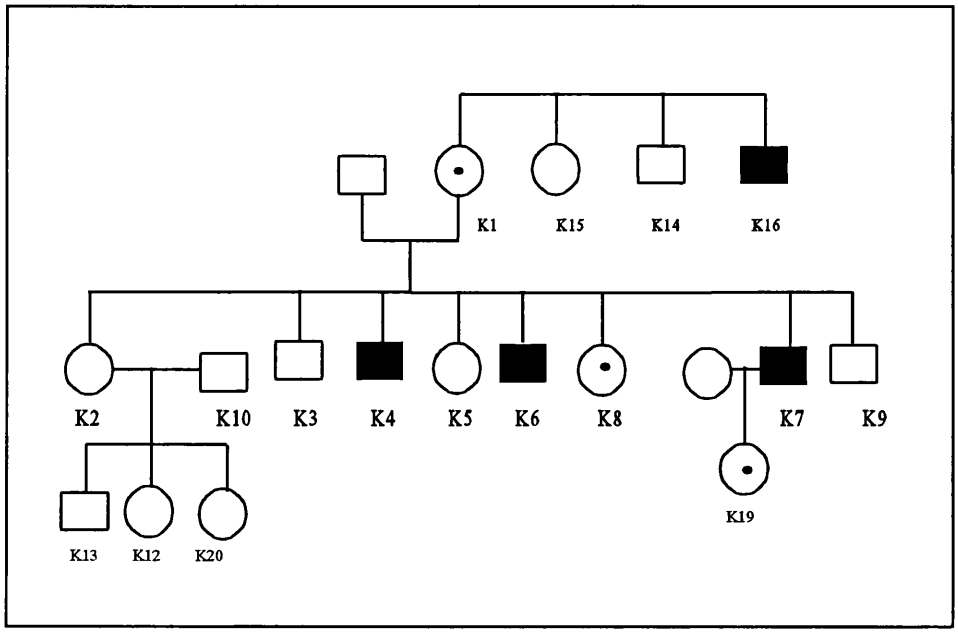


Fig. 7.3 Family W pedigree with XKS

Experimental protocol:

1. The PCR reactions were prepared as follows: total volume 25µl

2µl 10x reaction buffer,

1µl MgCl

0.5µl dNTPs

0.5µl each primer

0.125µl Taq polymerase (Bioline UK)

5µg of DNA

Concentrations of reagents were the same as the PCR reactions 7.2.11

2. The PCR reaction was performed for 25 cycles after which time the controls included in each reaction were tested on an agarose gel to ensure that the correct size products had been amplified.

3. 0.1µl of P^{32} (1uCi=37KBq, stock is 18.5MBq in 50µl, therefore 1µl= 0.37MBq need 0.1µl which is 370KBq) was added to each tube together with 0.9µl of 10x buffer. The PCR reaction was then performed for a further 5 cycles.

4. 2µl of the final PCR product was taken and added to 2.5µl of denaturant (10% SDS, 10mM EDTA). The tubes were then vortexed.

5. 3.5µl of STOP dye was added to the denatured DNA and the mixture heated for 5mins at 95°C and then snap frozen on ice.

6. 3µl of sample was loaded onto a pre run 7.5% acrylamide gel.(see appendix B)

7. The gel was electrophoresed for 15 hours at 15 Watts.

8. The gel was dismantled and dried before putting down with photographic film and leaving to autoradiogprah for 48 hours.

7.2.13 DNA Sequencing direct from PCR products.

DNA sequencing was performed according to the chain termination method designed by Sanger. The DNA to be sequenced acts as a template for the enzymatic synthesis of new DNA starting at a defined primer binding site. A mixture of deoxy and dideoxynucleotides was used in this reaction, at a concentration that creates a finite probability that a dideoxynucleotide will be incorporated in place of a usual deoxynucleotide. Incorporation of a dideoxynucleotide will block further chain elongation, resulting in a population of

truncated fragments of varying lengths. The identity of the nucleotide on the end of the chain can be identified by running four separate reactions, each with only one dideoxynucleotide (ddATP, ddCTP, ddGTP and ddTTP). The resulting population of molecules can then be separated according to size on an acrylamide gel.

In this case DNA sequencing was performed using a kit purchased from Amersham Life Sciences- Product number 70170. Samples were taken from a group of patients whose mutations had already been characterised to optimise the method before using it on new patients. An example of its application was to determine whether A21(Fig. 7.1) the new born child of Family A was affected with KS or not, the family already having one affected male child (A19).

1. Once the PCR had been performed and checked, the product underwent a two step enzyme treatment in order to remove residual single stranded primers and single stranded DNA (Exonuclease 1). The shrimp alkali phosphatase removed remaining dNTPs from the mixture. These enzymes were active at 37°C for 15 mins and inactivated by heating to 80°C for 15 mins

5µl of PCR amplification mixture was taken and 1µl of exonuclease 1 was added to it and incubated at 37°C for 15 minutes and then inactivated.

1µl of Shrimp alkaline phosphatase was then added and incubated in the same way as the other enzyme.

2. 5µl of treated PCR product was taken and 1µl of primer (10pmol/µl) added and the volume made up to 10µl with sterile water. The mixture was heated to 100°C for 3 minutes and then placed on ice to cool for 5 minutes. This allowed the primer to anneal to the template.

3. The annealed primer to template was then labelled with S^{35} ATP in the following reaction.

Buffer	2µl
DTT 0.1M	1µl
1:5 diluted labelling mix	2µl
radio labelled dATP	0.5µl
Sequenase DNA polymerase	2µl

The reaction was mixed and left at room temperature for 5 minutes.

4. This labelling reaction was terminated by adding 3.5µl of the labelling reaction to 2.5µl

of each termination mix; dATP, dCTP, dGTP and dTTP. The termination mixes had been aliquoted out into different coloured tubes and pre-warmed to 37°C in a water bath prior to use.

5. The label and termination mixes were incubated at 37°C for 10 minutes and the reaction was stopped by adding 4µl of STOP solution.

6. The samples were then ready to be stored at -20°C or used immediately.

7.2.13.1 Preparation of electrophoresis equipment

Equipment constructed in our laboratory was used as well as apparatus manufactured by Flowgen and Gibco BRL were used. The plates were 45cm long and spacers were 0.4mm thick. The combs used were sharkstooth combs. Glass plates were cleaned with warm soapy water and then with distilled water and finally with ethanol and then dried. One of the plates was coated with dimethldichlorosilane (Repli coat BDH). This would later allow the plates to be separated more easily and ensure that the gel stayed on the plate that has not been coated. Plastic spacers were then placed between the two glass plates and the plates sealed together using insulating tape.

7.2.13.2 Preparation of polyacrylamide gel

A denaturing 6% acrylamide gel was prepared. (see Appendix B)

The gel was pre run once the comb had been placed in the gel and flushed out using 1X glycerol tolerant buffer. Sample buffer was loaded into the wells to ensure that the comb was correctly positioned and that the wells had no air bubbles. The gel was pre-run for 30 minutes at 45Watts.

The DNA samples to be sequenced were placed on the dry heating block at 100°C for 3 minutes and then placed on ice before being loaded onto the gel. The wells of the gel were flushed out before loading and then 2µl of sample was loaded in to each well. One set of reactions was loaded at a time (ie. 4 tubes A,C,G, and T) and the gel run at constant 45Watts power for 6 hours.

Once the gel had run, it was removed from the tank and the glass plates separated so that the gel remained on the non-silonised glass plate. The gel was lowered into a tank containing 10% methanol 5% acetic acid in water, and it remained in the tank for 20 minutes to remove

the urea from the gel.

The glass plate was removed from the tank and the gel carefully blotted onto a piece of 3MM paper. The gel was covered in Saran wrap and placed on a vacuum dryer at 80°C for one hour.

The gel was then placed in a photographic cassette O/N with fast film and left at room temperature.

7.2.14 Automated sequencing of PCR products.

Since the number of samples being handled was considerable and the time in which to process them was short, a number of samples were sent to be sequenced on an ABI automated sequencer.

Unlike manual sequencing methods, which use a radioactive label to visualize the banding pattern by autoradiography, automated sequencing uses a scanning laser to detect DNA fragments labelled with fluorescent dyes. The dye is added to the dideoxynucleotides, the reaction is performed in one tube, and run on one lane of the gel. Each dideoxynucleotide has another coloured dye linked to it so that when it terminates a strand of DNA each nucleotide fluoresce another colour and the results are seen as different coloured peaks on a chromatograph.

The samples sequenced were from the group of patients with XKS that had been screened for mutations by PCR and found to have all their exons present. The reason for sequencing all the exons in these patients was, given their phenotypic features, a mutation was suspected in their *KAL* gene.

1. The PCR reaction for each exon was performed as above.
2. The products were passed through a QIAquick column to remove any oil, unused Taq polymerase and primers before sequencing.
3. The DNA was eluted off in 50µl of T.E buffer.

The DNA samples were sent away and the results were sent in the form of coloured chromatographs.

7.2.15 Restriction fragment length polymorphism (RFLP) analysis to identify carriers of XKS.

For each of the families known to us to harbour a point mutation within a particular exon of *KAL* a detailed restriction map of that exon was constructed (Families A, W, D and B Figs.7.1, 7.2 and 7.3). In three out of four pedigrees the mutation was predicted to destroy an enzyme restriction site. The RFLP pattern generated in each individual within these pedigrees identified affected males, unaffected males and carrier females (Odile Grau and Remy Griffais, 1994).

1. DNA was extracted from whole blood and 100µg was included in a PCR reaction in order to amplify the exon of interest.
2. PCR products were purified using QIAquick columns (Quiagen) and the DNA was eluted in 50µl of Tris buffer.
3. 10µl of purified DNA was digested with 10 units of restriction enzyme and incubated at appropriate temperatures for two hours. (Bsc 1 at 65°C and Rsa 1 and Age 1 at 37°C.)
4. 5µl of uncut DNA was loaded onto a 2% gel alongside 15µl of digested DNA, and the gel was electrophoresed , stained and photographed.(see appendix B)

7.3 Results of mutation analysis of patients with X-linked and sporadic KS.

7.3.1 Transformation of KAL cDNA followed by small and large scale plasmid DNA preparations.

Competent cell were successfully transformed with the *KAL* cDNA. The cells grew well and large amounts of plasmid DNA were successfully isolated from the maxi-preps. This DNA was digested with the appropriate enzymes to ensure that *KAL* had been isolated.

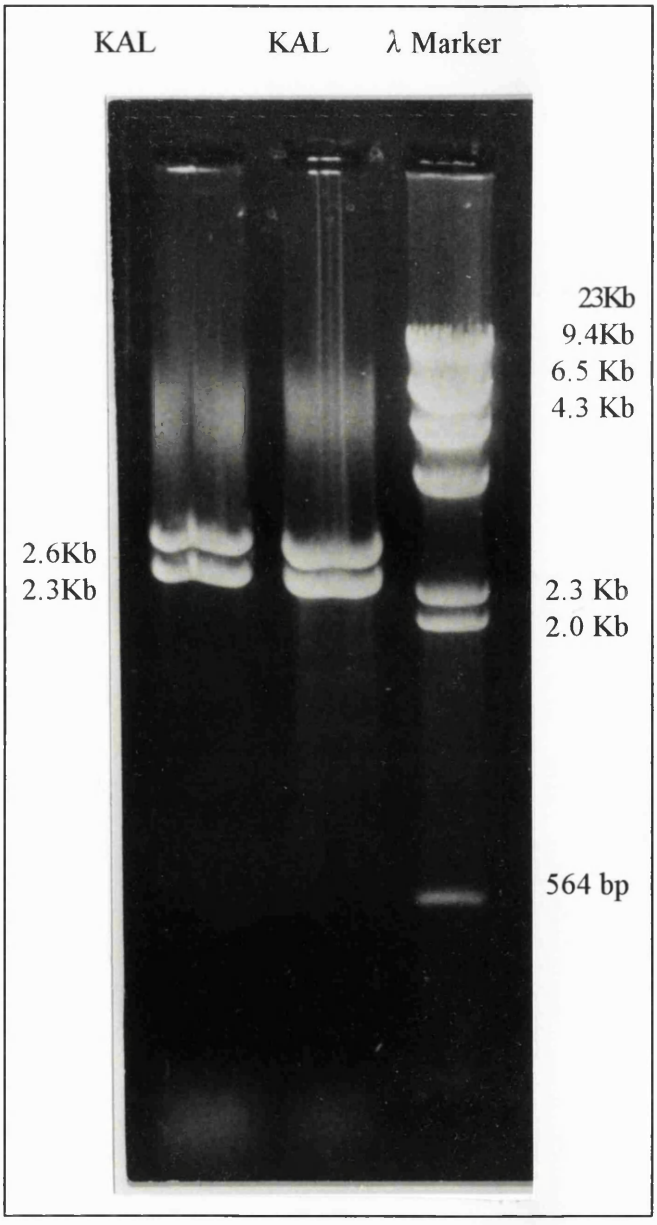


Fig 7.4 Bam H1 and Cal digests of KAL cDNA inserted in Bluescript vector.

7.3.2 Hybridisation of KAL cDNA to southern blot of patients DNA

DNA from patient with XKS and sporadic KS was digested with HindIII (Lanes 1 to 5) and EcoR1 (Lanes 9 to 13) and then run on an agarose gel with 1 Kb ladder molecular weight marker O/N. (Fig 7.5). The DNA on the gel was then transferred by Southern blotting.

Fig 7.5 Agarose gel with EcoR1 and Hind 111 digested DNA used for Southern blot

The blot was probed with the *KAL* cDNA and the following result was obtained (Fig 7.6). NC, PD and DB showed no hybridisation of the probe suggesting that *KAL* is deleted in these patients. However, AT and ANN showed a variety of bands suggesting that *KAL* had hybridised to the DNA on the blot. The pattern of bands seen with the two different enzymes is different as expected since the enzymes would have cleaved the DNA into different sized fragments. The probe also hybridised to the marker, although washed to high stringency. Background hybridisation was evident and it is possible that further washes would have decreased this background. In spite of this, it was possible to identify which of these patients had deletions of KAL.

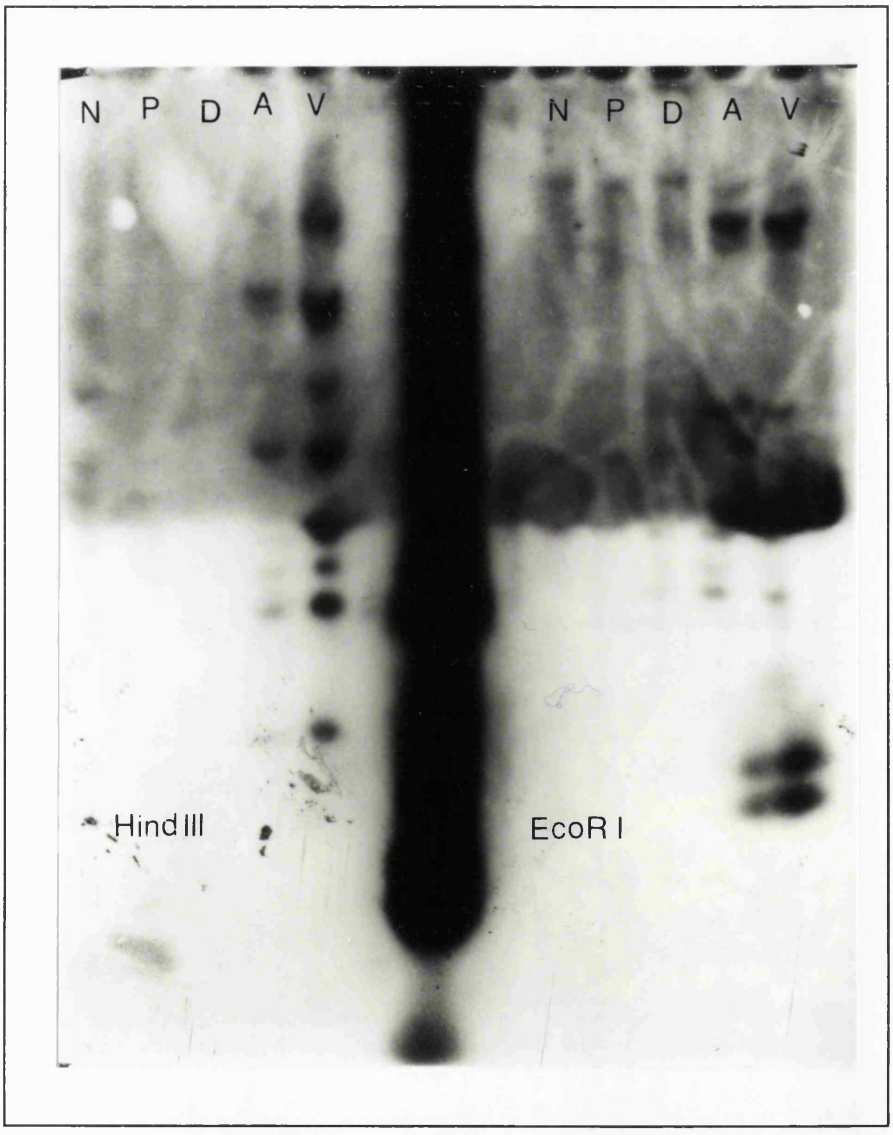


Fig. 7.6 Southern blot of gel in fig.7.5 probed with KAL cDNA

7.3.3 Polymerase Chain reaction-PCR

This method was used to identify patients with deletions of either individual exons of *KAL* or the whole gene. This method does not identify any mutations in the sequence eg. single base deletions or substitutions or other such point mutations. It only allows one to determine whether the exon is present or not. More detailed information about the sequence of each individual exon can be obtained by sequencing the entire exon.

7.3.3.1 Optimising the PCR reaction for Exon 1

In this experiment two factors were changed: the annealing temperature and the concentration of $MgCl_2$ included in the reaction mixture.

The temperature was varied in a step wise fashion ie. a gradient from 60°C to 65°C. The concentration of $MgCl_2$ was varied from 0.8mM to 2.0mM.

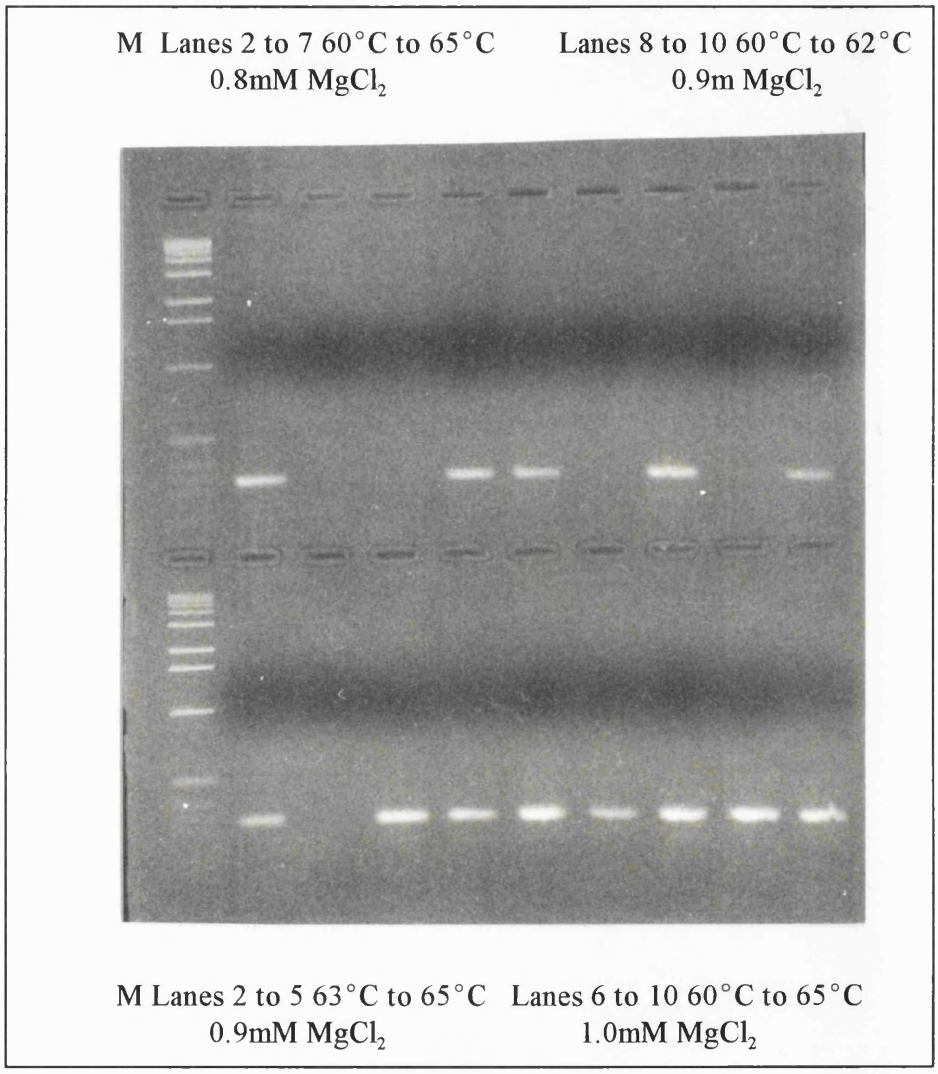


Fig 7.7 A $MgCl_2$ conc 0.8mM to 1.0mM Temp. gradient 60°C to 65°C.

1.0mM is the first concentration in which bands are seen evenly regardless of temperature. The band at 63°C is a little more intense than the others. (M- molecular weight marker)

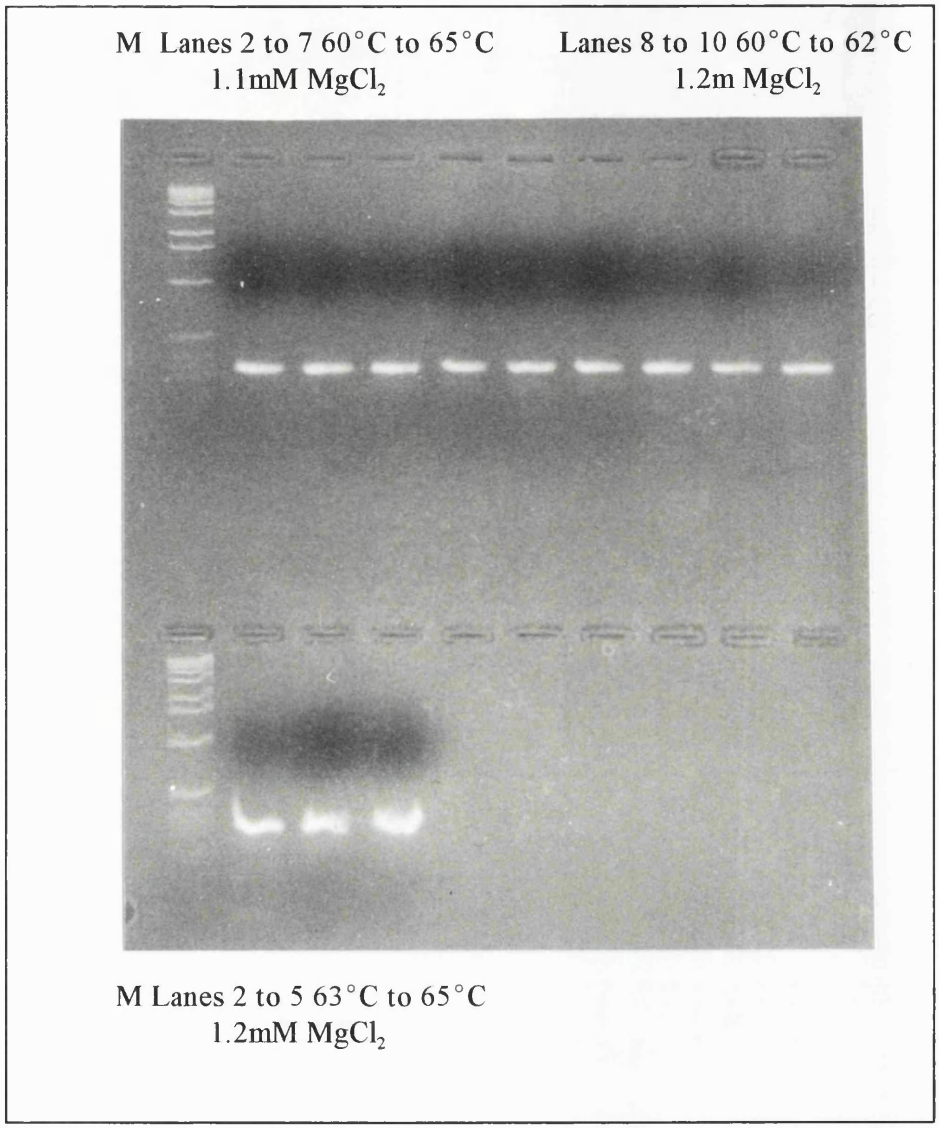


Fig 7.7 B $MgCl_2$ conc 1.1mM to 1.2mM, across a Temp. gradient 60°C to 65°C.

