Molecular Genetic Studies on Disorders of Calcium and Phosphate Homeostasis.

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Abstract of Thesis

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The aim of my project has been to investigate the molecular basis for the mineral disorders of familial hypoparathyroidism and X-linked hypophosphataemic rickets. For familial hypoparathyroidism, autosomal dominant, autosomal recessive and X-linked recessive forms of inheritance have been established. For four pedigrees which show an autosomal mode of inheritance (two recessive and two dominant), I have investigated the parathyroid hormone (PTH) gene on chromosome 11p for abnormalities which may be associated with the pathology of the disease. In order to facilitate segregation analysis in these families, I have characterised a novel tetranucleotide polymorphism at the PTH locus. By segregation analysis and direct DNA sequencing of the PTH gene and its associated promoter, I have identified a donor splice site mutation at the exon 2/intron 2 boundary of the PTH gene in one pedigree with autosomal recessive hypoparathyroidism and have demonstrated that this mutation cosegregates with hypoparathyroidism in this family. In order to characterise the effect of this mutation upon PTH mRNA processing, as parathyroid tissue was not available, I have used the sensitivity of the polymerase chain reaction to detect the illegitimate or non-tissue specific transcription of the PTH gene in total RNA isolated from cultured lymphocytes from both unaffected and affected individuals from this family. Analysis of the PTH transcript from affected individuals demonstrated that this mutation caused exon skipping to occur and that the PTH mRNA lacked exon 2 of the coding sequence, thereby causing parathyroid hormone deficiency. Analysis in the other three remaining pedigrees allowed the exclusion of the PTH gene as the cause of hypoparathyroidism.

For the X-linked recessive hypoparathyroidism, the gene for which has been localised to the long arm of the X
chromosome (Xq26-27), I have used the flanking markers, 4D.8 (locus DXS98) and pCDRl (locus CDR) from this region for pulsed field gel electrophoresis studies to generate physical map data around this locus. The markers 4D.8 and pCDRl have been used to screen the ICI yeast artificial chromosome (YAC) library, and I have investigated and characterised the YAC clones obtained from this region containing the gene.

In addition, I have investigated the molecular basis of X-linked hypophosphataemic rickets. Using an interspecific backcross segregating for hyp, the putative murine homologue of X-linked hypophosphataemic rickets, I have localised the mouse calbindin D9K gene to the region of the hyp locus and have investigated the possible role of this gene in this disorder of phosphate homeostasis.
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CHAPTER ONE:
INTRODUCTION
1. INTRODUCTION

The homeostatic control of calcium and phosphate in man and all terrestrial vertebrates is principally regulated by parathyroid hormone and the active forms of vitamin D. The actions of parathyroid hormone and vitamin D are both cooperative and coordinated in order to regulate the total body content of the two ions, and to control the equilibrium between the insoluble mineral phase of bone and the soluble fractions of calcium and phosphate in the extracellular fluids of the body.

In addition to parathyroid hormone and vitamin D, the recently identified parathyroid hormone-related peptide (PTHrp), which shares N-terminal homology with parathyroid hormone, has been postulated to have a role in foetal mineral homeostasis and the regulation of maternal bone resorption and intestinal calcium absorption during lactation.

1.1 PARATHYROID HORMONE

The physiological role of parathyroid hormone (PTH), through concerted actions on its target organs of the kidney, bone and intestine, is to increase the flow of calcium into the extracellular fluid and thereby regulate the level of the ion in the plasma. In accordance with this, the production of parathyroid hormone is regulated by the concentration of calcium in the plasma. A decrease in the level of plasma calcium rapidly stimulates the production of PTH and conversely the secretion of the hormone is inhibited by an increase in plasma calcium (Sherwood et al 1966, Habener et al 1976, Mayer et al 1976).