Exon 1 is amplified in each experiment. However when this was repeated in order to confirm the results only 1mM $MgCl_2$ at 63°C gave a PCR product ie. Fig 7.4 C

(M- molecular weight marker)

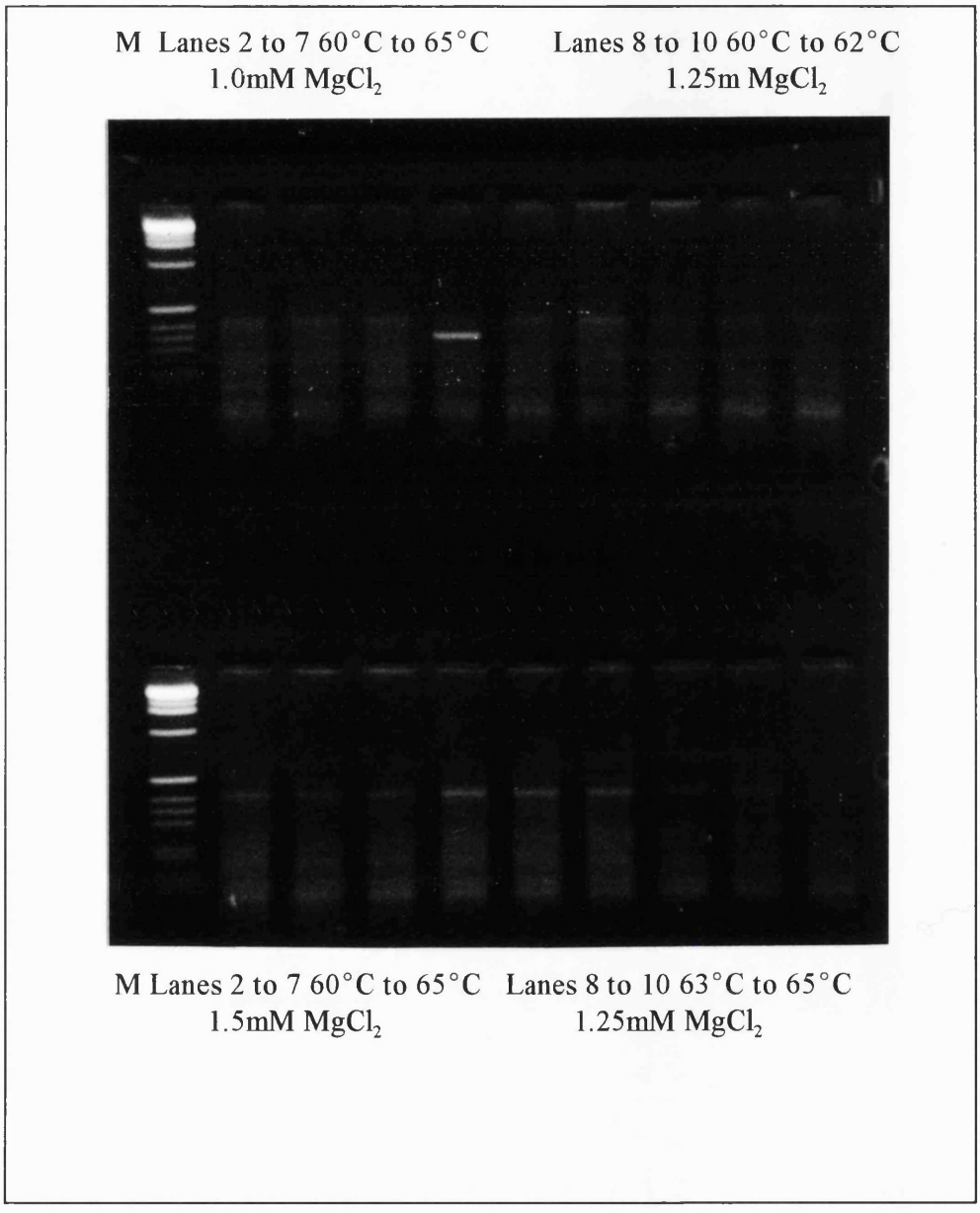


Fig 7.7 C $MgCl_2$ conc 1.0mM to 1.25mM across a Temp. gradient 60°C to 65°C.

The only temperature and concentration where a PCR product could be identified was 63°C and 1mM $MgCl_2$. (M- molecular weight marker)

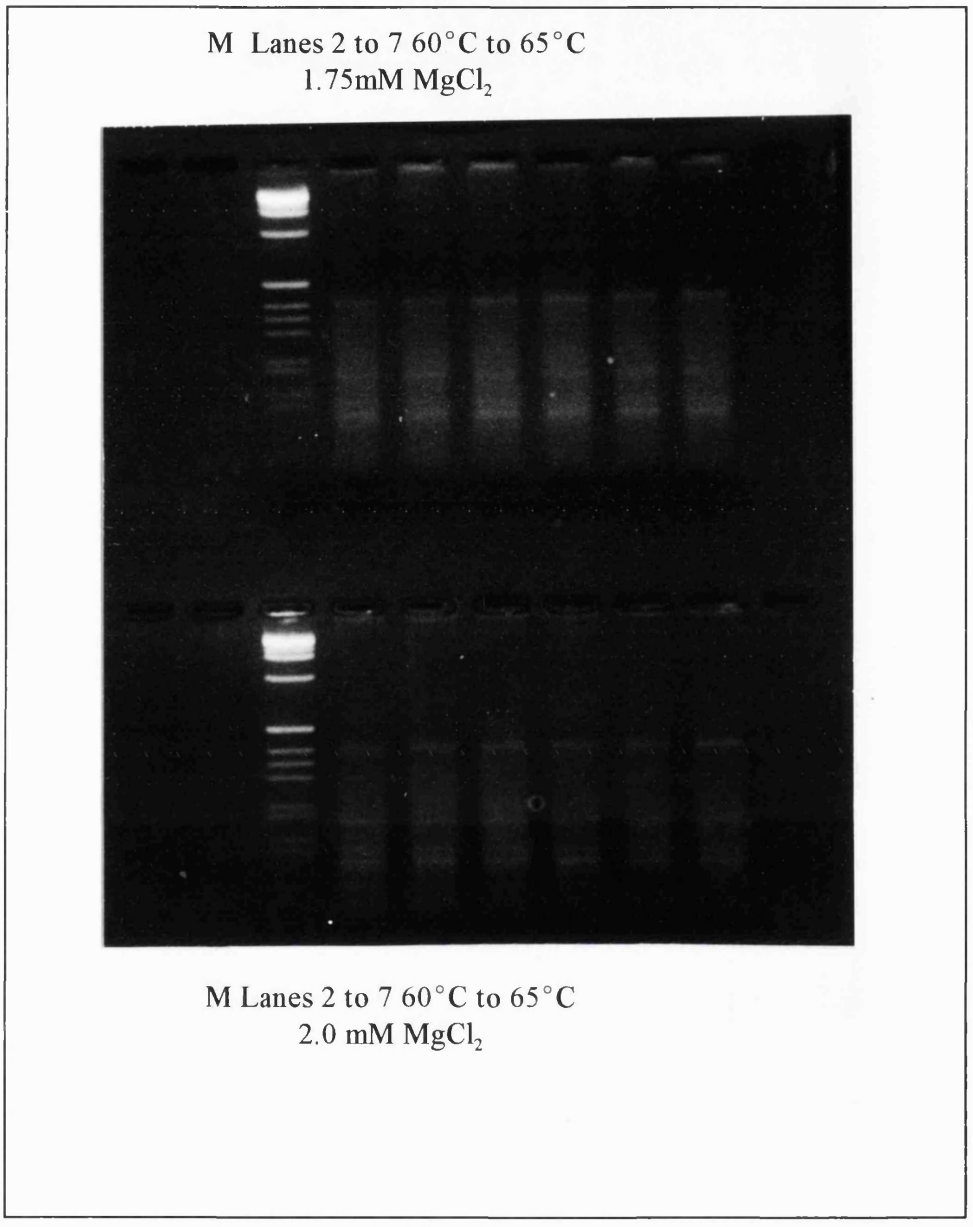


Fig. 7.7 D $MgCl_2$ conc 1.75mM to 2.0mM across a Temp. gradient 60°C to 65°C.

No amplification was seen at the higher concentrations of $MgCl_2$.

(M- molecular weight marker)

From this experiment it was concluded that:

a) The optimal temperature for the amplification of Exon 1 of KAL is 63°C.

b) The optimal concentration of $MgCl_2$ is 1mM per reaction.

7.3.3.2 Optimising the PCR conditions for Exon 2 of KAL

Since the primer concentration, annealing temperature and $MgCl_2$ concentration needed was optimised for Exon 2 and the other 14 exons of *KAL* (as above) the only other factor to be optimised was the concentration of dNTPs to be used for each reaction. The concentration was increased from 2mM to 10mM per reaction.

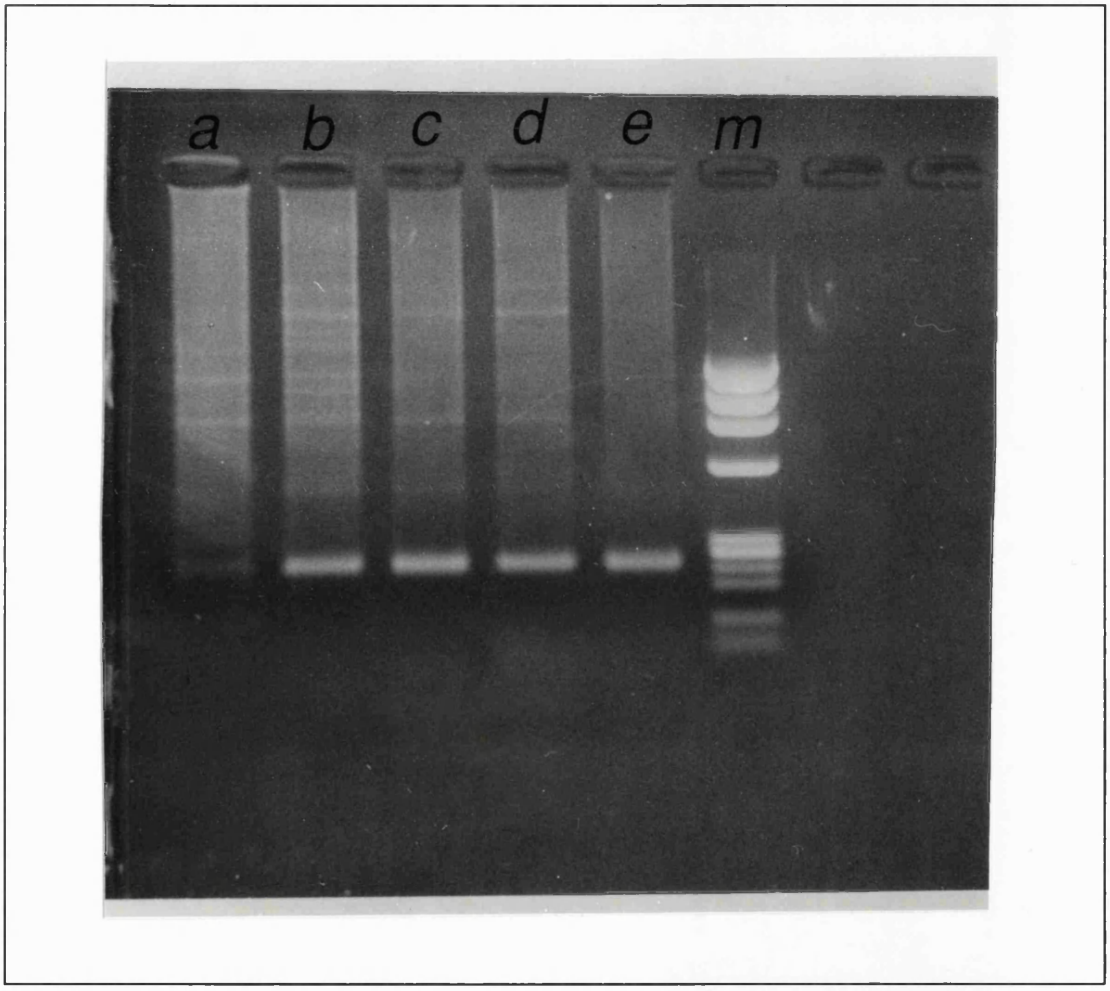


Fig 7.8 PCR reactions of Exon 2 of KAL with increasing concentrations of dNTPs from 2mM to 10mM.

From this experiment it was concluded that 10mM of dNTPs per reaction is the optimal concentration of dNTPs to be used in the PCR reaction.

7.3.3.3 Design of new PCR primers for Exons 11 and 14 of *KAL*

The sequences were carefully compared and found a GTG in the X sequence which was TTA in the Y chromosome sequence. This was therefore the site of the new reverse primers, (The original forward primer was retained.)

New reverse primer GCTTACACATCTGTGCAATTTCAC

Y: GATCTCATCTTATGCATCATCATCCACATCATTACAAGCCTTCTCCAGAAA
X: GATCTCATCTTAAGCATCGTCATCCACATCATTACAAGCCTTCTCCAGAAA

Y:GATAC*TAA*ACTGTTCAAAAAGATTTTTTAAAATTGCACAGATGTGTAAGCT
X:GATAC*TAA*ACTGTTCAAAAAGATTTTGTGAAATTGCACAGATGTG**TAAGCT**

Y:TGTTGAACTTCGGCCACAAGACATGCATACTTCCAGAGGCAGTGGTAACT
X:**TGTTGAACTTCGGCC**ACGAGACATGCACACTTCCAGAGGCAGTGGGAACT

Y:GCTCAGAGGCCCGGACTCTCCTAT
X:GCTCAGAGGCCCGGACTCTCCTAT

Fig 7.9 Exon 14 intron/exon sequences for X and Y chromosomes.

TAA-STOP codon

Underlined sequence is the new PCR primer. The sequence in bold is the old PCR primer

For Exon 11 the new forward primer sequence was CATCCTTGTTGGATGGAATATG

The reverse primer was retained. This new sequence had two bases different from the Y sequence.

X:TATATAGGAAAACATCCTT**G**TTGGATGGAATAT**G**ATTTCAATTCTTGCCAG
Y:TATATAGGAAAACATCCTT**A**TTGGATGGAATAT**T**ATTTCAATTCTTGCCAG

X:TGTTTTTT
Y:TGTTTTTT

Fig.7.10 Exon 11 intron/exon sequence for X and Y chromosomes.

underlined sequence is the new PCR primers.

7.3.3.4 PCR screen for presence of the 14 *KAL* exons in patients with X-linked KS, sporadic KS and IHH.

	SKS (n=18)	XKS (n=20)	IHH (n=7)	female KS (n=3)	STS (n=1)	renal agenesis (n=1)
All 14 exons present	17	12	7	3	1	-
1 exon deleted only	1	1	-	-	-	1
total gene deletion	-	6	-	-	-	-
Other	-	1	-	-	-	-

Table 7.5 Results of PCR screen of 14 Exons of KAL

Of the patients with SKS only one had an exon missing- exon 1. This deletion might have also involved the promoter region although this cannot be proven.

Of the patients with XKS, there were three separate families that each had two members with complete deletions for *KAL*. There was one person with XKS who had a translocation of Xp22.3 (the last 4 exons of KAL were deleted). He also had other abnormalities associated with the contiguous gene defects associated with Xp22.3 including steroid sulphatase deficiency. The one patient who had an exon missing was missing exon 11. The female patients had all 14 exons present. This is expected since it is not thought that *KAL* on Xp22.3 is responsible for their KS but an as yet unidentified autosomal gene .

These results show that both females with KS and most of the patients presenting with the sporadic form of KS (no family history) have an intact *KAL* gene and that the cause of their clinical features does not lie in a lesion in *KAL* but probably on some autosomal locus. Patients with XKS however, have in this study been shown to harbour a number of genetic lesions, ranging from complete deletions of KAL to point mutations or translocation involving some or all of KAL. The patients who appear to have all the exons present by PCR may in that case harbour a point mutation in one of the exons but the only way to identify the exact cause of KS in these patients would be sequence each exon in turn.

7.3.4 Single Stranded Conformation Polymorphism-SSCP

In the first experiment where DNA samples were taken from families A, W, and D (fig. 7.1 to 7.3) the only set of samples in which any differences could be seen was family D.

A difference could be seen between the carriers, the normals and the affected members of this family. (Fig 7.13). It was therefore decided to perform SSCP on the group of patients with XKS and SKS looking only at exon 9 as this was the only exon to which there was a reliable control.

A group of 120 patients were included in this study of Exon 9 of KAL together with members from Family D. No band shifts were observed in any of the patients examined for exon 9.

This method failed to provide any information about possible mutations in a group of 120 KS patients. Possible modifying the experimental conditions would improve the sensitivity of this method.

Fig. 7.11 SSCP of Exons 5,6 and 9 of KAL in patients with KS and other normal and carrier family members.

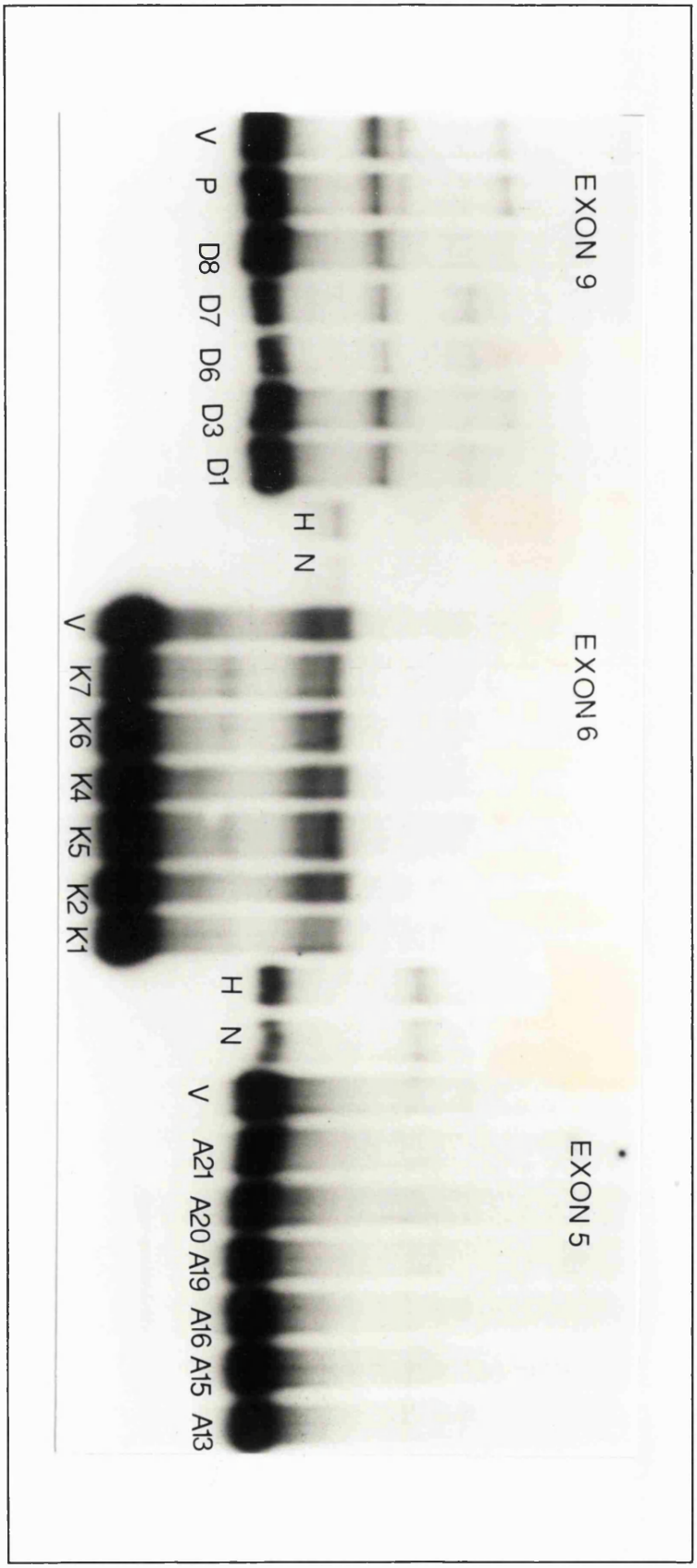


Fig. 7.12 SSCP of Exon 9 in Family D

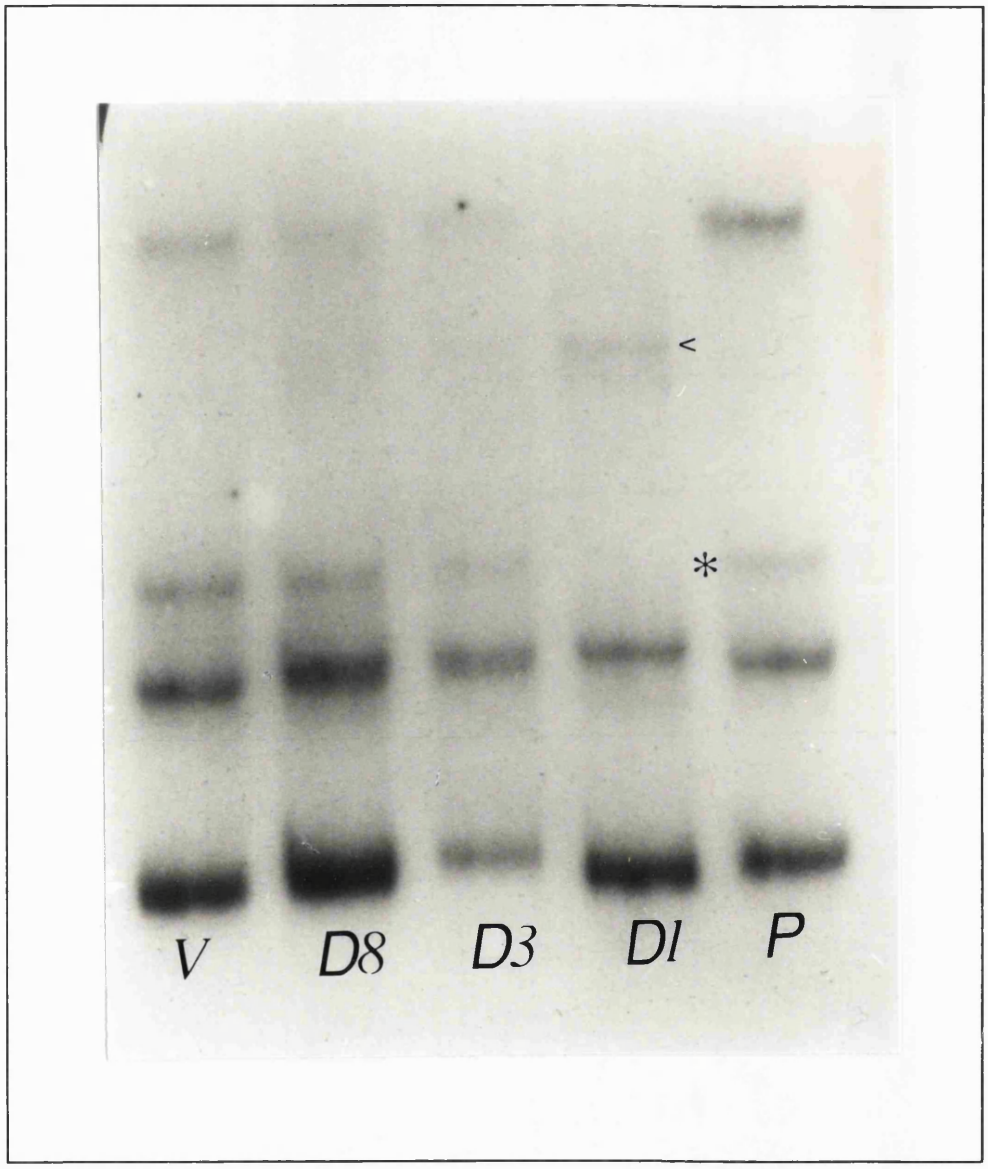
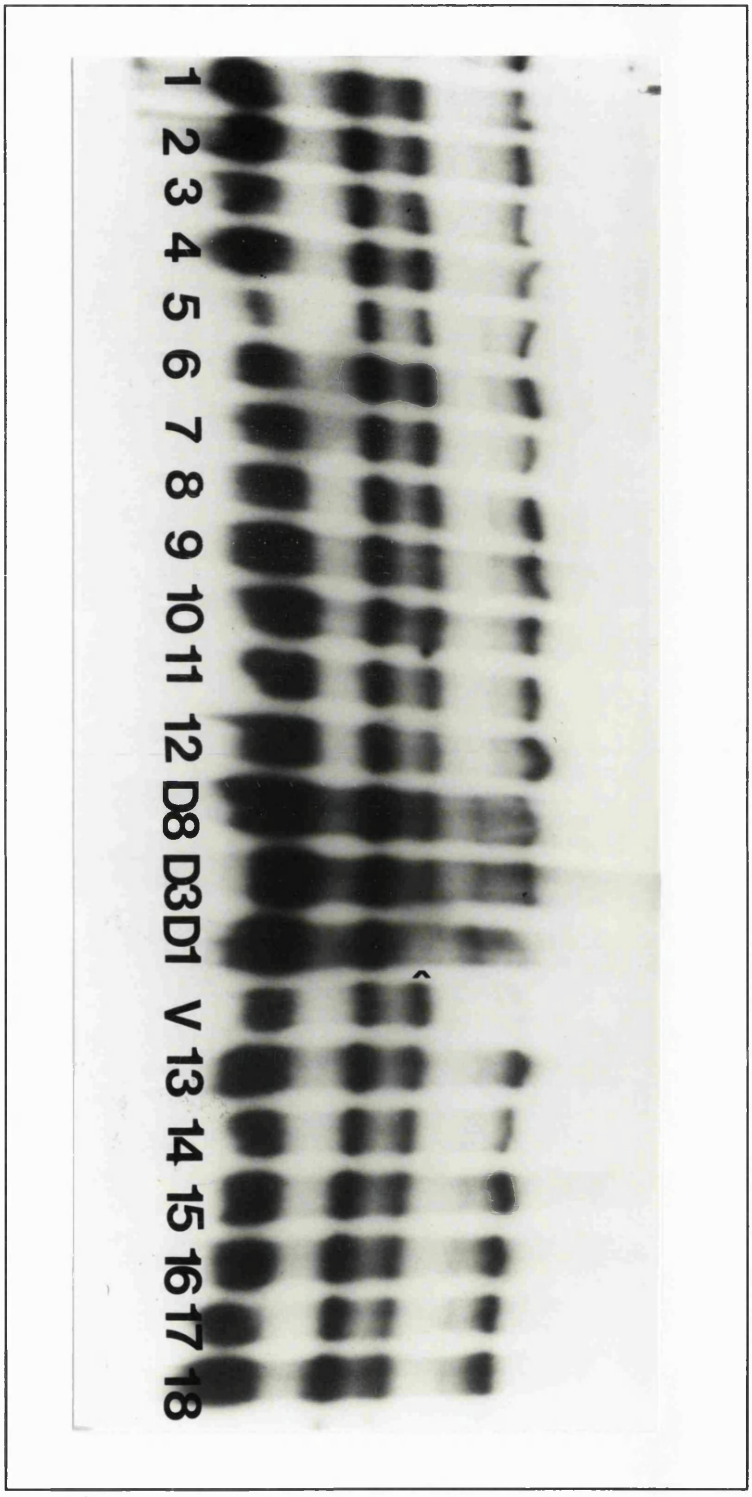


Fig. 7.13 SSCP for Exon 9 of a group of unrelated patients with SKS and XKS.



7.3.5 DNA sequencing of patients with KS.

The patient whose sample was sequenced manually was found to carry the same mutation as had been previously seen in his family ie. a base substitution of a G for an A in exon 5.

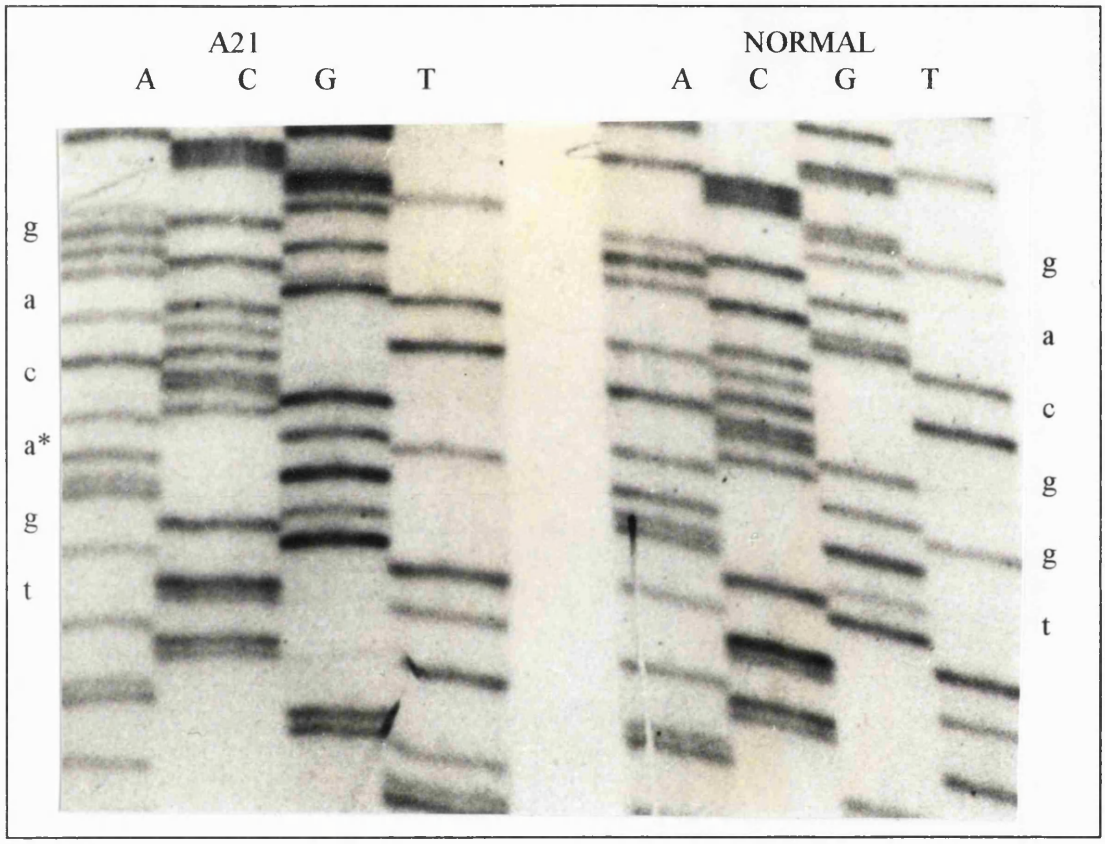


Fig 7.14 A21 DNA sequence compared to normal sequence, A21 has G to A base change.

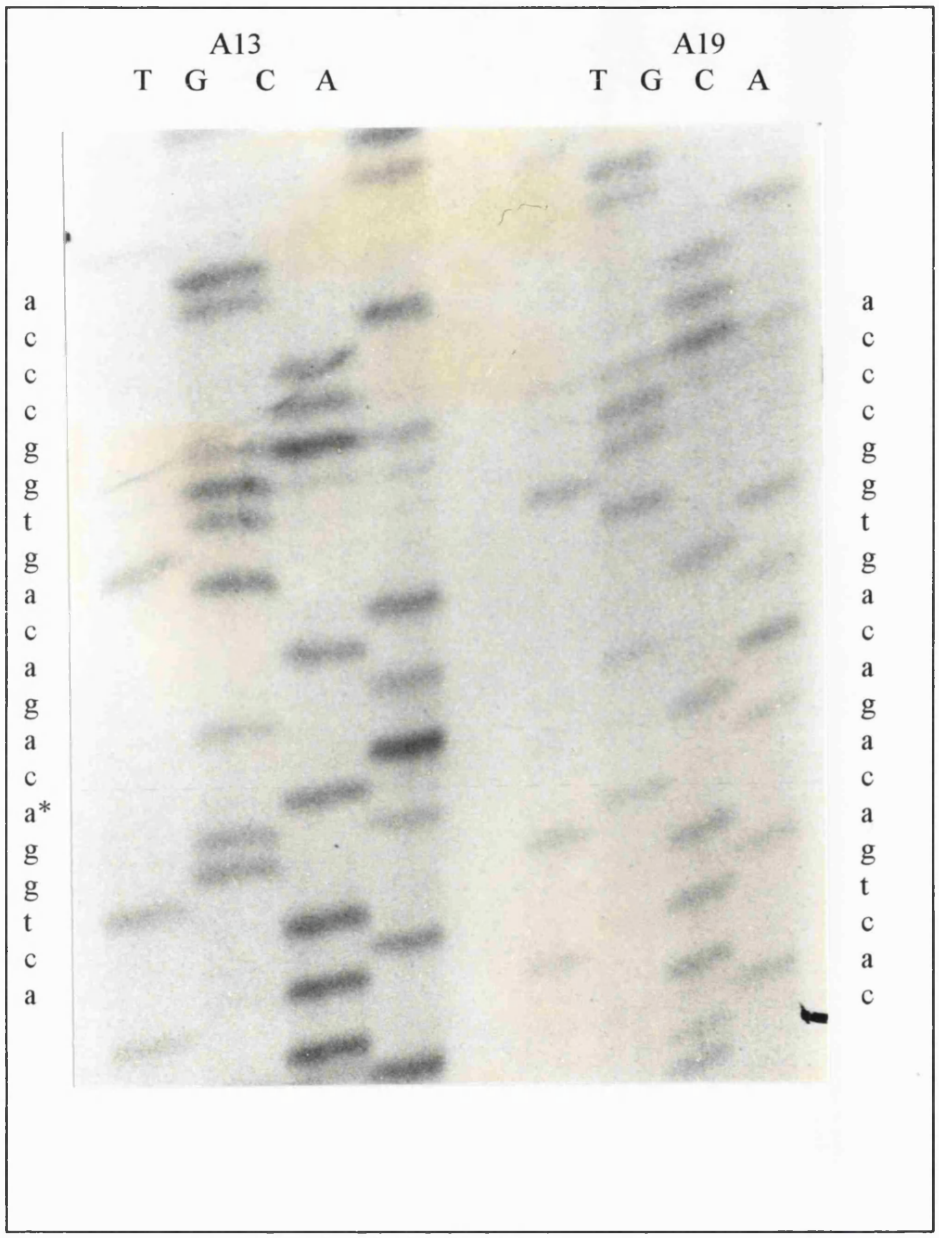


Fig. 7.15 A19 sequence (G to A substitution) compared to carrier mother A13. (both G and A sequences present).

Of the samples sent for automated sequencing the following results were seen:

Subject	Genotype	Phenotypic anomaly	Inheritance
S13	no coding sequence mutation	Bimanual synkinesis (BS), cryptorchid.	clear X-linked pedigree
K7 ---------- K4	Exon 6: G to A_{924} base substitution causing STOP	none apparent ---------- BS, Left kidney absent, cryptorchid	clear X-linked pedigree
JB	Exon 12: deletion of C_{1847} causing frameshift & STOP	BS, cryptorchid, R sensorineural deafness	mutation inherited from mother.
H1 ---------- H2 ---------- H3	no coding sequence mutation	BS, absent L kidney, cryptorchid ---------- BS, absent R kidney.	clear X-linked pedigree
JH	Exon 11 deleted	marfinoid habitus, cryptorchid	brother is also affected.

Table 7.6 Results of DNA sequencing in a group of patients with all 14 exons of KAL present.

From this study the following conclusions could be drawn:

1) There were two families (H1, H2, H3 and S13) were no coding mutation could be identified even by sequencing each of the 14 coding exons, suggesting that the mutation may lie either in the promoter region or in the 3' untranslated region. Alternatively there may be no coding mutation at all, but a translation defect later on.

2) Two new mutations were identified in this study, the deletion of exon 11 in JH and the point mutation of exon 12 in JB. (Fig 7.17 & 7.18)

3) The mutations seen in K2 and K7 were already known but were confirmed in this study.

Fig 7.16 A DNA sequence of Exon 12 of normal control-forward

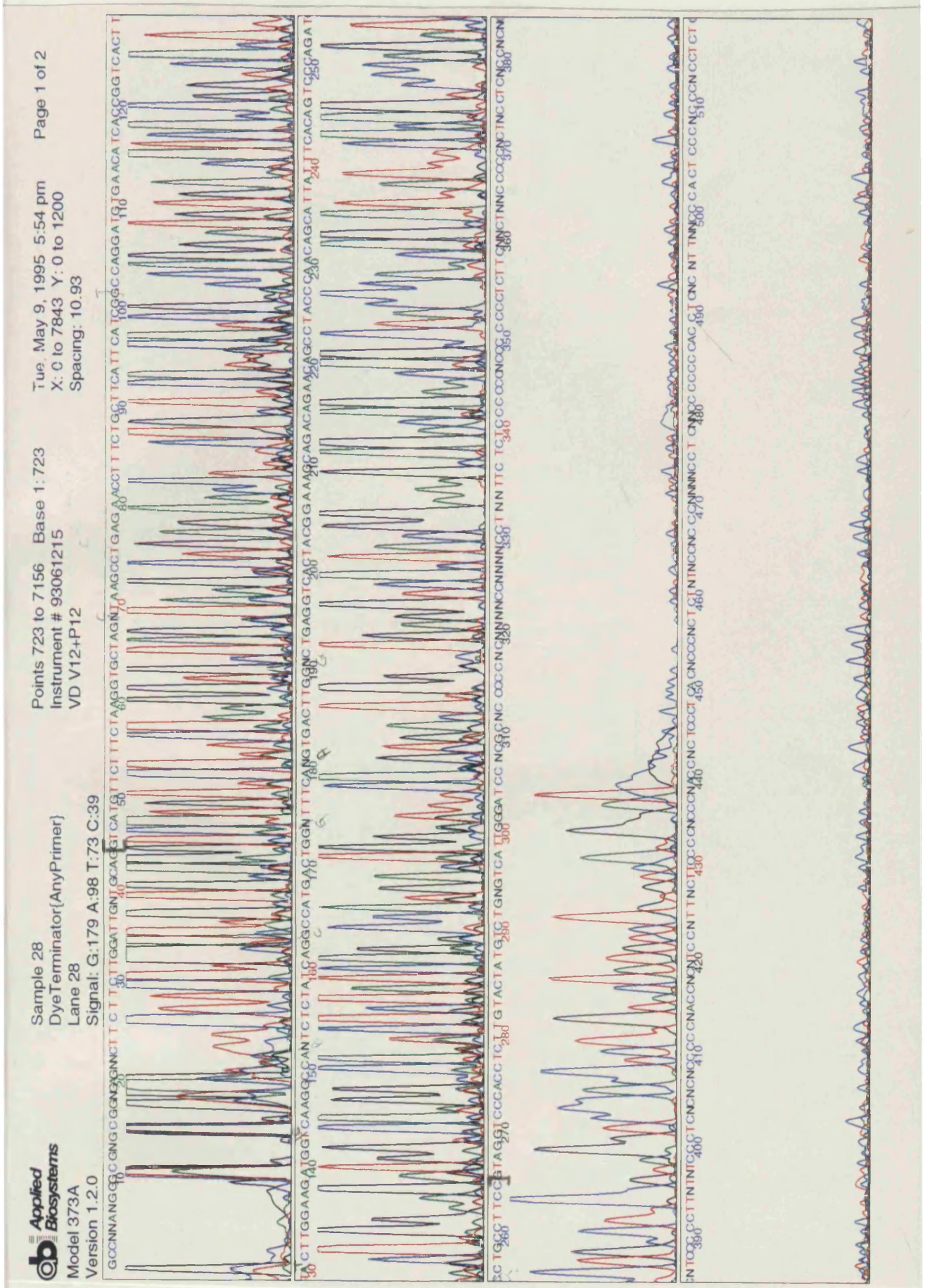
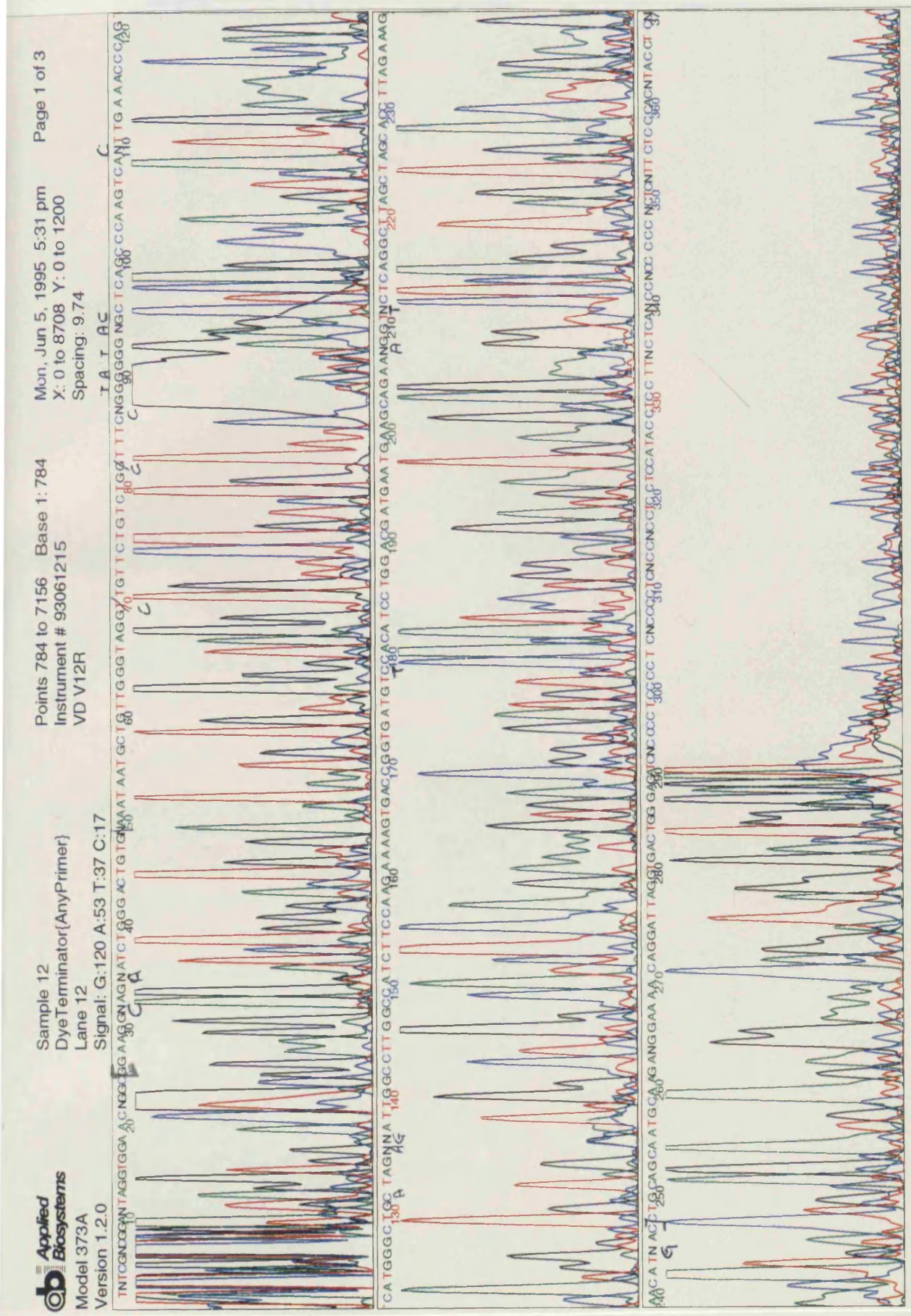


Fig. 7.16 B DNA sequence of Exon 12 of normal control- reverse



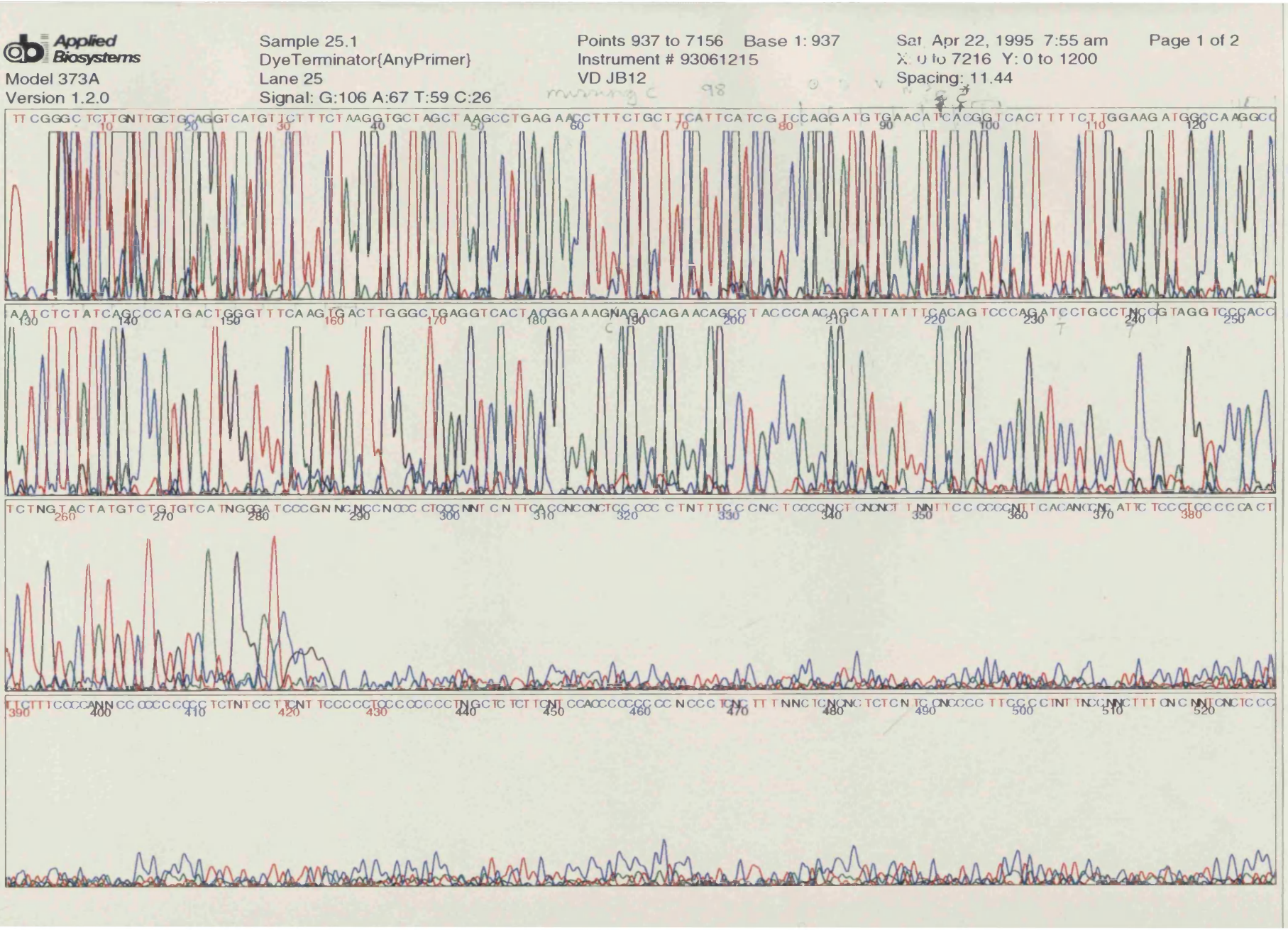


Fig. 7.17 A DNA sequence of Exon 12 of JB - forward C missing at position 1847.

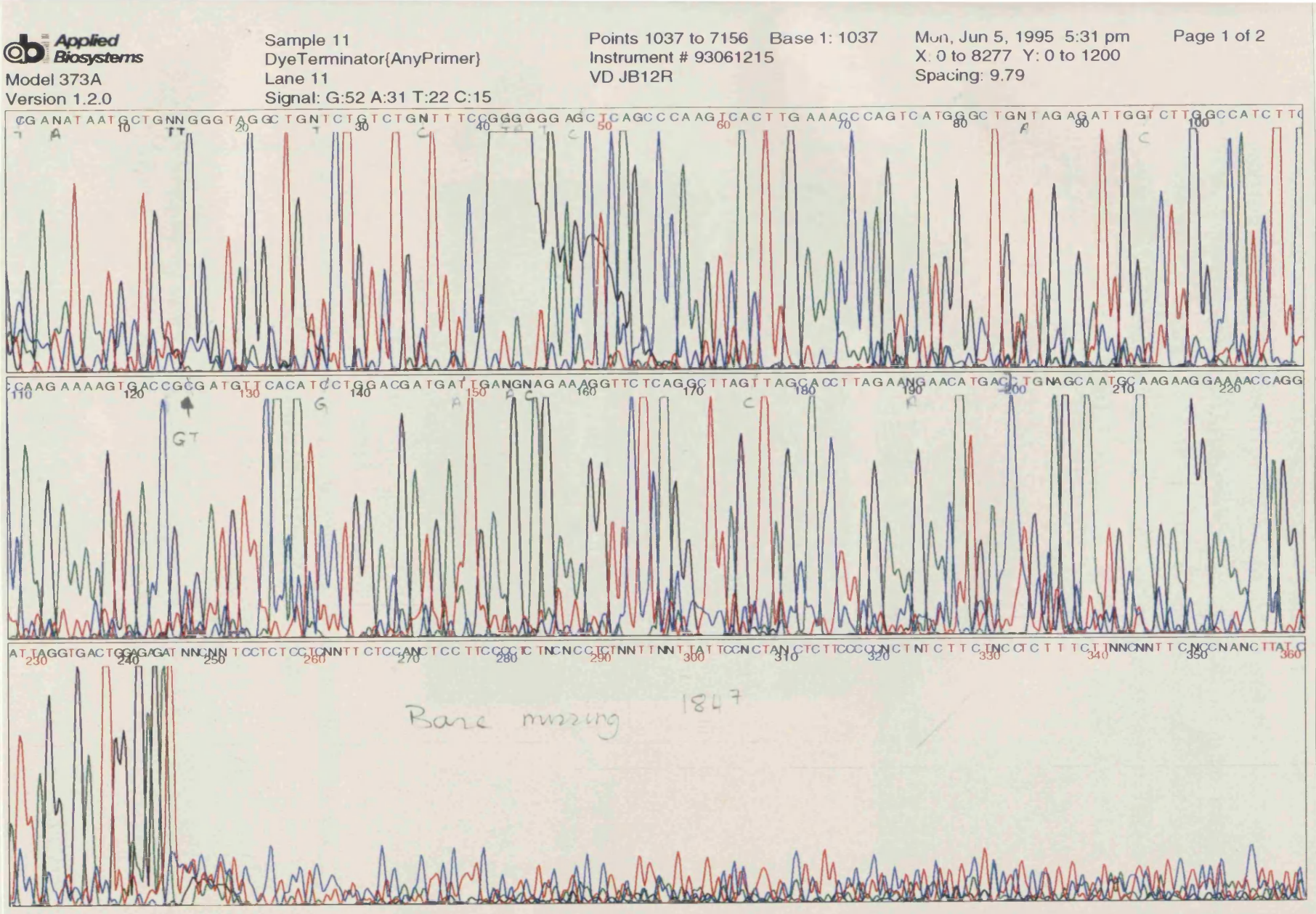


Fig. 7.17 B DNA sequence of Exon 12 of JB-reverse

7.3.6 RFLP analysis of patients and carriers of KS.

In family A affected individuals were known to harbour a base substitution in exon 5 (G for an A at position 861). This disrupts a Bsc 1 site so that the PCR products of exon 5 were no longer cleaved by the enzyme. Normal family members retained this site so that cleavage of exon 5 PCR products induced two bands (241bp and 81bp), whereas three bands were seen in carriers, this being a combination of normal and affected RFLP patterns (ie both cut 241 and 81bp, and uncut 295bp fragments of exon 5 appeared on the gel.) fig. 7.19

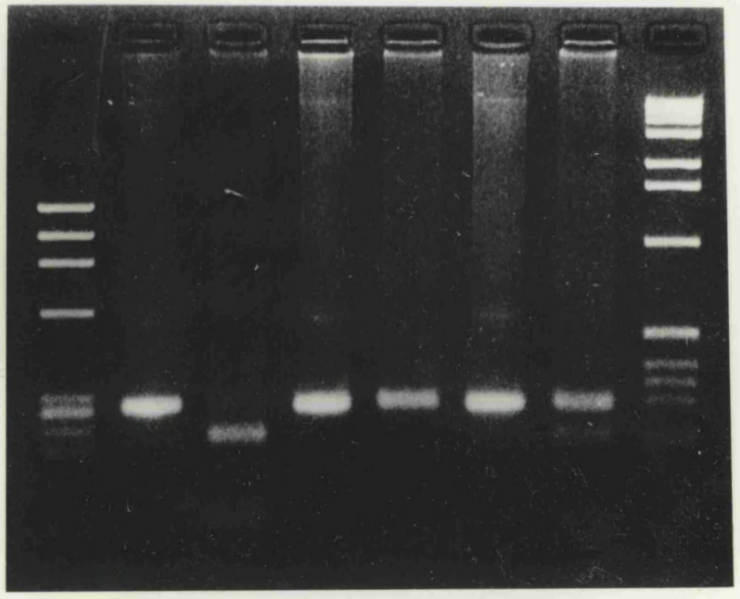


Fig 1: Family A exon 5 base substitution G to A disrupts Bsc 1 site.
Lane 1: Φ174 RF DNA/Hae III Lane 8: 1Kb ladder

Similarly affected individuals of family W harbour a base substitution in exon 6 (G for A at position 924) (see sequencing of patients above)which disrupts a Rsa 1 site (Fig 7.20). Normal individuals retained the Rsa 1 site so that cleavage of Exon 6 with the enzyme gave products of 130bp and 98bp, whereas carriers had three bands, this being a combination of affected and normal (238bp, 130bp and 98bp).

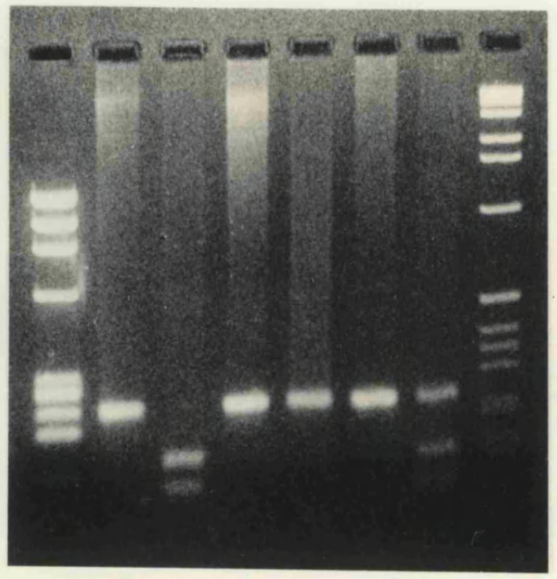


Fig 2: Family W exon 6 base substitution G to A disrupts Rsa 1 site.
Lane 1:Φ174 RF DNA/Hae III Lane 8: 1Kb ladder

In family B a hitherto unpublished mutation (deletion of 1847 C in exon 12) was identified in the proband. This mutation disrupts the Age 1 restriction site.(Fig 7.21)

In this family the normal members showed two bands when cleaved with Age1 (190bp and 120bp). The carriers showed a combination of the normal and patient's RFLP patterns (310bp, 190bp and 120bp)

This method has proven to be very useful, since it is fast and technically less demanding than sequencing each patients DNA.

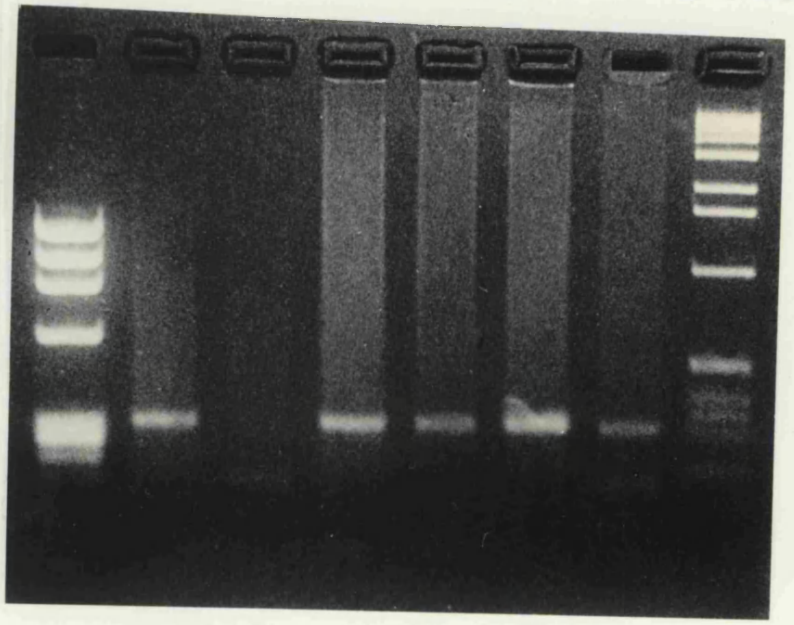


Fig 3: Fam B exon 12 C deleted disrupts Age 1 site.
Lane 1:Φ174 RF DNA/Hae III Lane 8: 1Kb ladder

7.4 Discussion

Performing mutation analysis on a group of patients with XKS and sporadic KS have enabled the following observations to be made.

i) It shed light on the fact that patients with sporadic KS are very unlikely to have complete deletions of KAL or even an exon missing. It is likely that the cause of their KS may not even lie in a mutation in *KAL*. However, this will remain unproven until such time as an autosomal gene for KS is identified.

ii) Complete deletions of KAL in patients with XKS is not common occurring in only 30% of the patients studied.

iii) Although there were several different point mutations seen (Exons 5, 6 and 12) each mutation was seen only once, ie. no two unrelated patients had the same mutation. This finding has made it impossible to identify a mutational "hot spot". Although no hot spot was identified the mutations found, all lie in an area of the gene which codes for protein that has a high degree of homology to fibronectin type III repeats ie. a neural cell adhesion molecule. The absence of a hot spot make speculations about the functionally important parts of the KAL protein impossible. However, this finding supports the unproven hypothesis, that the role of the KAL protein is one of an adhesion molecule.

iv) SSCP as a method has a limited use, since if a shift were to be seen, it would be necessary to sequence the exon of interest in order to characterise the mutation. However it does allow one to screen large numbers of patients in a short time. The sensitivity of this method is questionable however as two sets of families with known mutations in different exons of KAL failed to show a band shift when subjected to SSCP and only one family gave a result, giving this method only 33% sensitivity, far too low to be of value. Possibly modifying the method by, for example, changing the concentration of polyacrylamide in the gels or running the gels for a longer period of time in order to improve resolution of the samples, could improve sensitivity.

v) Looking at the phenotype of the patients, it has not been possible to draw any correlations between genotype and phenotype of the patients studied.

Chapter 8

FINAL DISCUSSION

Kallmann's Syndrome, although rare, comes about due to a unique accident of nature involving two distinctly different developmental systems, namely the olfactory and urogenital systems. This syndrome is a result of the failure of migration of the olfactory axons and the GnRH neurones. The heterogeneity of clinical and genetic features is noteworthy. The clinical features associated with KS apart from the IHH and anosmia have been found to vary depending on the mode of inheritance, ie. synkinesis is strongly associated with the X-linked form of KS as is renal agenesis whereas cleft palate and other midline defects are associated with the sporadic form of KS. The modes of transmission of KS are as diverse as the clinical features including autosomal dominant, autosomal recessive, X-linked and the most frequently occurring sporadic KS.

In this thesis the answer to several questions regarding Kallmann's Syndrome was sought, regarding the XKS gene and protein in both human development and patient.

Kallmann protein expression in the developing human and its role.

In order to identify areas of Kallmann protein expression monoclonal antibodies were raised in mice to a short sequence of the putative KAL sequence using a multiple antigenic peptide. The original question still remains unanswered because the antibody generated lacked the necessary specificity and further epitopes of this protein needed to be investigated. Furthermore the absence of the Kallmann protein has made validating antibodies produced difficult. The method employed clearly demonstrated that high affinity antibodies could be generated using multiple antigenic peptides. Increasing available data on protein sequences, their folding and homologies to other proteins would enable further epitopes of KAL to be studied. However, this was beyond the scope of this investigation. From this experiment it is clear that the antibody cross reacts with another protein. Whether this cross reactivity is total or whether the antibody has some affinity to KAL protein could only be proven if the identity of the cross reacting protein were known. In which case a higher affinity and specificity antibody to it could be used to block out the cross reactivity, thereby revealing specific KAL binding.

An alternative strategy to finding the role of KAL protein in development could include expressing KAL and then raising antibodies to the protein. Using bacterial systems such as E.Coli, the KAL cDNA could be transformed into the cells, transcribed into RNA and translated into protein using the E.Colis' own machinery. This may be difficult to purify, since E.Coli has no facility for glycosylating proteins. Therefore creating a fusion protein between KAL and some other marker protein and then transforming E.Coli and generating fusion proteins is likely to be a more profitable way of producing KAL in sufficient amount enabling purification. The purified protein could be employed as an antigen in mice or rabbits to produce primary polyclonal antibodies and then if necessary monoclonal antibodies. In vitro systems could also be used, such as rabbit reticulocyte lysate or wheatgerm cell free systems which would transcribe and translate protein in a coupled system. However, this method is not generally conducive to large scale protein production. These are some suggestions of ways in which KAL could be expressed and then isolated for use in generating antibodies in a similar way to the antibodies that were generated in this thesis. These antibodies could then be used to screen both foetal and adult tissues for areas and stages of KAL expression so that a map can be created enabling fuller understanding of the role of KAL. Furthermore isolating KAL protein would enable validation of the antibodies generated, using immunoprecipitation.

Investigation of the areas and stages of KAL gene expression in normal development.
Using a combination of *in situ* hybridisation and RT-PCR, it was possible to study gene expression in human foetal material between the ages of 45 days and 11 weeks. KAL expression was found in the developing kidney only at 45 days gestation, but later at 11 weeks gestation, transcripts was found in the olfactory bulb, retina and again in the kidney. These results correlated well with the clinical features of KS since olfactory bulb dysgenesis with subsequent defective neural migration causes anosmia and IHH. Additionally, renal agenesis occurs in 40% of patients. Assuming that transcription is followed by translation into the mature functioning protein, KAL expression therefore appears necessary for the normal development of the olfactory bulb and the kidney in the first trimester of human pregnancy. The interaction of KAL protein with one or more other factors cannot be discounted. Therefore studying different stages of human development is critical in gaining a clearer understanding of the role of KAL and the timing of its expression.
The only limiting factor in this experiment was the great difficulties involved in obtaining

foetal material of different gestational stages that was in an adequate condition for performing such experiments. Had material been more readily available, it would have been possible to draw up a map of both areas and stages of gene expression which is essential for reaching a greater understanding of the role of KAL and other genes involved in development. At present there is no organised infrastructure for the collection, storage and processing of human foetal material. This is a service that is seriously lacking within the community of developmental biologists and embryologists. Were this service to be available many questions about gene expression in the developing human could no doubt be answered.

The Kallmann patient.

The third area of study was the Kallmann's patients themselves, looking at the genetics of the disorder in a group of sporadic and X-linked patients. This part of the thesis addressed two principal issues.

i) Does a mutation hot spot exist? By identifying such a mutation and then locating the area of the protein to which it corresponds, it may be possible to identify regions of the protein which have greater functional importance than others. Individual mutations may affect parts of the protein critical to folding and therefore biological activity; mutations may affect domains or epitopes within a consensus sequence essential for binding to the receptor (eg. the androgen receptor and ligand binding domain (Olaf Hiort et al. 1993). Therefore identifying mutational hot spots would lead to a greater understanding of the function and functionally important regions of the protein. Variations in the types of mutations and the regions of the protein mutated may well explain the variations in phenotypes observed in this group of patients.

ii) Is there a relation between the phenotype of the patients and the genotype? Since not all patient share the same clinical features outside of the anosmia and hypogonadotrophic hypogonadism (eg. synkinesis and renal agenesis are not seen in all the patients,) do specific mutations and genetic variants lead to specific phenotypes?

The data obtained did not enable a precise conclusion to be drawn, although important pieces of information were obtained, including the fact that complete deletions of KAL were not seen in sporadic patients but only in 30% of XKS patients.

Over the course of this study, it was possible to identify what, if any, mutation was being harboured in the KS patient and also look at other family members and identify carriers of KS. Mutations found were in exons 5,6,9 and 12. These exons showed homology to

fibronectin type 3 repeats- a neural cell adhesion molecules. This data suggests that the NCAM part of KAL is important although the precise role of this part of the gene remains unclear. Obtaining information on carriers has enabled more information to be given to the patients and their families and offer for the first time, genetic counselling.

Overall this study has lead to a greater understanding of the Kallmann gene both in development and in the patient. The gene has an involvement in both the olfactory and renal systems as well as the migration of GnRH. This can be seen in the studies on expression of KAL as well as the migration pattern of GnRH and olfactory axons in the Kallmann foetus. The gene product is necessary for the later events, that occur in the developing olfactory bulb. The bulbs have been visualised by MRI in KS patients, suggesting that KAL is not necessary for the initial formation of the bulb, but the fact that they are aplastic suggests that KAL is involved in bulb histogenesis at a later stage, possibly synapsing of the olfactory neurones within the bulb and the subsequent migration of GnRH neurons along the pathway provided by the vomeronasal and terminal nerves (the accessory olfactory).

In this respect, neural cell adhesion molecule (NCAM) which shows homology to the putative KAL protein has been implicated in the migration of GnRH neurons into the forebrain along the accessory olfactory nerve (Schwanzel-Fukuda and Pfaff, 1991). When NCAM migration is interrupted by the use of anti NCAM antibodies the number of GnRH neurons migrating from the olfactory placode to the forebrain is greatly reduced in mice, strengthening the association between KAL and NCAM. The function of KAL in other parts of the central nervous system is currently unclear. However, the data presented suggest that KAL may be involved in axonal migration or neural maturation in the retina (Legouis et al. 1993b).

The above studies of KAL expression in the developing kidney lead to the suggestion that KAL has a role in the formation and maintenance of the developing kidney. However, KAL is likely to be acting in conjunction with other molecule, as bilateral renal agenesis is almost unknown in KS and only 40% of KS patients have a kidney missing. This suggests that some as yet unidentified factor or protein is involved in kidney formation.

In conclusion this study has revealed the fact that KAL is expressed in a variety of tissues in development. Some of these locations correlate well with the clinical features seen in the patients with mutations of KAL. The above investigations have underlined that there is much about KAL that remains unknown, that in fact this field is in its infancy.

The possibility that KAL protein is either cleaved by an enzyme in order to render it functional or that it acts in conjunction with another protein or proteins ie. NCAM cannot be discounted. At this stage, the role of KAL in development is thought to include cell-cell and cell-matrix interactions to provide pathways over which different sets of axons and neurons can migrate in the developing brain and urogenital system. This, however, may be only part of the picture.

At this stage, in order to reach a greater understanding of the role of KAL in human development obtaining a full length transcript of the protein is imperative. The reverse genetic methods used to isolate KAL have possibly caused more questions to be asked than can be answered using these methods. Isolation of a gene is only the beginning. Understanding its role both in development and in the adult requires isolation of the protein. Possibly reverting to more traditional methods of protein chemistry may lead to the isolation of the KAL protein. Extraction of total protein from olfactory bulbs, for example and then separating the proteins on the basis of size on an SDS-PAGE or Sephadex column. The appropriate sized protein could then either be sequenced or used in a bio-assay in order to confirm its identity.

Once the protein has been identified, the receptor to which it binds must be located. Adhesion molecules bind to integrins, often by a consensus sequeence, therefore the KAL receprtor may do the same.

Another possible strategy, which may lead to a greater understanding of the role of KAL in development may be to concentrate on identifying a KAL homologue in the mouse. Although the pseudoautosomal region on which KAL lies is not present in the mouse the mouse does have olfactory bulbs, suggesting that either KAL or another gene is present in the mouse. From the above studies it is clear that KAL can be compensated for by anothr as yet unidentifed gene(s). The autosomal locus may be capable of this compensation and therefore needs to be identified too!

APPENDIX A - STOCK SOLUTIONS USED

A1 General use solutions

0.5M EDTA

186.1g EDTA

dissolved in 800ml of water

pH was adjusted to 8.0 with NaOH, volume was made up to 1 Litre with water.

5M NaCl

292.2g NaCl

dissolved in 800ml of water, once dissolved volume was made up to 1 Litre with water

20 X SSC

350.5g NaCl (3M)

176.5g Sodium citrate (0.3M)

dissolved in 1 Litre of water, volume made up to 2 Litres with water.

10% SDS-Sodium dodecyl sulphate

50g SDS

450ml of water was added and the solution warmed gently in microwave to dissolve.

Once cool the volume was made up to 500ml with water.

TE buffer

0.60g Tris (10mM)

0.186g EDTA(1mM)

250ml of water was and pH adjusted to 8.0 with HCl.

volume was made up to 500ml with water.

1M Tris

121.1g Tris

dissolved in 800ml of water, pH was adjusted to desired value by adding Hcl.

A2 Western blotting solutions

Separating gel buffer

18.15g Tris in 100ml H_2O

pH to 8.8 with HCl

stored at 4°C

Stacking gel buffer

6g Tris in 100ml H_2O

pH to 6.8 with HCl

stored at 4°C

Sample buffer

20ml glycerol-20% (use 80% stock diluted with water)

50ml 20%SDS stock

7.5ml 1M Tris pH6.8

0.01% Bromophenol blue

100mM DTT-made up fresh on the day

volume was made up to 100ml using water

stored at 4°C

Reservoir buffer

3g Tris

14.27g glycine

2g SDS

volume was made up to 1 Litre using water

Transfer buffer

5.81g Tris

2.93g Glycine

0.375g SDS

200ml Methanol

volume was made up to 1 Litre using water

Gel stain

40% methanol

10% acetic acid

0.025% coomassie blue

volume was made up to 1 Litre with water

Gel destain

40% methanol

10% acetic acid

volume was made up to 1 Litre with water

Tris buffered saline-TBS

6.04g Tris

21.91g NaCl

2 Litres of water was added and pH adjusted to 7.5 with HCl

volume was made up to 2.5 Litres with water.

TTBS

1ml of Tween was added to 2 Litres of TBS

Blocking solution

3g of dried milk powder in 60ml of TTBS

Antibody buffer

2ml blocking solution

8ml TTBS

antibody

A3 *In situ* hybridisation and RT-PCR solutions

All solutions were prepared with DEPC water or PBS ie. 0.5ml of DEPC per Litre of water then solutions were autoclaved.

Phosphate buffered saline-PBS

5 pellets of dry PBS per Litre (Sigma)

DEPC was added (0.5ml per Litre of water) and autoclaved.

4% Paraformaldehyde-PFA

40g of PFA in 1 Litre of PBS, the solution was warmed gently in order to allow the PFA to dissolve. A few drops of NaOH were added to completely dissolve the PFA., this was prepared in the fume cupboard.

0.83% Saline solution

11.36ml of 5M NaCl

388.64ml of DEPC water

Proteinase K solution

20ug/ml of Proteinase K (Sigma) from 10mg/ml stock

50mM Tris-HCl (BDH) from 1M stock

5mM EDTA from 0.5M stock

Column buffer (For separation of ribo probe)

0.1% SDS in TE buffer

NTE solution

0.5M NaCl from 5M stock

10mM Tris from 1M stock

5mM EDTA from 0.5M stock

DTT 10mM

10mM is needed: 1M=154g in 1 Litre

0.1M=15.4g in 1 Litre

0.01M=1.54g in 1 Litre

however 1.54g in 10ml = 1M. This solution was added to any solution over 37°C, 1ml was added for every 100ml of solution.

50% Formamide solution

50% formamide

2X SSC from 20X SSC stock

10mM DDT

STOP solution (30% Na thiosulphate)

150g Na thiosulphate

350 ml H_2O

stored in dark room wrapped in foil at room temperature.

Fixative

1% glycerol

1% acetic acid

Solution D

25ml guanidinium thiocyanate

6.25µl Na citrate

1.25ml 10% sarcosyl

180µl mercapthoethanol.

A4 Solutions for DNA extraction.

Lysis buffer

109.4g Sucrose

1.20g Tris

1.02g $MgCl_2.6H_2.0$

10ml Triton

500ml of water was added, mixed pH adjusted to 7.5 with HCl.
volume was made up to 1 Litre with water.
Stored at 4°C for no more than a few days.

NaCl-EDTA
4.38g NaCl (0.075M)
8.93g EDTA (0.024M)
500ml of water was added, mix and pH adjusted to 8.0 with NaOH.
volume was made up to 1 Litre with water.

Proteinase K
25mg Proteinase K
dissolved in 2.5ml of T.E.buffer.
Stored frozen at -20°C and thawed only when needed.

Chloroform/Octanol-24:1
240ml chloroform
10ml octanol
mixed and stored in a glass container.

Phenol
Recrystallised phenol was thawed in a 65°C water bath.
100ml was decanted into a dark glass container and 100ml of Tris buffer added,
this was gently mixed and left over night before using.
Stored at 4°C . Phenol was only used in the fume hood.

Sodium acetate
40.80g Sodium acetate-trihydrate
80ml of water was added and pH adjusted to 5.2 with glacial acetic acid.
volume was made up to 100ml with water.

A5 Solutions for agarose gel electrophoresis

10 X TAE running buffer

7.20g EDTA

96.80g Tris

700µl EtBr

EDTA and Tris were dissolved in 1 Litre of water, pH was adjusted to 7.7 with glacial acetic acid.

volume was made up to 2 Litres with water.

EtBr was added to solution.

Loading buffer

0.25% Bromophenol blue,

0.25% Xylene cyanol

40% w/v sucrose in water

stored at 4°C

A6 Solutions for southern blotting

0.25M HCl

9.115ml conc HCl

volume was made up to 1 Litre with water.

Denaturing solution

40g NaOH (0.5M)

176g NaCl (1.5M)

NaCl was added to 1 Litre of water and allowed to dissolve before adding NaOH.

volume was made up to 2 Litres with water.

Neutralization solution

176g NaCl (1.5M)

240g Tris (0.5M)

0.185g EDTA (0.001M)

175ml HCl-conc

volume was made up to 2 Litres with water.

A7 Solutions for hybridisation of DNA probe.

20 X SSPE

175.3g NaCl (3.6M)

27.59g sodium phosphate(0.2M)

7.44g EDTA (0.001M)

800ml of water was added and pH adjusted to 7.4 with NaOH.

volume was made up to 1 Litre with water.

100 X Denhardt's solution

2.0g Ficol 400 (2%)

2.0g PVP polyvinylpyrrolidone (2%)

2.0g BSA bovine serum albumin

Ficol was added to PVP and 60ml of water and gently heat to dissolve.

BSA was dissolved in 20ml of water, and added to Ficol and PVP once cool.

volume was made up to 100ml with water and stored at -20°C.

Prehybridisation solution

6.25ml 20 X SSPE (5X)

1.25ml 100 X Denhardts solution(5X)

1.25ml 10% SDS

volume was made up to 25ml with distilled water.

50µl of boiled and snap frozen herring sperm was added DNA just before adding prehybe solution to membrane.

A8 Solutions for Transformation of KAL cDNA

LB-Leurier broth

20g LB

dissolve in 1 Litre of water and autoclave.

LB agar

15g Bacto agar

1 Litre LB

autoclave on liquid cycle and then allowed to cool a little, flask was swirled regularly to ensure the agar remained dissolved .

Once cool ampicillin was added, 100μg/ml needed

500ml of LB agar X 100 = 50000μg of ampicillin or 50mgs. This is one ampicillin vile, dissolve ampicillin in 1ml of sterile water and add to the agar.

The agar was then poured (Flame mouth of flask before pouring) out into petri dishes - about 30ml per dish and left for at least two hours to set.

Plates were dried in a class 1 hood before storing at 4°C inverted.

SOC

97ml water

2.0g Bactotryptone

0.5g Yeast extract

1ml NaCl (1M)

0.25ml KCl (1M)

this solution was autoclaved before use.

A9 Solutions for small scale preparation of plasmid DNA

Solution 1-Lysis buffer

0.3g Tris (25mM)

0.37g EDTA (10mM)

0.9g Glucose (50mM) added after autoclaving

dissolved in 100ml of water, and autoclaved store at 4°C.

Solution 2

0.2ml NaOH (0.2M)

0.5ml SDS (1%)

volume was made up to 5ml with sterile water *must be prepared fresh at the time of use*

Solution 3

60ml Potassium acetate (5M-29.44g in 60ml)

11.5ml Glacial acetic acid

28.5ml water

this solution was autoclaved and store at 4°C.

APPENDIX B-PREPARATION OF GELS

10% Ammonium persulfate-APS

0.1g of APS in 1ml of sterile water

this is prepared fresh at the time of use,

can be stored for up to 1 week at 4°C.

B1 Protein gels for Western blotting

1. The BioRad apparatus was used for this experiment. Both glass plates were cleaned before use. Spacers were placed on either side of larger glass plate and smaller plate was layered on top.
2. Plates were slid into the perspex holder and aligned using pouring stand. Screws were tightened on the perspex holder.
3. The rubber seals were placed on the base of the gel pouring stand and plates were clipped into gel pouring stand ensuring that there was a seal made.
4. Reservoir buffer was run in between the plates to ensure that there were no leaks.

10% Separating gel

125ml 40% acrylamide solution

1. 875ml separating gel buffer

4. 365ml water

75ul 10%SDS

10ul Temed *

50ul 10%APS *

* these were added last as they cause gel polymerisation

500ul to 1ml of isobutanol was layered onto the gel, which was then left to polymerise for at least one hour.

5% Stacking gel

Prepared on the day of use

0.812ml 40% acrylamide solution

0.94ml stacking gel buffer

4.625ml water

75ul 10%SDS

7.5ul Temed*

37.5ul APS*

The comb was placed and care was taken not to allow any air bubbles. The gel was left for an hour to fully polymerise.

B2 Agarose gels

A 1% gel was prepared as follows:

1. 1g of agarose was dissolved in 100ml of running buffer-1X TAE by gently heating in a microwave oven. Care was taken not to allow the gel to boil too vigorously while ensuring full dissolution of the powdered agarose.
2. The solution was allowed to cool a little until it could be handled. It was then poured into a gel casting tray, care was taken to eliminate air bubbles and to produce an even gel . Combs were inserted into the gel and the gel was left for one hour to set. (Could be stored in the fridge O/N).
3. Once set the combs were removed as was the casting tape and the gel was placed in the electrophoresis tank and covered with 1x running buffer.
4. Samples were mixed with 10% volume of gel loading dye, loaded into the gel and electrophoresed at 100 volts for an hour. A molecular weight marker was always loaded onto the gel.
5. The gel was visualised on a UV transilluminator and photographed. The ethidium bromide (EtBr) in the running buffer and gel intercalates between stacked base pairs of the DNA molecules in the gel and emits ultraviolet induced fluorescence. EtBr has a higher affinity for double stranded than single DNA.

Photographs were taken using a Polaroid camera.

B3 Polyacrylamide gels

Equipment constructed in our laboratory was used as well as apparatus manufactured by Flowgen and Gibco BRL were used. The plates were 45cm long and spacers were 0.4mm thick. The combs used were sharkstooth combs. Glass plates were cleaned with warm soapy water and then with distilled water and finally with ethanol and then dried. One of the plates was coated with dimethldichlorosilane (Repli coat BDH). This would later allow the plated to be separated more easily and ensure that the gel stayed on the plate that has not been coated. Plastic spacers (0.4mm thick) were then placed between the two glass plates and the plates sealed together using insulating tape.

A denaturing 6% acrylamide gel was prepared for DNA sequencing.
7.5ml 40% acrylamide solution (National Diagnostics)
5ml 20X glycerol tolerant buffer
25g Urea
volume was made up to 50ml with water.
The solution was gently warmed while being stirred at the same time to solubilise the urea.
12.5µl of TEMED and 50µl of 10% APS were added to the gel mixture when ready to pour.
A bladder syringe was used to pour the gel. The glass plate was tilted at an angle and care was taken not to allow any air bubbles to form. Once poured the comb was put in place at the top of the gel and clips were used to seal the whole gel together which was then left to set for a minimum of 1 hour or O/N.

A non-7.5% acrylamide gel was prepared for SSCP
7.5ml of 10X TAE buffer
14ml of 40%acrylamide
3ml ie. 4% 0.5M EDTA
3.75ml ie.5%glycerol
10% APS and Temed were added last just before the gel was poured
this gel was non-denaturing as it did not contain any urea.
The plates and pouring was as for a sequencing gel.
For SSCP the gel must allow the DNA to re-nature!

REFERENCES

A.Batch, D.M.Williams, H.R.Davies, B.D.Brown, B.A.J.Evans, I.A.Hughes and M.N.Patterson. (1992) Androgen receptor gene mutations identifieed by SSCP in fourteen subjaect with androgen insensitivity syndrome. *Hum. Mol. Genet.* **1**, 497-503.

Andria, G., Ballabio, A., Parenti, G., Di Maio, S. and Piccirillo, A. (1984) Steroid sulphatase deficiency is present in patients with the syndrome 'ichthyosis and male hypogonadism' and with 'Rud syndrome'. *J. Inherit. Metab. Dis.* **7 Suppl 2**, 159-160.

Anthony C.Casamassima, Patrick L.Wilmot, Betsy K.Vibert and Lawerence R.Shapiro (1993) Kallmann Syndrome Associated With Compleex Chromosome Rearrangement. *Am. J. Med. Genet.* **45**, 539-541.

Anu Suomalainen, Emma Ciafaloni, Yasutoshi Koga, Lena Peltonen, Salvatore DiMauro and and Eric A.Schon. (1992) Use of single stranded conformation polymorphism analysis to detect point mutations in human mitochondrial DNA. *Journal of Neurolgical Sciences* **111**, 222-226.

Atta M Abbasi, Kerry A.Chester, Andrew J.S.Macpherson, Geoffrey M.Boxer, Richard H.J.Begent and Alan D.B.Malcolm (1992) localization of CEA messinger RNA by *in situ* hybridisation in normal colonic mucosa and colorectal adenocarcinomas. *Journal of Pathology* **168**, 405-411.

Ballabio, A., Parenti, G., Napolitano, E., Di Natale, P. and Andria, G. (1985) Genetic complementation of steroid sulphatase after somatic cell hybridization of X-linked ichthyosis and multiple sulphatase deficiency. *Hum. Genet.* **70**, 315-317.

Ballabio, A., Parenti, G., Tippett, P., Mondello, C., Di Maio, S. and Tenore, A. (1986) X-linked ichthyosis, due to steroid sulphatase deficiency, associated with Kallmann syndrome (hypogonadotropic hypogonadism and anosmia): linkage relationships with Xg and cloned DNA sequences from the distal short arm of the X chromosome. *Hum. Genet.* **72**, 237-240.

Ballabio, A., Sebastio, G., Carrozzo, R., Parenti, G., Piccirillo, A., PersicoMG. and Andria, G. (1987) Deletions of the steroid sulphatase gene in "classical" X-linked ichthyosis and in X-linked ichthyosis associated with Kallmann syndrome. *Hum. Genet.* **77**, 338-341.

Ballabio, A., Parenti, G., Carrozzo, R., Coppa, G., Felici, L., Migliori, V., Franceschini, P. and Andria, G. (1988) X/Y translocation in a family with X-linked ichthyosis, chondrodysplasia punctata, and mental retardation: DNA analysis reveals deletion of the steroid sulphatase gene and translocation of its Y pseudogene. *Clin. Genet.* **34**, 31-37.

Ballabio, A., Bardoni, B., Carrozzo, R., Andria, G., Bick, D., Campbell, L., Ferguson-Smith, M.A., Gimelli, G., Fraccaro, M. and et al. (1989) Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. *Proc. Natl. Acad. Sci. USA.* **86**, 10001-10005.

Bardin, C.W. (1971) Hypogonadotropic hypogonadism in patients with multiple congenital defects. *Birth Defects: Original Article Series* **7**, Original A-Original 8.

Bick, D., Franco, B., Sherins, R.J., Heye, B., Pike, L., Crawford, J., MaddalenaA., Incerti, B., Pragliola, A., Meitinger, T. and et al. (1992) Brief report: intragenic deletion of the KALIG-1 gene in Kallmann's syndrome [see comments]. *N. Engl. J. Med.* **326**, 1752-1755.

Birnbacher, R., Wandl-Vergesslich, K. and Frisch, H. (1994) Diagnosis of X-recessive Kallmann syndrome in early infancy. Evidence of hypoplastic rhinencephalon. *Eur. J. Pediatr.* **153**, 245-247.

Bossy.J (1980) Development of olfactory and Related Strucutres in Staged Himan Embryos. *Anat. Embryol* **161**, 225-236.

Cheung, R., Ferreira, L.C. and Youson, J.H. (1991) Distribution of two forms of somatostatin and peptides belonging to the pancreatic polypeptide family in tissues of larval lampreys, Petromyzon marinus L.: an immunohistochemical study. *Gen. Comp. Endocrinol.* **82**, 93-102.

Conrad, B., Kriebel, J. and Hetzel, W.D. (1978) Hereditary bimanual synkinesis combined with hypogonadotropic hypogonadism and anosmia in four brothers. *J. Neurol.* **218**, 263-274.

Cowen, M.A. and Green, M. (1993) IL-1b and Kallmann's syndrome: a variant model of schizophrenia? [editorial]. *Biol. Psychiat.* **33**, 71-72.

Crowley, W.F., Jr. and Jameson, J.L. (1992) Clinical counterpoint: gonadotropin-releasing hormone deficiency: perspectives from clinical investigation. [Review]. *Endocr. Rev.* **13**, 635-640.

Danek, A., Heye, B. and Schroedter, R. (1992) Cortically evoked motor responses in patients with Xp22.3-linked Kallmann's syndrome and in female gene carriers. *Ann. Neurol.* **31**, 299-304.

David T.Moran, Luis Monti-Bloch, Larry J.Stensaas and David L.Berliner (1995) Structure and Function of the Human Vomeronasal Organ. In: Richard L.Doty, (Ed.) *Handbook of Olfaction and Gustation*, pp. 793-820. Dekker]

De Morsier, G. (1954) Etudes sur les dysraphies cranio-encephaliques. I Agenesie des lobes olfactifs (telencephaloschizis lateral) et des commisures calleuse et anterieure (telencephaloschizis median). La dysplasie olfacto-genitale. *Schweiz. Arch. Neurol. Psychiat.* **74**, 309-361.

del Castillo, I., Cohen-Salmon, M., Blanchard, S., Lutfalla, G. and Petit, C. (1992) Structure of the X-linked Kallmann syndrome gene and its homologous pseudogene on the Y chromosome. *Nature Genet.* **2**, 305-310.

El Amraoui, A. and Dubois, P.M. (1993) Experimental Evidence for an Early Commitment of Gonadotrophin Releasing Hormone Neurons, with Special Regard to Their Origin from the Ectoderm of Nasal Cavity Presumptive Teritory. *Neuroendocrinology* **57**, 991-1002.

Evain-Brion, D., Gendrel, D., Bozzola, M., Chaussain, J.L. and Job, J.C. (1982) Diagnosis of Kallmann's syndrome in early infancy. *Acta Paediatr. Scand.* **71**, 937-940.

Frawley, L.S. and Boockfor, F.R. (1991) Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. [Review]. *Endocr. Rev.* **12**, 337-355.

Giancarlo Perenti, Maria Grazia Rizzolo, Monica Ghezzi, Salvatore Di Maio, Maria Pia Sperandeo, Barbara Inceerti, Brunella Franco, Andrea Ballabio and Generoso Andria (1995) Variable Peneetrance of Hypogonadism in a Sibship With Kallmanns Syndrome Due to a Deletion of the KAL Gene. *Am. J. Med. Genet.* **57**, 476-478.

Hardelin, J.-P., Levilliers, J., del Castillo, I., Cohen-Salmon, M., Legouis, R., Compain, S., Bouloux, P.-M.G., Kirk, J.M.W., Moraine, C. and et al. (1992) X chromosome-linked Kallmann syndrome: stop mutations validate the candidate gene. *Proc. Natl. Acad. Sci. USA.* **89**, 8190-8194.

Hardelin, J.-P., Levilliers, J., Blanchard, S., Carel, J.C., Leutenegger, M., Bouloux, P.-M.G. and Petit, C. (1993a) Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. *Hum. Mol. Genet.* **2**, 373-377.

Hardelin, J.-P., Levilliers, J., Young, J., Pholsena, M., Legouis, R., Kirk, J.M.W., Bouloux, P.-M.G., Petit, C. and Schaison, G. (1993b) Xp22.3 deletions in isolated familial Kallmann's syndrome. *J. Clin. Endocrinol. Metab.* **76**, 827-831.

Hardman, T., Kolatsi, M., Winyard P J D., Towers P.R and Woolf, A.S. (1994) *Eexp. Nephrol* **2**, 211-219.

Hawkins, J.R., Taylor, A., Berta.P, Levilliers.J, Van der Auwera.B and Goodfellow.P.N (1992) Mutational analysis of SRY nonsense and missense mutations in XY sex reversal. *Hum Genet* **88**, 471-474.

Hermanussen, M. and Sippell, W.G. (1985) Heterogeneity of Kallmann's syndrome. *Clin. Genet.* **28**, 106-111.

Hipkin, L.J., Casson, I.F. and Davis, J.C. (1990) Identical twins discordant for Kallmann's syndrome. *J. Med. Genet.* **27**, 198-199.

Humphrey, T. (1940) The development of the olfactory and the accessory olfactory formations in human embryos and fetuses. *J. Comp. Neurol.* **73**, 431-468.

Humphrey, T. (1960) Structure and Function of the Cerebral Cortex, In: Anonymous Amsterdam: Elsevier]

Incerti, B., Guioli, S., Pragliola, A., Zanaria, E., Borsani, G., Tonlorenzi, R., Franco, B., Wheeler, D., Ballabio, A. and et al. (1992) Kallmann syndrome gene on the X and Y chromosomes: implications for evolutionary divergence of human sex chromosomes. *Nature Genet.* **2**, 311-314.

Johnson, C.L., Charmley, P., Yen, P.H. and Shapiro, L.J. (1991) A multipoint linkage map of the distal short arm of the human X chromosome. *Am. J. Hum. Genet.* **49**, 261-266.

Jones, J.R. and Kemmann, E. (1976) Olfacto-genital dysplasia in the female. *Obstet. Gynecol. Annu.* **5**, 443-466.

Kallmann, F.J., Schoenfeld, W.A. and Barrera, S.E. (1944) The genetic aspects of primary eunuchoidism. *Am. J. Mental Def.* **48**, 203-236.

Kanai, T. (1940) Uber das kombinierte Vorlommen des partiellen Reichlappendefects mit dem eunuchoidismus. *Okajimas Folia anat. japon* **19**,

Kirk. J.M.W, D.B.Grant, M.O.Savage., G.M.Besser and P.M.G.Bouloux (1994) Identification of olfactory dysfunction in carriers of X-linked Kallmann's Syndrome. *Clin. Endocrinol. Oxf.* **41**, 577-580.

Kirk, J.M.W., Grant, D.B., Besser, G.M., Shalet, S., Quinton, R., Smith, C.S., White, M., Edwards, O.M. and Bouloux, P.-M.G. (1994) Unilateral renal aplasia in X-linked Kallmann's Syndrome. *Clin. Genet.* **46**, 260-262.

Klein G, Langegger, M., Timpl, R. and Ekblom, P. (1988) *Cell* **55**, 331-341.

Klein, V.R., Friedman, J.M., Brookshire, G.S., Brown, O.E. and Edman, C.D. (1987) Kallmann syndrome associated with choanal atresia. *Clin. Genet.* **31**, 224-227.

Klingmuller, D., Dewes, W., Krahe, T., Brecht, G. and Schweikert, H.U. (1987) Magnetic resonance imaging of the brain in patients with anosmia and hypothalamic hypogonadism (Kallmann's syndrome). *J. Clin. Endocrinol. Metab.* **65**, 581-584.

Klink, A., Meindl, A., Hellebrand, H. and Rappold, G.A. (1994) A patient with an interstitial deletion in Xp22.3 locates the gene for X-linked recessive chondrodysplasia punctata to within a one megabase interval. *Hum. Genet.* **93**, 463-466.

Knorr, J.R., Ragland, R.L., Brown, R.S. and Gelber, N. (1993) Kallmann syndrome: MR findings. *Am. J. Neuroradiol.* **14**, 845-851.

Larsen William James (1993) Anonymous *Human Embryology*,

Lee, W.C., Ferrero, G.B., Chinault, A.C., Yen, P.H. and Ballabio, A. (1993) A yeast artificial chromosome contig linking the steroid sulfatase and Kallmann syndrome loci on the human X chromosome short arm. *Genomics* **18**, 1-6.

Legouis, R., Hardelin, J.-P., Levilliers, J., Claverie, J.M., Compain, S., Wunderle, V., Millasseau, P., Le Paslier, D., Cohen, D., Caterina, D., Bouguerelet, L., Delemarre-Van der Waal, H., Lutfalla, G., Weissenbach, J. and Petit, C. (1991) The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* **67**, 423-435.

Legouis, R., Lievre, C., Leibovici, M., Lapointe, F. and Petit, C. (1993a) Expression of the kal gene in multiple neuronal sites during chicken development. *Proc. Natl. Acad. Sci. USA.* **90**, 2461-2465.

Legouis, R., Lievre, C.A., Leibovici, M., Lapointe, F. and Petit, C. (1993b) Expression of the KAL gene in multiple neuronal sites during chicken development. *Proc. Natl. Acad. Sci. USA.* **90**, 2461-2465.

Levy, C.M. and Knudtzon, J. (1993) Kallmann syndrome in two sisters with other developmental anomalies also affecting their father. *Clin. Genet.* **43**, 51-53.

Lieblich, J.M., Rogol, A.D., White, B.J. and Rosen, S.W. (1982) Syndrome of anosmia with hypogonadotropic hypogonadism (Kallmann syndrome): clinical and laboratory studies in 23 cases. [Review]. *Am. J. Med.* **73**, 506-519.

Lobie, P.E., Breipohl, W., Lincoln, D.T., Garcia-Aragon, J., Waters, M.J., Main, K., Price, D.A., Savage, M.O. and Skakkebaek, N.E. (1993) Localization of the growth hormone receptor/binding protein in skin Decreased sweating in seven patients with Laron syndrome. *J. Clin. Endocrinol. Metab.* **77**, 821-823.

Lutz, B., Kuratani, S., Rugarli, E.I., Wawersik, S., Wong, C., Bieber, F.R., Ballabio, A. and Eichele, G. (1994) Expression of the Kallmann Syndrome gene in human fetal brain and in the manipulated chick embryo. *Hum. Mol. Genet.* **3;10**, 1717-1723.

Lyle G Best, Walter A.Wasdahl, Linda M.Larson and Jeean Sturlaugson (1990) Chromosome Abnormality in Kallmann Syndrome. *Am. J. Med. Genet.* **35**, 306-309.

Males, J.L., Townsend, J.L. and Schneider, R.A. (1973) Hypogonadotropic hypogonadism with anosmia -Kallmann's syndrome. A disorder of olfactory and hypothalamic function. *Arch. Intern. Med.* **131**, 501-507.

Meindl, A., Hosenfeld, D., Bruckl, W., Schuffenhauer, S., Jenderny, J., Oppermann, H.C., Swensson, O., Bouloux, P.-M.G. and Meitinger, T. (1993) Analysis of a terminal Xp22.3 deletion in a patient with six monogenic disorders: implications for the mapping of X linked ocular albinism. *J. Med. Genet.* **30**, 838-842.

Meitinger, T., Heye, B., Petit, C., Levilliers, J., Golla, A., Moraine, C., Sippell, W.G.,

Murken, J. and Ballabio, A. (1990) Definitive localization of X-linked Kallman syndrome (hypogonadotropic hypogonadism and anosmia) to Xp22.3: close linkage to the hypervariable repeat sequence CRI-S232 [published erratum appears in Am J Hum Genet 1990 Nov;47(5):883]. *Am. J. Hum. Genet.* **47**, 664-669.

Meng Inn Chuah and Farbman, A.I. (1995) Developmental Anatomy of the Olfactory System. In: Richard L.Doty, (Ed.)

Menon, V., Butt, W.R., Clayton, R.N., Edwards, R.L. and Lynch, S.S. (1984) Pulsatile administration of GnRH for the treatment of hypogonadotrophic hypogonadism. *Clin. Endocrinol. Oxf.* **21**, 223-232.

Merriam, G.R., Beitins, I.Z. and Bode, H.H. (1977) Father-to-son transmission of hypogonadism with anosmia: Kallmann's syndrome. *Am. J. Dis. Child.* **131**, 1216-1219.

Monti-Graziadei, A.G. and Graziadei, P.P.C. (1992) Sensory Reeinnervation After Partial Removal of the Olfactory Bulb. *The Journal of Comparative Neurology* **316**, 32-44.

Moran, D.T., Jafek, B.W. and Rowley, J.C. (1991) The vomeronasal (Jacobson's) organ in man: ultrastructure and frequency of occurrence. *J. Steroid Biochem. Molec. Biol* **39**, 545-552.

Muske, Linda E. (1993) Evolution of Gonadotrophin Reeleasing Hormone (GnRH) Neuronal Systems. *Brain Behav Evol* **42**, 215-230.

Nakashima, T., Kimmelman, C.P. and Snow, J.B. (1985) Vomeronasal organs and nerves of Jacobsen in the human fetus. *Acta Otolaryngol. Stockh.* **99**, 266-271.

Nowakowski, H. and Lenz, W. (1961) Gentic aspects in male hypogonadism. *Rec Prog Horm Res* **17**, 53-95.

Odile Grau and Remy Griffais (1994) Diagnosis of mutations by the PCR doublee RFLP method (PCR-dRFLP). *Nucleic Acid Research* **22**, 5773-5774.

Olaf Hiort, Qian Huang, Gernot H.G.Sinnecker, AB Sadeghi-Nejad, Klaus Kruse, Hubert J.Wolfe and David W.Yandell (1993) Single Strand Conformation Polymorphism Analysis of Androgen Receptor Genee Mutations in Patients with Androgen Insensitivity Syndromes: Application for Diagnosis, Genetic Counseling and Therapy. *Endocrinology and Metabolism* **77**, 262-267.

Oldberg ST (1932) Ein Beitrag zur frage der Arhinencerphalie. *Uppsala Lak for Forh* **38**,

Oppermann, D., Happ, J. and Mayr, W.R. (1987) Stimulation of spermatogenesis and biological paternity by intranasal (low dose) gonadotropin-releasing hormone (GnRH) in a male with Kallmann's syndrome: intraindividual comparison of GnRH and gonadotropins for stimulation of spermatogenesis. *J. Clin. Endocrinol. Metab.* **65**, 1060-1066.

Parr, J.H. (1988) Midline cerebral defects and Kallmann's syndrome. *J. R. Soc. Med.* **81**, 355-356.

Patterson, G. and Bonnier, G. (1937) Inherited Sex-Mosaic in Man. *Hereditas* **23**,

Pawlowitzki, I.H., Diekstall, P., Schadel, A. and Miny, P. (1987) Estimating frequency of Kallmann syndrome among hypogonadic and among anosmic patients. *Am. J. Med. Genet.* **26**, 473-479.

Pearson, A.A. (1941a) The development of the nervus terminalis in man. *J. Comp. Neurol.* **75**, 39-66.

Pearson, A.A. (1941b) The development of the olfactory nerve in man. *J. Comp. Neurol.* **75**, 199-217.

Petit, C., Levilliers, J. and Weissenbach, J. (1990) Long-range restriction map of the terminal part of the short arm of the human X chromosome. *Proc. Natl. Acad. Sci. USA.* **87**, 3680-3684.

Posnett D.N., McGrath H. and Tam P.J (1988) Studies of th pore-forming domain of a voltage-gated potassium channel protein. *Proc. Natl Acad Sci* **85**, 5409-5413.

Posnett, D., McGrath, H. and Tam, J.P. (1988) A Novel Method for Producing Anti -Peptide Antibodies. Production of Sit Specific Antibodies to the T cell Antigen Receptor B-Chain. *Journal of Biological Chemistry* **263**, 1719-1725.

Prager, D. (1993) EDITORIAL: X-chromosome-linked Kallmann's Syndrome: pathology at the molecular level. *J. Clin. Endocrinol. Metab.* **76**, 824-826.

Randall Moorman, J., Barbara Crain and Dennis Osborne (1984) Kallmann's Syndrome with Associated Cardiovascular and Intracranial Anomalies. *The American Journal of Medicine* **77**, 369-372.

Ronnekleiv, O.K. and Resko, J.A. (1990) Ontogeny of Gonadatropin Releasing hormone containing neurons in early fetal development of rhesus macaques. *Endocrinology.* **126**, 498-511.

Rosen, S.W., Gann, P. and Rogol, A.D. (1979) Congenital anosmia: detection thresholds for seven odorant classes in hypogonadal and eugonadal patients. *Ann. Otol. Rhinol. Laryngol.* **88**, 288-292.

Rugarli, E.I., Lutz, B., Kuratani, S.C., Wawersik, S., Borsani, G. and Ballabio, A. (1993) Expression pattern of the Kallmann syndrome gene in the olfactory system suggests a role in neuronal targeting. *Nature Genet.* **4**, 19-26.

Sainton, P. (1902) Un cas d'eunuchisme familial. *Nouv. Icon. Salpetr.* **25**, 272-277.

Santen, R.J. and Paulsen, C.A. (1973) Hypogonadotropic eunuchoidism. I. Clinical study of the mode of inheritance. *J. Clin. Endocrinol. Metab.* **36**, 47-54.

Schmitz, F., Holbach, M. and Drenckhahn, D. (1993) *Histochemistry* **100**, 473-479.

Schott, G.D. and Wyke, M.A. (1981) Congenital mirror movements. *J. Neurol. Neurosurg. Psychiat.* **44**, 586-599.

Schwanzel-Fukuda, M., Bick, D. and Pfaff, D.W. (1989) Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. *Mol.Brain Res.* **6**, 311-326.

Schwanzel-Fukuda, M., Abraham, S., Crossin, K.L., Edelman, G.M. and Pfaff, D.W. (1992) Immunocytochemical demonstration of neural cell adhesion molecule (NCAM) along the migration route of luteinizing hormone-releasing hormone (LHRH) neurons in mice. *J. Comp. Neurol.* **321**, 1-18.

Schwanzel-Fukuda, Marlene and Pfaff, Donald (1995) Strucutre and Function of the Nervus Terminalis. In: Richard L.Doty, (Ed.) *Handbook of Olfaction and Gustation*, pp. 835-865. Dekker]

Schwanzel-Fukuda, M., Reinhard, G.R., Abraham, S., Crossin, K.L., Edelman, G.M. and Pfaff, D.W. (1994) Antibody to neural cell adhesion molecule can disrupt the migration of luteinizing hormone-releasing hormone neurons into the mouse brain. *J. Comp. Neurol.* **342**, 174-185.

Schwanzel-Fukuda, M. and Pfaff, D.W. (1989) Origin of Luteinizing hormone releasing hormone neurons. *Nature* **338**, 161-164.

Schwanzel-Fukuda, M. and Pfaff, D.W. (1991) Migration of LHRH immunoreactive neurons from the olfactory placode rationalizes olfacto-hormonal relationships. *J. Steroid Biochem. Molec. Biol* **39**, 565-572.

Sette, A., Buus, S., Appella, E., Smith, J.A., Chesnut, R., Milees, C., Sonia M.Colon and Howard Grey (1989) prediction of major histocompatibility complex binding reegions of protein antigens by sequence patteern analysis. *Proc. Natl. Acad. Sci* **86**, 3296-3300.

Sherwood, N.M., Lovejoy, D.A. and Coe, I.R. (1993) Origin of mammalian gonadotropin-releasing hormones. *Endocr. Rev.* **14**, 241-254.

Sinclair, A., H, Berta, P., Palmer, M.S., Hawkins, R., Griffiths, B.L., Smith, M.J., Jamie W.Foster, Anna-Maria Frischauf, Robin Lovell-Badge and and Peter Goodfellow (1990) The gene from the human sex-determining region encodes a protein with homology to a conserved DNA binding motif. *Nature* **346**, 240-244.

Sunohara, N., Sakuragawa, N., Satoyoshi, E., Tanae, A. and Shapiro, L.J. (1985) A new syndrome of anosmia, ichthyosis, hypogonadism, and various neurological manifestations with deficiency of steroid sulphatase and arylsulphatase C. *Ann. Neurol.* **19**, 174-181.

Tagatz, G., Fialkow, P.J., Smith, D. and Spadoni, L. (1970) Hypogonadotropic hypogonadism associated with anosmia in the female. *N. Engl. J. Med.* **283**, 1326-1329.

Takeda, T., Takasu, N., Yamauchi, K., Komiya, I., Ohtsuka, H., Nagasawa, Y. and Yamada, T. (1992) Magnetic resonance imaging of the hypoplasia of the rhinencephalon in a patient with Kallmann's syndrome. *Intern. Med.* **31**, 394-396.

Tam, J.P. (1988) Synthetic peptide vaccinee desigb: Synthesis and propertiees of a high-dnsity multiple antigenic peptide system. *Proc. Natl. Acad. Sci* **85**, 5409-5413.

Truwit, C.L., Barkovich, A.J., Grumbach, M.M. and Martini, J.J. (1993) MR imaging of Kallmann syndrome, a genetic disorder of neuronal migration affecting the olfactory and genital systems. *Am. J. Neuroradiol.* **14**, 827-838.

Turnbull, A.J., Law, A. and Peters, T.J. (1988) Ulcerative colitis associated with Kallmann's syndrome. *J. R. Soc. Med.* **81**, 354-355.

Vogl, T.J., Stemmler, J., Heye, B., Schopohl, J., Danek, A., Bergman, C., Balzer, J.O. and Felix, R. (1994) Kallmann syndrome versus idiopathic hypogonadotropic hypogonadism at MR imaging. *Radiology* **191**, 53-57.

Webster, D., France, J.T., Shapiro, L.J. and Weiss, R. (1978) X-linked ichthyosis due to steroid-sulphatase deficiency. *Lancet.* **1**, 70-72.

Wegenke, J.D., Uehling, D.T., Wear, J.B., Jr., Gordon, E.S., Bargman, J.G., Deacon, J.S. and Opitz, J.M. (1975) Familial Kallmann syndrome with unilateral renal aplasia. *Clin. Genet.* **7**, 368-381.

Weidenreich, F. (1914) Uber partiellen Riechlappendefekt und Eunuchoidismus beim Menschen. *Z. Morphol. Anthropol.* **18**, 157-190.

White, B.J., Rogol, A.D., Brown, K.S., Lieblich, J.M. and Rosen, S.W. (1983) The syndrome of anosmia with hypogonadotropic hypogonadism: a genetic study of 18 new families and a review. *Am. J. Med. Genet.* **15**, 417-435.

Whittall R., Gudnason V, Weavind G.P, Day L.B, Humphries S.E. and Day I.N.M (1995) Utilities for high throughput use of the single stranded conformational polymorphism method: screeening of 791 patients with familial hypercholesterolaemia for mutations in eexon 3 of the low deensity lipoprotein reeceptor gene. *J Med Geneetics* **32**, 509-515.

Wray, S., Grant, P. and Gainer, H. (1989) evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc. Natl. Acad. Sci. USA.* **86**, 8132-8136.

Yousem, D.M., Turner, W.J., Li, C., Snyder, P.J. and Doty, R.L. (1993) Kallmann syndrome: MR evaluation of olfactory system. *Am. J. Neuroradiol.* **14**, 839-843.

Zhang, Y., McMahon, R., Charles, S.J., Green, J.S., Moore, A.T., Barton, D.E. and YatesJR. (1993) Genetic mapping of the Kallmann syndrome and X linked ocular albinism gene loci. *J. Med. Genet.* **30**, 923-925.

PUBLICATIONS

Abstracts and oral communacations

1."Frequency of gene deletion in patients with Idiopathic Hypogonadotrophic Hypogonadism and Kallmanns Syndrome." G.Matfin, **V.Duke,** P.Munroe, G.M Besser, C.Petit and P.M.G.Bouloux. March 1993 British Endocrine Society- Annual meeting - Liverpool. Poster

2."Foetal expression of Kallmann gene product using monoclonal antibody immunohistochemistry." **V M Duke,** P B Munroe, G Matfin, D Read, G M Boxer, W,Nicholas, R H J Begent and P M G Bouloux. November 1993 Royal Society Of Medicine Endocrine Division -London. Poster

3."Investigation of Lipoprotein Lipase as a candidate gene for Dyslipidemic Hypertension." March 1994 London Hypertension Society 5th Annual Symposium-Wellcome Building London. Poster.

4. "Investigation of expression of Kallmann gene mRNA in the developing foetus using in situ hybridisation." **V M Duke**, P J D Winyard, H Dewchand, A S Woolf, P V Thorogood & P M G Bouloux. March 1994. British Endocrine Society-Annual meeting-Bournmouth. Poster

5. "KAL gene expression during Kidney development explains renal aplasia in Kallmann's syndrome." P.J.D Winyard, **V.M.Duke,** P.Soothill, P.V.Thorogood, J.T Norman, P.M.G.Bouloux and A.S.Woolf. April 1994. Renal Association of Great Britain Annual meeting. Poster

6."Spatial Expression of KAL gene mRNA in the developing human foetus." July 1994. 3rd International Congress of Neuroendocrinology-Budapest. Oral communication

7. Diagnosis of Kallmann's Syndrome at age 18 months by demonstration of base substitution in KAL gene by direct PCR sequencing. **VM Duke**, P A De Zoysa, R Quinton, PMG Bouloux. March 1995. British Endocrine Society-Annual meeting Warwick. Poster.

8. Mutation analysis in patients with Kallmann's Syndrome using Polymerase Chain Reaction. **VM Duke**, P A De Zoysa, R Quinton, PMG Bouloux. April 1995. Medical Research Society, Cambridge. Poster.

9."KAL a gene mutated in Kallmann's Syndrome is expressed in the first trimester of human development." **VM Duke**, PJD Winyard, PV Thorogood, P Soothill, PMG Bouloux and AS Woolf. June 1995 77th American Endocrine Society meeting, Washington. Oral communication.

Publications:

Deletion analysis maps ocular albinism proximal to the steroid sulphatase locus. P.M.G.Bouloux, J.Kirk, P.Munroe, **V.Duke**, A.Meindl, A.Hilson, D.Grant, N.Carter, D.Betts, T.Meitinger and G.M.Besser. (1993) Clinical Genetics Vol **43** pg 169-173

Kallmann's Syndrome - Clinical Experience. P.M.G.Bouloux, **V.M.Duke**, M.A.Hall-Craggs, P.Manning, R.Quinton, H.S.Jacobs. (1994) Current Opinions in Obstetrics and Gynaecology. **6:**301-303.

Treatment of male infertility. Quinton R, **Duke V**, Kirk J, Bouloux P. Letter, Lancet **334**(8916):192 Jul 1994

KAL a gene mutated in Kallmann's Syndrome is expressed in the first trimester of human development. **VM Duke**, PJD Winyard, PV Thorogood, P Soothill, PMG Bouloux and AS Woolf. (1995) Molecular and Cellular Endocrinology. **110**:73-79

CLINICAL GENETICS
ISSN 0009-9163

Deletion analysis maps ocular albinism proximal to the steroid sulphatase locus

Bouloux, P-MG, Kirk J, Munroe P, Duke V, Meindl A, Hilson A, Grant D, Carter N, Betts D, Meitinger T, Besser GM. Deletion analysis maps ocular albinism proximal to the steroid sulphatase locus.
Clin Genet 1993: 43: 169–173. © Munksgaard, 1993

We describe a pedigree in which four male members are affected by a contiguous gene abnormality involving the short arm of the X chromosome (Xp22.32). Bivariate flow cytometry of lymphoblastoid cell lines from two of these individuals and a normal male showed a 6–7 megabase deletion in affected males, and high resolution chromosomal G-banding of an obligate heterozygote showed the deletion to reside in the Xp22.32 region. Affected members had X-linked ichthyosis due to steroid sulphatase deficiency, Kallmann's syndrome, but no ocular albinism. In two out of four affected individuals studied, there was unilateral renal agenesis. Deletion analysis using the Xp22.32 markers MIC2, DXS31, DXS 89, GMGX9, DXS278, DXS143, and DXS9 showed that the deletion extended from DXS31 to DXS143 (inclusive). The absence of ocular albinism in this pedigree shows conclusively that the X-linked ocular albinism gene resides proximal to the DXS143 locus. Further, the inconstant association of unilateral renal agenesis with X-linked Kallmann's syndrome, even when the latter is caused by a complete deletion of the gene, suggests that the absence of the X-linked Kallmann gene can be compensated in renal development.

P-M. G. Bouloux, J. Kirk, P. Munroe, V. Duke, A. Meindl[3], A. Hilson, D. Grant[5], N. Carter[1], D. Betts[2], T. Meitinger[3] and G. M. Besser[4]

Academic Department of Medicine, Royal Free Hospital, [1]Department of Pathology, Downing Site Cambridge, [5]Hospital for Sick Children, Great Ormond Street, London, [2]ICRF, St Bartholomew's Hospital, [3]Abt. Päd. Genetik KinderPoliklinik der Universität München, Germany and [4]Department of Endocrinology, St Bartholomew's Hospital, London, UK

Key words: deletion interval – Kallmann's syndrome – ocular albinism – unilateral renal agenesis

Dr Pierre Bouloux MD BSc MRCP, Academic Department of Medicine, Royal Free Hospital School of Medicine, Pond Street, London NW3 2QG, UK

Received 13 August, revised version received 25 November, accepted for publication 10 December 1992

Kallmann's syndrome (KS) describes the association of hypogonadotrophic hypogonadism with anosmia, due to olfactory lobe agenesis (Kallmann et al. 1944, de Morsier 1954). The condition can occur sporadically, or be inherited as an X-linked recessive condition; more rarely it occurs as an autosomal or recessive trait. The incidence of Kallmann's syndrome is 1 in 10000 in males and 1 in 50000 in females (Jones & Kemmann 1976, Pawlowitzki et al. 1987). The X-linked form of the condition is associated with renal agenesis, and mirror movements of the hands (hereditary bimanual synkinesis) (Schwankhaus et al. 1989). Kallmann's syndrome rarely occurs as part of a contiguous syndrome abnormality (Schmickel 1986), usually the result of an interstitial or terminal deletion involving Xp22.3. Depending on the size of the deletion, affected individuals demonstrate two or more abnormalities including chondrodysplasia punctata, mental retardation, X-linked ichthyosis and the Kallmann syndrome (Ballabio et al. 1987, 1989). The investigation of individuals with contiguous gene syndromes has played a major role in establishing the order of genes in the Xp22.3 region; this enabled narrowing of the Kallmann interval to 67 kb, which proved the starting point for the eventual cloning of a candidate gene for the X-linked Kallmann gene (Legouis et al. 1991).

We describe a pedigree with an interstitial deletion in the Xp22.32 associated with a contiguous gene abnormality. Affected males demonstrated mild mental subnormality, X-linked ichthyosis (XLI: due to steroid sulphatase deficiency) and KS. DNA analysis has been performed and the boundaries of the microdeletion mapped. The results have implications for assignment of the locus for X-linked ocular albinism.

Materials and methods

Clinical features

There are a number of contiguous gene abnormalities affecting the short arm of the X-chromosome, and these have been described previously (Ballabio et al. 1989, Petit et al. 1990). In the Xp22.32 region, interstitial deletions have been associated with two or more concurrent abnormalities such as mental retardation, short stature, chondrodysplasia punctata, X-linked ichthyosis (XLI: due to steroid sulphatase deficiency), and KS. The pedigree described here (Fig. 1) spans two generations, and the clinical details have been published previously (Pike et al. 1989). The index case (W1) presented at birth with undescended testes which required orchidopexy in late childhood. In earlier childhood, ichthyosis occurred affecting predominantly the limbs, particularly distally. Mild mental subnormality was present. He was of normal stature, despite pubertal failure, and endocrine investigation revealed a low gonadotrophin level, associated with low basal testosterone levels. There was no gonadotrophin response to an iv bolus of 100 μg GnRH, a modest rise in LH/FSH following a 500 μg iv GnRH dose, and no LH/FSH response to a standard clomiphene test (50 mg bd orally for 7 days). High resolution contrast enhanced CT scan (GE9800) of the pituitary/hypothalamic region failed to reveal a structural lesion. The testes were less than 2 ml, and the penis was small. Anosmia was present on testing with seven odorants at a variety of concentrations (Rosen et al. 1968). Bimanual synkinesis was demonstrable. Three other males in the pedigree (W9, W10, W11) were similarly affected, with synkinesis, micropenis and small testes (< 2 ml) prior to treatment. All affected members underwent a detailed ophthalmological examination, which failed to reveal any stigmata of ocular albinism.

Bivariate flow cytometry (Fig. 2)

Lymphocytes from affected and an unaffected male within the pedigree were isolated from whole blood and transfected with Epstein Barr virus from a marmoset cell line. The lymphoblastoid cell lines so produced were grown in RPMI medium, to which was added fetal calf serum. Metaphase chromosomes isolated from lymphoblasts from W1, W4 (normal male control), and W11 were stained with the fluorescent dyes Hoescht 33258 (HO) and chromomycin A3 (CA). They were then passed into a dual beam flow cytometer (van den Engh et

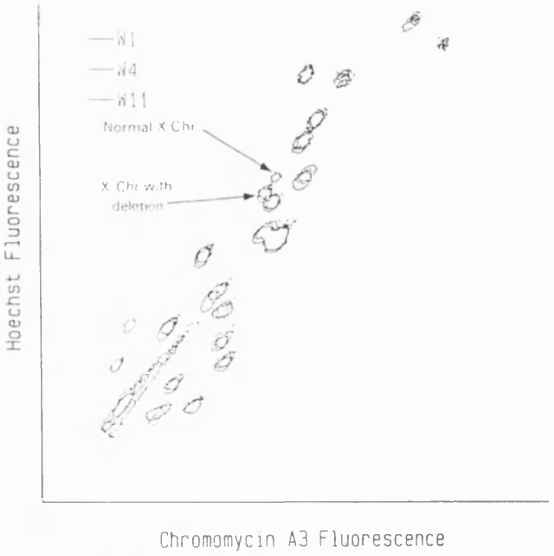


Fig. 2. Flow karyogram of metaphase chromosomes from W1, W4 (normal X chromosome) and W11 stained with Hoechst 33258 and chromomycin A3. The plot shows that W1 and W11 have reduced Hoechst 33258 and chromomycin A3 fluorescence, the result of a deleted Xp22.32 fragment.

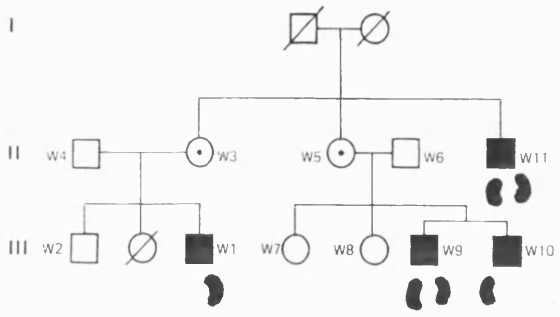


Fig. 1. Family tree showing affected males (solid squares) and unaffected males and females (hollow squares and circles), and obligate carriers (circles with central dot). Renal status of individuals shown. W1 and W10 have unilateral renal agenesis (DMSA scanning).

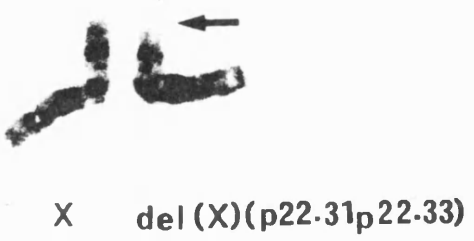


Fig. 3. High resolution G banding of obligate female carrier showing interstitial deletion of X chromosome.

Table 1 Deletion analysis of patients (W1, W9) with contiguous gene abnormality and obligate carriers (W5, W3)

	Phenotype	Mic2	DXS31	DXS89	GMGX9	DXS278	DXS143	DXS9
W1	*XLI/Kal*	+	−	−	−	−	−	+
W9	*XLI/Kal*	+	−	−	−	−	−	+
W5	*Carrier*	+	+	+	+	+	+	+
W3	*Carrier*	+	+	+	+	+	+	+

al. 1985, 1988), and the HO and CA fluorescence intensities of approximately 30 000 chromosomes were quantified. The position of the peaks showed a bivariate distribution, with the flow karyotype reflecting both the relative DNA content and the base composition of the chromosomes. An iterative bivariate Gaussian distribution-fitting procedure was used to assign peak positions in flow karyotypes objectively. The distance between the origin and the projection of a peak onto a line running through the origin and the peak for chromosome 4 is linearly correlated to chromosome DNA content. To estimate deletion size, the peak position of the derivative X in W1, W11 was compared with that of a normal X (W4) in the same karyotype. The flow karyotypes were normalized using the average HO and CA intensities of all the autosomes except 9–12 to the mean position of normal X chromosomes from 30 unrelated individuals to compare the deleted X chromosome against the mean position of a normal X-chromosome.

High resolution G banding (Fig. 3)

High resolution G banding was performed by tryptic immersion of the karyogram obtained from a metaphase preparation of lymphoblastoid cell lines, and banded using Leishmann's stain. The karyogram from an obligate carrier is shown in Fig. 3.

DNA extraction and analysis

Total human DNA was extracted from whole blood and 5 μg digested to completion by the appropriate restriction enzyme. The resulting fragments were separated according to molecular weight using agarose gel electrophoresis (0.85%). Transfer of the DNA to Hybond N filters, hybridization to radio-labelled DNA probes and autoradiography were performed using standard protocols (Southern 1975).

Probes

Analysis by Southern blotting of DNAs of the members of the present 2-generation family was performed (Table 1) with the polymorphic X-specific probes MIC 2 (PsG1; Rouyer et al. 1986), DXS31 (Mandel et al. 1989), DXS89 (Ahrens et al. 1985), DXS278 (Knowlton et al. 1989), GMGX9 (Gillard et al. 1987), DXS143 (Middlesworth et al. 1985) and DXS9 (Murray et al. 1982).

Results

Previously cloned Xp22.3 probes were used to confirm the deletion detected with high resolution G-banding and bivariate flow cytometry and to map the approximate boundaries (Table 1). The bivariate flow cytometry of affected individuals gave a deletion size of approximately 2%, corresponding to about 6–7 megabases. Deletion mapping showed that the microdeletion extended from MC1A to the DXS143 loci, but did not affect the RC8 locus.

Discussion

The investigations performed in this family have revealed a large interstitial deletion of 6–7 megabases in affected male individuals, the deletion starting proximal to MIC2 involving DXS31, and extending to involve the DXS143 locus but ending distal to DXS9. The affected inviduals manifested the full complement of a contiguous gene abnormality including X-linked ichthyosis, Kallmann's syndrome (anosmia, hypogonadotrophic hypogonadism), and mild mental retardation. The KS was associated with hereditary bimanual synkinesis in all affected males, and unilateral renal agenesis in two out of four. There was no nystagmus, and there were no ocular pursuit movement abnormalities. Detailed ophthalmological examination of affected males and an obligate heterozygote failed to reveal any stigmata of ocular albinism.

There have been several reports of concurrent XLI and Kallmann's syndrome existing as part of a contiguous gene deletion (Ballabio et al. 1989); one report has documented a case of XLI, and ocular albinism in the same individual (Schnur et al. 1989), leading to a proposed assignment of the ocular albinism locus near to the STS locus. This report conflicts with the accepted map of this region (Ballabio et al. 1986, 1989).

Schnur et al. (1989) proposed that the ocular albinism gene was in close proximity to the steroid

sulphatase locus, and suggested that the polymorphic marker DXS278 could be used for linkage analysis of ocular albinism. These data are clearly at variance with the present report, demonstrating deletion of the marker DXS143, which is proximal to DXS278. Our patient and the obligate carriers did not have any manifestations of ocular albinism; thus, by implication, the X-linked OA gene must lie proximal to DXS143.

The putative Kallmann gene has recently been cloned (Franco et al. 1991, Legouis et al. 1991), and appears to encode a protein with cell adhesion properties. The neurobiological defect in Kallmann's syndrome appears to be a failure of cellular migration, whereby gonadotrophin releasing hormone (GnRH) neurones fail to move from their site of origin in the medial olfactory placode into the septo-preoptic nucleus of the hypothalamus in early embryonic life. Simultaneously, there is a failure of the olfactory nerve and bulb to develop. The Kallmann gene product has homology with the NCAM family of proteins (Legouis et al. 1991), which appear to play a fundamental role in the peregrination of nerve cell bodies, and the development of axon pathways. The absence of this gene and its product gives a plausible explanation for some of the central nervous abnormalities in Kallmann's syndrome (bimanual synkinesis, pursuit movement abnormalities). One hypothesis for the synkinesis proposes that there is defective pyramidal decussation of corticospinal pathways (Conrad et al. 1978); another suggests that the abnormality may reside in an abnormality of transcallosal inhibitory fibres. With respect to the putative role of the gene in renal development, the lack of 100% penetrance in respect of the unilateral renal agenesis demonstrated in this family (Fig. 1) is less easily explained. Although the present report as well as others (Wegenke et al. 1975) suggest a role of the X-linked Kallmann gene in renal development, it seems that its absence can be compensated.

In summary, we have reported a family with an extensive interstitial Xp22.32 deletion demonstrated on both bivariate chromosomal flow cytometry and high resolution G banding, extending from DXS31 to DXS143 inclusive. The absence of ocular abnormalities in this pedigree has implications for the mapping of the X-linked ocular albinism gene, which must lie proximal to DXS143.

Acknowledgements

We are grateful for financial support from the Joint Research Board of St Bartholomew's Hospital, London, which enabled these studies to be carried out.

References

Ahrens P, Albertsen H, Riis Vestergaard SR, Bolund L, Kruse TA. Anonymous X chromosomal probes revealing DNA polymorphisms, one of which is a deletion of more than 3 kb. Cytogenet Cell Genet 1985; 40: 567.

Ballabio A, Parenti G, Tippett P, Mondello C, Di Maio S, Tenore A, Andria G. X-linked ichthyosis due to steroid sulphatase deficiency, associated with Kallmann's syndrome (hypogonadotrophic hypogonadism and anosmia): linkage relationship with Xg and cloned DNA sequences from the distal short arm of the X chromosome. Hum Genet 1986; 72: 237–249.

Ballabio A, Sebastio G, Carozzo R, Parenti G, Piccirillo A, Persico MG, Andria G. Deletions of the steroid sulphatase gene in 'classical' X-linked ichthyosis and in X-linked ichthyosis associated with Kallmann's syndrome. Hum Genet 1987; 77: 338–341.

Ballabio A, Bardoni B, Carrozzo R, Andria G, Bick D, Campbell L, Hamel B, Ferguson Smith MA, Gimelli G, Fraccaro M. Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. Proc Natl Acad Sci USA 1989; 86: 10001–10005.

Conrad B, Kriebel J, Hetzel WD. Hereditary bimanual synkinesis combined with hypogonadotrophic hypogonadism and anosmia in four brothers. J Neurol 1978; 218: 263–274.

de Morsier G. Etudes sur les dysraphies cranio-encephaliques. I. Agénésie des lobes olfactifs (telencephaloschizis latéral) et des commissures calleuses et anterieures (telencephaloschizis median). La dysplasie olfacto-genitale. Schweiz Arch Neurol Neurochir Psychiatr 1955; 74: 309–361.

Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carrozzo R, Maestrini E, Pieretti M, Taillon-Miller P, Brown CJ, Willard HF, Lawrence C, Graziella Persico M, Camerino G, Ballabio A. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. Nature 1991; 353: 529–536.

Gillard EF, Affara N, Yates JRW, Goudie DR, Lambert J, Aitken DA, Ferguson-Smith MA. Detection of a DNA sequence in eight of nine families with X-linked ichthyosis (steroid sulphatase deficiency). Nucl Acid Res 1987; 15: 3977–3985.

Jones JR, Kemmann E. Olfacto-genital dysplasia in the female. Obstet Gynecol Ann 1976; 5: 443–446.

Kallmann FJ, Schoenfeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. Am J Ment Defic 1944; 48: 203–236.

Knowlton RG, Nelson CA, Brown VA, Page DC, Donis-Keller H. An extremely polymorphic locus on the short arm of the human X-chromosome with homology with the long arm of the Y chromosome long arm. Nucl Acid Res 1989; 17: 423–437.

Legouis R, Hardelin JP, Levilliers J, Claverie J-M, Compain S, Wunderle, V, Millaseau P, Le Paslier D, Cohen D, Caterina D, Weissenbach J, Petit C. The candidate gene for X-linked Kallmann syndrome encodes a protein related to adhesion molecules. Cell 1991; 67: 423–435.

Mandel J-L, Willard HF, Nussbaum RL, Davies KE, Romeo G. Report of the committee on the genetic constitution of the X chromosome (Human Gene Mapping 9.5: update to the 9th International Workshop on Human Gene Mapping). Cytogenet Cell Genet 1989; 49: 107–128.

Middlesworth W, Bertelson C, Kunkel LM. An RFLP detecting single copy X-chromosome fragment, dic56, from Xp22-Xpter (HGM8 assignment no. DXS143). Nucl Acid Res 1985; 13: 5723.

Murray JM, Davies K, Harper S, Meredith L, Mueller CR, Williamson R. Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy. Nature 1982; 350: 69–71.

Pawlowitzki IH, Diekstall P, Schadel A, Mimy P. Estimating the frequency of Kallmann syndrome among hypogonadal and among anosmic patients. Am J Hum Genet 1987; 26: 473–479.

Petit C, Melki J, Levilliers J, Serville F, Weissenbach J, Maroteaux P. An interstitial deletion in Xp22.3 in a family with X-linked recessive chondrodysplasia punctata and short stature. Hum Genet 1990; 85: 247–250.

Pike MG, Hammerton M, Edge J, Atherton DJ, Grant DB. A family with X-linked ichthyosis and hypogonadism. Eur J Paediatr 1989; 148: 442–444.

Rosen SW, Gann P, Rogol AD. Congenital anosmia: detection thresholds for seven odorant classes in hypogonadal and eugonadal patients. Ann Otol 1979; 88: 288–292.

Rouyer F, Simmler MC, Johnsson C, Vergnaud G, Cooke HJ, Weisenbach J. A gradient of sex linkage in the pseudoautosomal region of the human sex chromosomes. Nature 1986; 319: 291–295.

Schmickel RD. Contiguous gene syndromes: a component of recognizable syndromes. J Paediatr 1986; 109: 231–241.

Schnur RE, Trask BJ, van den Engh Ger, Punnett HH, Kistenmacher M, Tomeo MA, Naids RE, Nussbaum RL. An Xp22 microdeletion associated with ocular albinism and ichthyosis: approximation of breakpoints and estmation of deletion size by using cloned DNA probes and flow cytometry. Am J Hum Genet 1989; 45: 706–720.

Schwankhaus JD, Currie J, Jaffe MJ, Rose SR, Sherins RJ. Neurologic findings in men with isolated hypogonadotrophic hypogonadism. Neurology 1989; 39: 223–229.

Southern E. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98: 503–517.

van den Engh GJ, Trask BJ, Gray JW, Langlois RG, Yu LC. Preparation and bivariate analysis of suspensions of human chromosomes. Cytometry 1985; 6: 92–100.

van den Engh GJ, Trask BJ, Landsdorp P, Gray JW. Improved resolution of flow cytometric measurements of Hoechst- and chromomycin-A3-stained human chromosomes after addition of citrate and sulfite. Cytometry 1988; 9: 266–270.

Wegenke JD, Uehling DT, Wear JB, Gordon ES, Bargman JG, Deacon JSR, Hermann JPR, Opitz J. Familial Kallmann syndrome with unilateral renal aplasia. Clin Genet 1975; 7: 368–381.

ELSEVIER

Molecular and Cellular Endocrinology 110 (1995) 73–79

Molecular and Cellular Endocrinology

KAL, a gene mutated in Kallmann's syndrome, is expressed in the first trimester of human development

Veronique M. Duke[a,*], Paul J.D. Winyard[b], Peter Thorogood[b], Peter Soothill[c], Pierre M.G. Bouloux[a], Adrian S. Woolf[b]

[a]*Division of Endocrinology, Royal Free Hospital School of Medicine and University College London Medical School Joint Departments of Medicine, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2QG, UK*
[b]*Developmental Biology Unit, Institute of Child Health, London WC1N 1EH, UK*
[c]*Mothercare Clinical Genetics and Fetal Medicine Unit, Institute of Child Health, London WC1N 1EH, UK*

Received 13 January 1995; accepted 16 February 1995

Abstract

Kallmann's syndrome (KS) is characterised by the association of anosmia and isolated hypogonadotrophic hypogonadism (IHH). Mutations of the *KAL* gene which is located at Xp22.3 cause X-linked KS (XKS). In this study we used the reverse transcriptase polymerase chain reaction and in situ hybridisation to examine the developmental expression of KAL in the first trimester of pregnancy, the earliest stage of human gestation examined thus far. At 45 days after fertilisation KAL mRNA was detected in the spinal cord, the mesonephros and metanephros but not in the brain. Later in gestation, at 11 weeks, the gene was expressed in the developing olfactory bulb, retina and kidney. This expression pattern correlates with the clinical findings in XKS since olfactory bulb dysgenesis with subsequent defective neural migration causes anosmia and IHH. Additionally, renal agenesis occurs in 40% of patients. Therefore this study provides strong evidence that KAL expression is required for the normal development of the olfactory bulb and kidney in the first trimester of human pregnancy.

Keywords: Kallmann's syndrome; Development; Olfactory bulb; Nephrogenesis; In situ hybridisation; Reverse transcriptase polymerase chain reaction

1. Introduction

Mutations of the human *KAL* gene cause the X-linked form of Kallmann's syndrome (XKS). This disorder is characterised by anosmia, associated with malformation of the olfactory bulbs and sulci, and isolated hypogonadotrophic hypogonadism (IHH) caused by hypothalamic gonadotrophin releasing hormone (GnRH) deficiency (Bouloux, 1992). Patients most commonly present with delayed puberty and may also present during childhood with cryptorchidism and micropenis. It is considered that the failure of olfactory axons in the accessory olfactory nerves to project through the cribriform plate and establish synaptic connections within the developing olfactory bulb is central to the pathogenesis of these clinical features. GnRH neurons, originating in the primitive nasal area, consequently fail to migrate into the forebrain because this migration is normally guided by a scaffold provided by these olfactory nerves. In addition, up to 40% of XKS patients have unilateral absence (agenesis) of a kidney (Hardelin et al., 1994); *KAL* mutations are the only defined gene defects which cause severe renal malformations in humans (Hardman et al., 1994). Other clinical associations are cryptorchidism, micropenis and distal mirror movements of the upper limbs (bimanual synkinesis).

KAL spans 210 kb of genomic DNA in Xp22.3 and has 14 coding exons. It escapes X-inactivation and has a non-functional homologue on the Y chromosome (Yq11.2). The gene encodes a 680 amino acid protein which contains a signal peptide but no membrane insertion or anchorage sequences, suggesting that the protein is secreted. The putative KAL protein has homologies to both antipro-

* Corresponding author, Tel.: +44 171 794 0500, ext. 5059; Fax: +44 171 794 3472.

0303-7207/95/$09.50
SSDI 0303-7207(95)03518-C

teases and to cell–matrix and cell–cell adhesion molecules (Franco et al., 1991; Legouis et al., 1991; Hardelin et al., 1993).

The clinical features of XKS alluded to above are highly likely to have their origins in the aberrant expression of KAL during early human development. In this study we have investigated the tissue-specific developmental expression of KAL. We found KAL transcripts in the olfactory bulb and the kidney at critical times in organogenesis and these data are consistent with roles for the gene in the normal development of diverse organ systems in the first trimester of pregnancy.

2. Materials and methods

2.1. Collection of embryos

After consent from individual patients and from the hospitals ethical committees, normal human fetuses were collected from six chemically induced termination's performed at 45 days (2), 48 days and 11 weeks (3) after fertilisation, critical periods of development when organogenesis is occurring. The exact stage of gestation of the samples was determined by standard criteria as assessed by the examination of external morphology (Larsen, 1993).

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Unless otherwise stated chemicals were obtained from Sigma (Poole, Dorset, UK) and embedding reagents were supplied by British Drug House (Glasgow, UK). One embryo was collected in ice-cold L15 medium, then dissected within half an hour of delivery. RNA was extracted from one 45-day gestational human embryo from the whole brain, eye, spinal cord, heart, liver, limbs, gonads, mesonephros and metanephros by the acid phenol-chloroform method (Chomczynski and Sacchi, 1987) and 300 ng of RNA from each organ was subjected to reverse transcription using random primers and reverse transcriptase (Gibco BRL-Paisley, UK) as recommended by the manufacturer. cDNA was then amplified by PCR. The specific primers for KAL were 5′ GCT GGA CGA GTC GCT GTC TGC CGG 3′ (sense primer corresponding to nucleotides 100–123 of KAL cDNA Petit et al. 1991) and 5′ CCC CCT GCT TCA CCA ACA GGA TGT 3′ (antisense primer corresponding to nucleotides 394 to 371). For each of the tissue samples RT-PCR for β actin, a ubiquitously expressed gene, was also performed in order to confirm the integrity of the extracted RNA. β actin primers were obtained from Clontech, Palo Alto, CA, USA. Specific primers were used together with 2.5 μg of cDNA, 2.5 units of Taq polymerase (Bioline UK, Finchley, UK), 0.75 mM each of dATP, dCTP, dGTP and dTTP, 50 mM $MgCl_2$ in a final volume of 50 μL The amplification times were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and ending with a 72°C for 10 min.

The KAL cDNA (courtesy of C. Petit) and β actin template were included as positive controls. The PCR products were electrophoresed through a 1.5% agarose gel and visualised after ethidium bromide staining. Amplified products were 295 bp for KAL and 838 bp for β actin. Confirmation of the specificity of PCR amplification using the above KAL primers was obtained by (i) performing a nested PCR reaction with the primers: 5′CGT CCA GCG CGC TCC TGC GCC TCC 3′ (sense primer corresponding to nucleotides 128–151 of KAL cDNA; Petit et al., 1991) and 5′ TGA GGA ACT CAC AGC TGG TCA AGC 3′ (antisense primer corresponding to 367–344) to generate a product of 239 bp and (ii) digesting KAL products from the outer set of PCR primers with restriction enzymes; *Hin*dIII digestion produced fragments of 90 and 205 bp and *Pst*I digestion generated

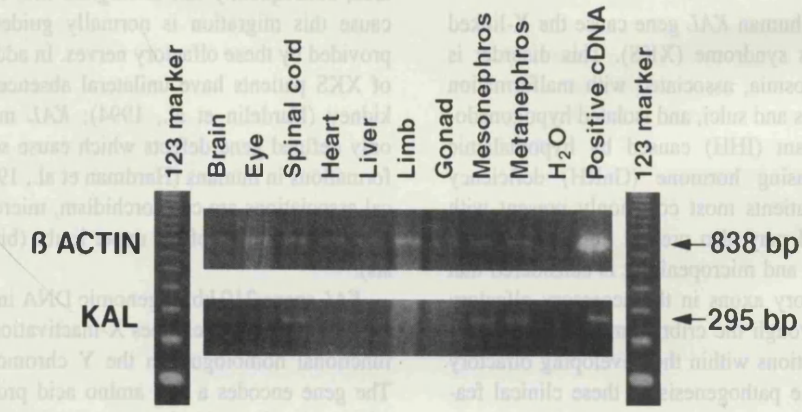


Fig. 1. RT-PCR for KAL and β actin on tissue from 45-day-old human fetus. Molecular weight markers indicate multiples of 123 base pairs, i.e. 123 bp, 246 bp, 369 bp, etc. Lanes contain tissue as marked. H_2O is negative control with cDNA omitted, KAL and β actin plasmid cDNA are positive controls. The KAL primers generate a 295 bp product and the β actin product is 838 bp.

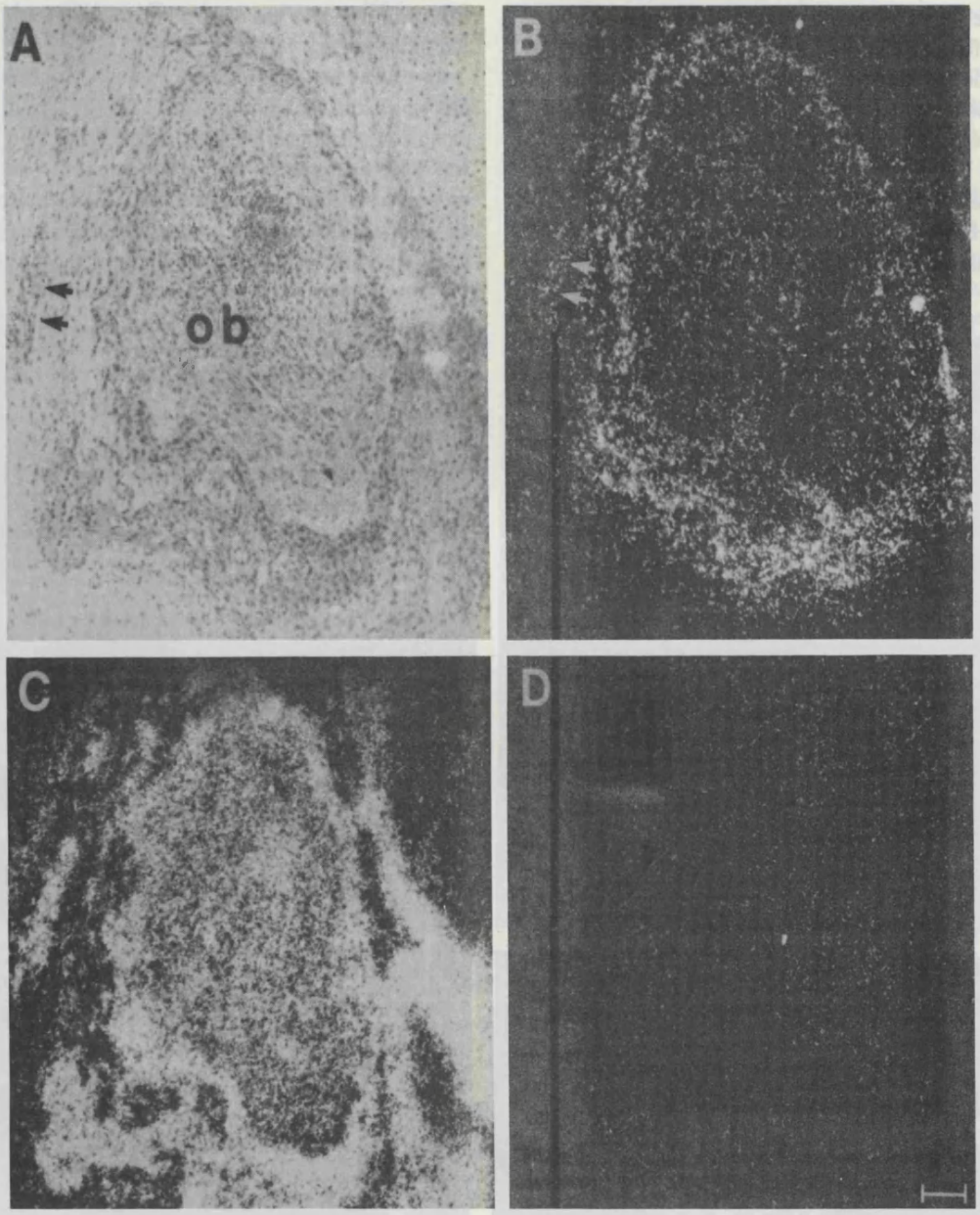


Fig. 2. KAL expression in the developing human olfactory bulb. (A) Bright field of a coronal section at 11 weeks gestation counterstained with haematoxylin and eosin. Olfactory neurons migrating into the bulb (ob) are arrowed. (B)–(D) In situ hybridisation with dark field illumination. (B) The strongest signal from the ^{35}S-labelled KAL antisense riboprobe was detected in the olfactory neurons situated in the olfactory layer of the bulb. Inner layers of the bulb (mitral and granule cells) showed a weak signal. (C) Hybridisation with the L14 lectin antisense riboprobe shows high levels of expression throughout the tissues as expected for this ubiquitously expressed gene and confirms the integrity of mRNA after embryo processing. (D) KAL sense riboprobe of the same areas showed no significant signal. Bar is 100 μm.

fragments of 66 and 229 bp (data not shown). These are the precise sizes expected.

2.3. *In situ hybridisation*

Embryos for in situ hybridisation (1 at 45 days, 1 at 48 days and 3 at 11 weeks) were fixed in ice-cold 4% paraformaldehyde in phosphate buffered saline (pH 7.0) overnight, then dehydrated through graded alcohols and Histo-clear (National Diagnostics, Atlanta, GA) before embedding in wax (Fibrowax). Sections (5 μm) were cut and placed on slides pretreated with 3-aminopropyl-triethoxy-silane and acetone. RNA riboprobe were prepared with [^{35}S]UTP (Amersham >l000 Ci/ mmol) as run-off transcripts. Full length human KAL cDNA (Legouis et al., 1991) subcloned into Bluescript SK– was linearised using *Bam*HI and T7 RNA polymerase to generate the

antisense riboprobe, or *Cla*I and T3 RNA polymerase to generate the KAL sense riboprobe. Additionally, riboprobes for *L14 Lectin*, a gene widely expressed at low levels during mammalian development (Poirier et al., 1992), was used as a rigorous control to test the integrity of mRNA after tissue processing. Probes were purified through columns of Sephadex G50, heated to 90°C for 5 min before layering onto the slides (250 000 cpm per slide). Hybridisation, washing and autoradiography were carried out as described by Wilkinson et al. (1987).

3. Results

3.1. Embryonic expression

As assessed by RT-PCR KAL mRNA was detected in the spinal cord, mesonephros and metanephros of a 45-day-old human embryo (Fig. 1, lower panel). No signal was detected in the whole brain, eye, heart, liver, limbs and gonads at this stage. β actin mRNA was detected in all tissues confirming the integrity of the RNA (Fig. 1, upper panel). In situ hybridisation experiments using the

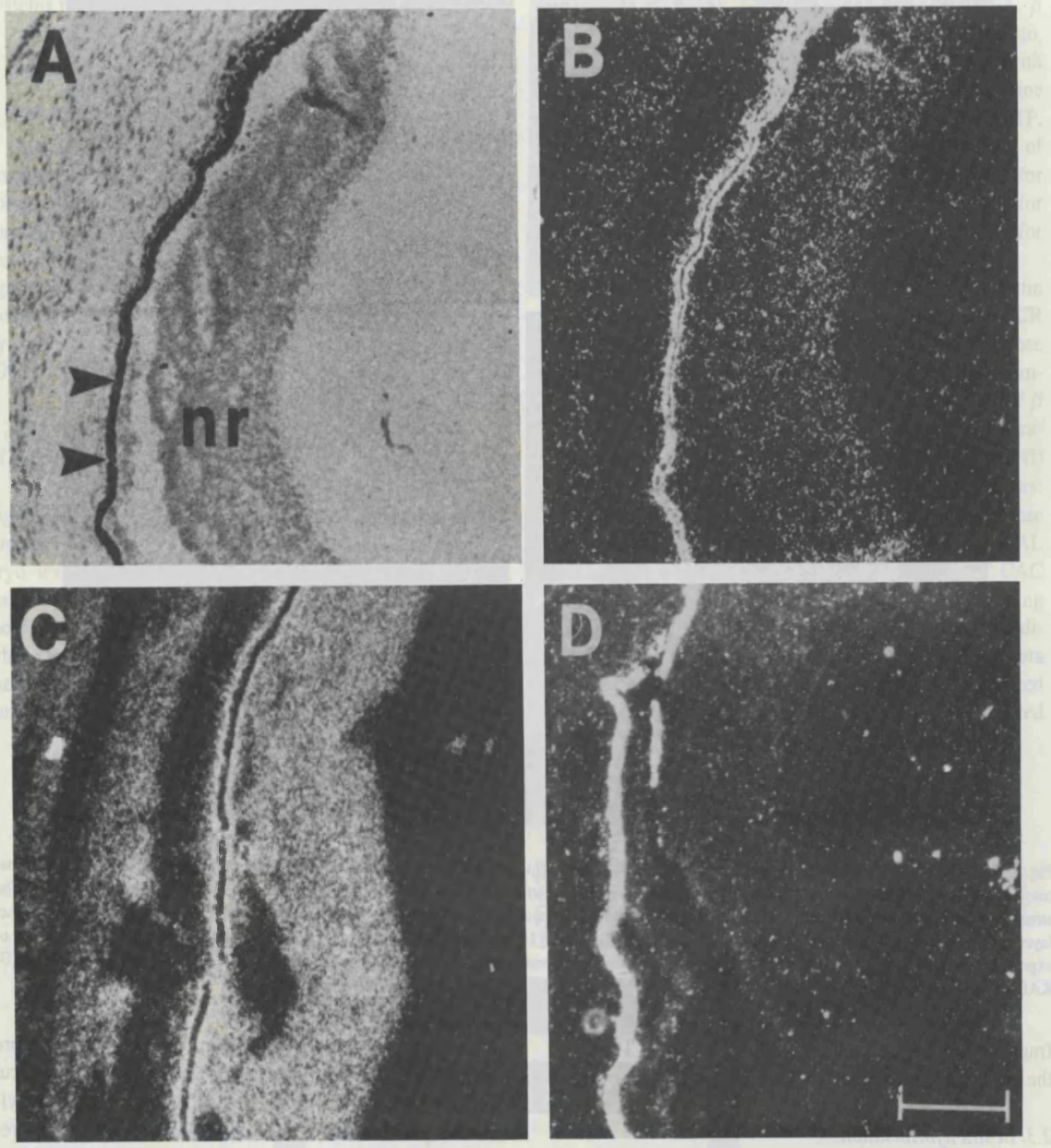


Fig. 3. KAL expression in the developing human retina. (A) Coronal section at 11 weeks gestation shows the developing retina (nr). (B)–(D) In situ hybridisation and dark field illumination. (B) The KAL antisense riboprobe hybridised with the mRNA in the neuroretina. (C) L14 Lectin antisense riboprobe shows widespread expression, confirming the integrity of mRNA. (D) Minimal signal with the KAL sense riboprobe in the eye. Note that the apparent signal in the pigmented retina (arrowheads in (A)) arises from melanin granules and is not due to binding of the riboprobe. Bar is 100 μm.

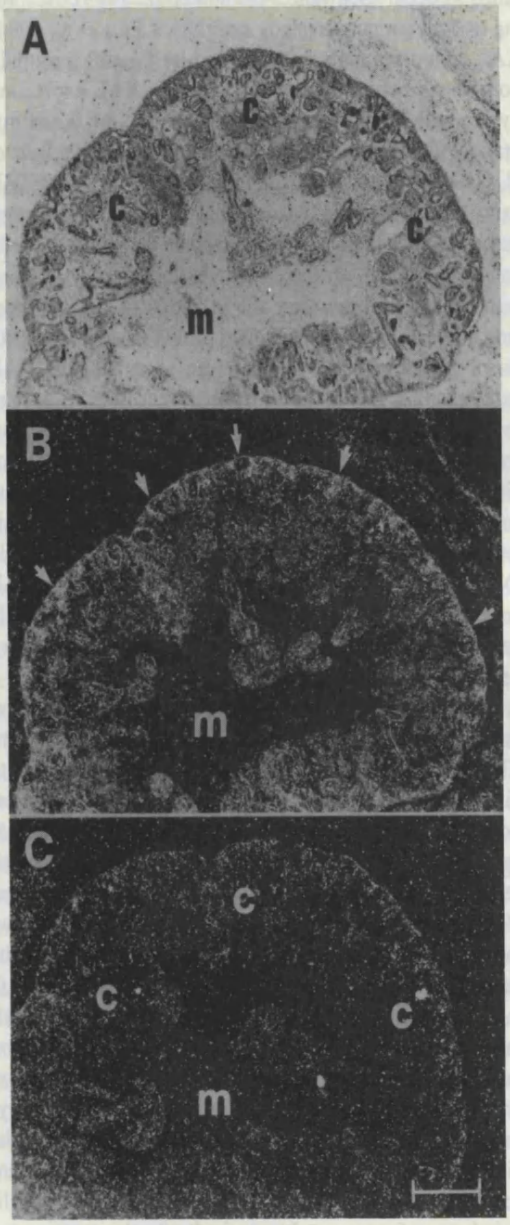


Fig. 4. KAL expression in the developing human kidney. (A) Bright field of section at 11 weeks gestation counterstained with haematoxylin and eosin showing the cortex (c) and medulla (m). (B,C) In situ hybridisation and dark field illumination. (B) The KAL anti-sense riboprobe hybridises with the mRNA in the outer cortex of the kidney (arrows). (C) KAL sense riboprobe of the same area showed no significant signal. Bar is 40 μm.

KAL antisense probes on two embryos (45 days and 48 days) failed to show any signal above that of the sense probe, even in tissues which expressed KAL transcripts as assessed by RT- PCR. In contrast, in situ hybridisation detected transcripts encoded by the L14 lectin gene in all tissues (data not shown). Therefore at this early stage of human development KAL mRNA appears to be selectively expressed in a few organ systems at very low levels.

3.2. Fetal expression

At 11 weeks of gestation KAL was expressed in the central nervous system in all samples which were examined by in situ hybridisation. With regard to the olfactory bulb, the highest signal was detected over the outermost layer of the bulb which is constituted by olfactory nerves (Fig. 2A,B). The inner layers of the bulb gave a weak signal; these areas comprise the mitral and granular cells. In contrast, the antisense probe for the L14 lectin showed a strong and equal signal over all layers of the bulb (Fig. 2C) and the KAL sense probe showed no significant hybridisation (Fig. 2D). The neuroepithelium of the developing retina also expressed KAL at week 11 of gestation (Fig. 3) although the low intensity and diffuse nature of the signal precluded a definitive conclusion regarding the specific cell type which expressed this gene. Transcripts were also detected in the intrinsic ocular muscles (not shown). Overall the intensity of the KAL signal was lower than that of L14 lectin, a gene ubiquitously expressed at low levels in gestation (Figs. 2 and 3). At 11 weeks gestation low levels of KAL transcript were detected in the outer cortex of the metanephric kidney where undifferentiated cells and nephron precursors are located (Fig. 4A,B). Mature nephrons and interstitial tissues located in the centre of the organ showed a signal which barely exceeded that of the sense probe (Fig. 4C).

4. Discussion

We reasoned that the key to reaching a better understanding of the pathogenesis of the clinical features of XKS would reside in establishing the normal expression of KAL during human development. Although recent studies have addressed KAL expression in avian development (Rugarli et al., 1993, Legouis et al., 1993) and also in late human gestation (19 weeks) (Lutz et al., 1994) this is the first report of KAL expression in early human gestation at a period when critical events of organogenesis are occurring. Using the techniques of in situ hybridisation and RT-PCR we found that KAL mRNA was expressed at low levels at 45 days in the spinal cord and excretory system but not in the brain. At 11 weeks after fertilisation KAL was detected by in situ hybridisation in the central nervous system (olfactory bulb and neuroretina) and the kidney. Therefore the gene is expressed throughout the period of early nephrogenesis. We were, however, unable to clearly define the time of onset of *KAL* expression within the olfactory bulb and neuroretina since there was no material available between the seventh and eleventh weeks of gestation.

4.1. Expression in the central nervous system

In this study we failed to detect KAL mRNA in the brain or the eye at 45 days gestation as investigated by the sensitive RT-PCR method, although expression was present in the spinal cord (Fig. 1). At this stage of develop-

ment corticospinal axons are just about to reach their decussation point in the lower medulla oblongata (Humphrey, 1960). Meanwhile olfactory nerve fibres are migrating from the epithelium of the olfactory ectoderm towards the forebrain (telencephalon) where the olfactory bulbs will later develop. Using in situ hybridisation we detected KAL transcripts at 11 weeks gestation in the developing olfactory bulbs (Fig. 2) where the highest expression appeared to be located over the outer olfactory nerve layer. At the same stage we also detected KAL transcripts at low levels in the neuroretina (Fig. 3). At the time of writing there is only one other report of KAL expression during human development; Lutz and coworkers examined the brains of 19-week-old human fetuses by in situ hybridisation and found that KAL was expressed in the olfactory bulb with the highest levels in the granule cell and olfactory neurone layers (Lutz et al., 1994). The same study reported that KAL was also expressed in the cerebellum and thalamus at that stage but organs outside the brain were not examined.

More extensive studies have been performed in developing chicks (Legouis et al., 1993; Rugarli et al., 1993). Chick *KAL* has a 77% homology to the human gene, with over 90% homology at the protein level. At stage 30 (equivalent to 38–45 days of human gestation), KAL transcripts can be detected by in situ hybridisation in the region of the presumptive olfactory bulb in the developing telencephalon. By stage 36 (equivalent to 52–57 days of human gestation) expression is found in the mitral neurons, the first layer to differentiate within the bulb. KAL expression is maintained in this layer during development but falls after birth.

It is of note that olfactory and granule cells appear to express the highest levels of KAL during human development (in this study and Lutz et al., 1994) whereas the mitral neurons are the site of highest expression in the chick olfactory bulb. These conclusions should be interpreted with two caveats in mind: (i) the limits of resolution of the radioactive in situ technique does not easily permit localisation of signal over any one cell and (ii) at the earlier stages of olfactory bulb development the cell layers are not well defined. Studies in the chick have also detected KAL transcripts within the developing neuroretina and spinal cord in concordance with our findings in the human (Legouis et al., 1993; Rugarli et al., 1993) (Figs. 1 and 3).

Assuming that the expression of KAL mRNA results in translation into functional protein, it becomes possible to speculate on the role of KAL in the context of other investigations regarding the normal and aberrant development of the olfactory bulb. In a 19-week gestation, human fetus aborted because of a large X chromosome deletion, including the KAL interval, initial migration of olfactory and GnRH neurons from the olfactory epithelium to the cribriform plate area was not impaired but onward migration of these neurons to the hypothalamus failed to occur (Schwanzel-Fakuda et al., 1989). According to both our current study and that of Lutz et al. (1994) KAL would normally be expressed by the olfactory bulb both at and before this stage. Therefore, KAL expression by the bulb is apparently not required for the initial migration of nerve fibres from the olfactory placode. Lutz et al. (1994) also made the important observation that KAL expression was maintained in the area of the olfactory bulbs in chick embryos in which the olfactory placode bad been ablated. In these animals no olfactory neurons were present and therefore the initiation of KAL expression in the bulb was independent of this innervation. With these studies in mind we therefore speculate that KAL expression in the olfactory bulb is necessary for later events, perhaps including the synapsing of olfactory neurons within the bulb and the subsequent migration of GnRH neurons along a pathway provided by the accessory olfactory (vomeronasal and terminal) nerves. In this respect it is of note that neural cell adhesion molecule (NCAM), which has some homology to the putative KAL protein, has been implicated in the migration of the GnRH neurons into the forebrain along the accessory olfactory nerve (Schwanzel-Fukuda, 1992, 1994).

The function of KAL in other parts of the central nervous system including the retina (Fig. 3) and spinal cord (Fig. 1) is currently unclear. The neural retina starts to differentiate at the end of the sixth week of human gestation into the outer neuroblastic layer which gives rise to rods and cones, and the inner neuroblastic layer which develops into ganglion and supporting cells (Larsen, 1993). Axons then sprout from the ganglion cells, growing towards the optic stalk and back into the brain. The retinal layers are not fully developed until the eighth month of gestation and therefore we suggest that KAL may be involved in axonal migration or neural maturation in the retina. Legouis et al (1993) also reported KAL expression in the developing chick retina between embryonic day 9.5 and 19 (equivalent to 52–57 days and the eighth month of human development, respectively): transcripts were noted in the amacrine, horizontal and ganglionic layers of neurones. Interestingly, the dystrophin gene is also expressed in the retina (Schmitz et al., 1993) and in the olfactory bulb (Goreki et al., personal communication). The dystrophin gene is mutated in patients with Duchenne Muscular Dystrophy (DMD) and abnormal electroretinograms (ERGs) have been reported in this condition (Fitzgerald et al., 1994). Whether a similar ERG abnormality occurs in XKS remains to be demonstrated. Conversely, in DMD patients there is no known clinical correlation with the observed dystrophin expression in the bulb. The mechanism underlying bimanual synkinesis in XKS was thought to be due to defective inhibitory neuronal communication between the two cerebral hemispheres (Danek et al., 1991). However, more recent work has suggested that defective decussation of the corticospinal tracts is responsible for this clinical manifestation of

XKS (Maystone et al., 1994). The latter hypothesis is supported by the RT-PCR signal for KAL which we found in the spinal cord in the present study (Fig. 1).

4.2. Expression in the developing excretory system

KAL expression was detected at 45 days gestation in the meso and metanephros using RT-PCR (Fig. 1). At this stage, however, mRNA levels were too low to be detected by the less sensitive method of in situ hybridisation. At this time in development the paired mesonephric kidneys are fully differentiated and they contain glomeruli. They will degenerate later in gestation although, in the male, remnants of the mesonephric duct give rise to the vas deferens. The metanephros is the precursor of the adult kidney and these organs can first be detected in the human at 35 days after fertilisation. At 45 days, when KAL transcripts can be detected by RT-PCR (Fig. 1), critical inductive interactions which are known to be essential for normal kidney development are occurring between the renal mesenchyme and the ureteric bud epithelium (Hardman et al., 1994). At 11 weeks of gestation low levels of KAL transcripts were detected in the outer cortex of the developing kidneys (Fig. 4). In this region nephrons continue to form until 34 weeks gestation. Up to 40% of XKS patients have unilateral absence of the kidney (Kirk et al., 1994) implicating KAL in nephrogenesis. Based on these clinical observations and on the results of this study we suggest that KAL has a role in the formation or maintenance of the developing kidney. The homology of the KAL protein to cell–cell and cell–matrix adhesion molecules could explain this role because similar functions have been assigned to both uvomorulin and to the laminin A chain (Klein et al., 1988; Hardman et al., 1994). These cell–cell and cell–matrix adhesion molecules are expressed in the transition of undifferentiated mesenchyme into polarised nephron epithelia. Our data confirm and extend the findings of a previous study in which KAL mRNA was reported to be expressed during chick nephrogenesis (Legouis et al., 1993).

In conclusion, we have shown that the KAL gene is expressed in diverse tissues in the first trimester of normal human development, and some of these locations correlate directly with the clinical pathologies found in XKS patients who harbour mutations of this gene. Further analysis of the precise roles of the KAL protein in central nervous system and renal development will require experiments in which gene expression or protein function is disrupted either in vivo or in vitro systems.

Acknowledgements

We thank C. Petit (Pasteur Institute, Paris, France) for the KAL cDNA, C.T.J. Chan (Institute of Child Health, London) for the L14 lectin cDNA, J.T. Norman (UCLMS) for the provision of one of the embryos used and to D. Lirenman and P. Hunt (Institute of Child Health) for technical advice. We thank R. Quinton for his helpful advice with the discussion. P.J.D.W. is an Action Research Training Fellow. A.S.W. is a National Kidney Research Fund Senior Fellow.

References

Bouloux, P.M.G., Munroe, P.B., Kirk, J.M.W. and Besser, G.M. (1992) J. Endocrinol. 133, 323–326.

Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.

Danek, A., Heye, B. and Schroedter, R. (1991) Ann. Neurol. 3, 299–304.

Fitzgerald, K.M., Cibis, G.W., Giamborne, S.A. and Harris, D.J. (1994) Clin. Invest. 93, 2425–2430.

Franco, B., Guioli, S., Pragliola, A., Incerti, B., Bardoni, B., Tonlorenzi, R., Carrozo, R., Maestrini, E., Pieretti, M., Taillon-Miller, P., Brown, C.J., Willard, H.F., Lawerence, C., Persico, M.G., Camerino, G. and Ballabio, A. (1991) Nature 353, 529–536.

Hardelin, J.P., Levilliers, J., Young, J., Pholsena, M., Legouis, R., Kirk, J., Bouloux, P., Petit, C. and Schaison, G. (1993) J Clin. Endocrinol. Metab. 76, 827–831.

Hardman, T., Kolatsi, M., Winyard, P.J.D., Towers, P.R. and Woolf, A.S. (1994) Exp. Nephrol. 2, 211–219.

Humphrey, T. (1960) in Structure and Function of the Cerebral Cortex, Elsevier, Amsterdam.

Klein, G., Langegger, M., Timpl, R. and Ekblom, P. (1988) Cell 55, 331–341.

Kirk, J.M.W., Grant, D.B., Besser, G.M., Shalet, S., Smith, C.S., White, M., Edwards, O. and Bouloux, P.M.G. (1994) Clin. Genet. 46, 260–262.

Larsen, W.J. (1993) Human Embryology, Churchill Livingstone, New York.

Legouis, R., Hardelin, J.P., Levilliers, J., Claverie, J.M., Compain, S., Wunderle, V., Millasseau, P., Le-Paslier, D., Cohen, D., Caterina, D., Bougueleret, L., Delemarre-Van de Waal, H., Lutfalla, G., Weissenbach, J. and Petit, C. (1991) Cell 67, 423–435.

Legouis, R., Ayer-Le Lievre, C., Leibovici, M., Lapointe, F. and Petit, C. (1993) Proc. Soc. Natl. Acad. Sci. USA 90, 2461–2465.

Lutz, B., Kuratani, K., Rugarli, E.I., Wawerski, S., Wong, C., Bieber, F.R., Ballabio, A. and Eichele, G. (1994) Hum. Mol. Genet. 3, 1717–1723.

Maystone, M.J., Harrison, L.M., Jobling, L., Quinton, R., Stephens, J.A. and Bouloux, P.M.G. (1994) Physiol. Soc. Abstracts December, 49–50.

Poirier, F., Timmons, P.M., Chan, C.T.J., Guenet, J.L. and Rigby, P.W.J. (1992) Development 155, 143–155.

Rugarli, E.I., Lutz, B., Kuranti, S.C., Wawersik, S., Borsani, G., Ballabio, A. and Eichele, G. (1993) Nature Genet. 4, 19–26.

Schwanzel-Fukuda, M., Bick, D. and Pfaff, D.W. (1989) Mol. Brain Res. 6, 311–326.

Schwanzel-Fukuda, M., Abraham, S., Crossin, K.L., Edelman, G.M and Pfaff, D.W. (1992) J. Comp. Neurol. 321, 1–18.

Schwanzel-Fukuda, M., Reinhard, G.R, Abraham, S., Crossin, K.L., Edelman, G.M. and Pfaff, D.W. (1992) J. Comp. Neurol. 342, 174–185.

Schmitz, F., Holbach, M. and Drenckhahn, D. (1993) Histochemistry 100, 473–479.

Wilkinson, D.G., Bailes, J.A. and McMahon, A.P. (1987) Development 99, 493–500.