In response to hypocalcaemia, an increase in the concentration of parathyroid hormone results in three principle actions (Figure 1.1). Firstly parathyroid hormone acts to increase the net rate of dissolution of bone mineral to release calcium and phosphate ions (together with other bone matrix materials) into the plasma. Secondly the hormone
acts in a divergent manner upon the kidney to increase the renal reabsorption of calcium and to directly inhibit the tubular reabsorption of phosphate, thereby ensuring the desired rise in plasma calcium levels whilst preventing hyperphosphataemia. The third action of parathyroid hormone is the indirect enhancement of the intestinal absorption of calcium. This effect is achieved by the hormone functioning as an activator of the 25(OH) vitamin D 1-alpha-hydroxylase enzyme in the proximal tubular cells of the kidney, thereby stimulating production of the active 1,25(OH)$_2$ vitamin D$_3$ metabolite, which increases the uptake of calcium and phosphate in the gut.
Figure 1.1: The physiological actions of parathyroid hormone (PTH) in calcium homeostasis. Parathyroid hormone acts on bone and kidney via specific receptors to raise the level of calcium in the extracellular fluid. In addition, PTH acts indirectly through enhancing the production of 1,25(OH)$_2$ vitamin D$_3$ to increase uptake of calcium in the intestine. Calcium in the extracellular fluid in turn exerts a negative feedback inhibition to reduce the rate of secretion of parathyroid hormone.
1.1.1 The Parathyroid Hormone Gene

Human parathyroid hormone is encoded by a single gene on the short arm of chromosome 11, band 11p15 (Mayer et al. 1983, Naylor et al. 1983). The nucleotide sequence of the human preproparathyroid hormone complementary DNA (cDNA) was described by Hendy et al. (1981), and demonstrates 90% nucleotide and 85% amino acid homology in coding sequence with the bovine PTH cDNA sequence (Kronenberg et al. 1979). The parathyroid hormone gene is approximately 4 Kilobases in size, and consists of three exons (or coding sequences) and two introns (Vasicek et al. 1983). The complete genomic sequence of the PTH gene, together with DNA sequence upstream from the transcription start site containing elements of the PTH promoter was published by Reis et al. (1990). Exon 1 of the PTH gene is 85 base pairs in size (Figure 1.2) and contains 5’ untranslated sequence. Exon 2, which is 90 base pairs in size, contains the initiation methionine codon (ATG) and encodes the 25 amino acid signal sequence together with the first three amino acids of the pro-sequence of the prepro-PTH molecule. Exon 3 is 612 base pairs in size and encodes the remaining 3 amino acids of the 6 amino acid pro-sequence and the 84 amino acid PTH peptide. The exon-intron boundary consensus sequences required for the post-transcriptional splicing of the PTH gene messenger RNA conform to the "gt-ag" rule proposed by Breathnach et al. (1978).

Two restriction fragment length polymorphisms or RFLPs (Figure 1.2), revealed by the enzymes PstI and TaqI, have been demonstrated to occur at the PTH locus (Schmidtke et al. 1984). Both polymorphisms are biallelic with reported allele frequencies of 0.578/0.422 for PstI and 0.628/0.372 for TaqI, and have been demonstrated by family studies to be inherited in a Mendelian manner (Schmidtke et al. 1984).
Figure 1.2: Schematic representation of the genomic organisation of the parathyroid hormone (PTH) gene. The PTH gene consists of 3 exons and 2 introns. Exon 1 contains 5' untranslated sequence, exon 2 contains the initiation codon (ATG) and encodes the 25 amino acid signal peptide together with the first three amino acids of the pro-sequence, and exon 3 encodes the remainder of the pro-sequence and the 84 amino acid PTH peptide. Two restriction fragment length polymorphisms are associated with the PTH gene with the enzymes TaqI and PstI (Schmidtke et al. 1984). The TaqI polymorphic site (denoted by a closed triangle) is within intron 2 of the PTH gene, and the PstI polymorphic site (open triangle) is situated approximately 1.7 Kilobase pairs downstream of the gene.
1.1.2 Transcriptional Control of the Parathyroid Hormone Gene

In addition to the control of secretion of PTH from the parathyroid glands, the level of extracellular calcium exerts an effect upon the rate of biosynthesis of parathyroid hormone. Studies performed by Russell et al (1983) have demonstrated that increases in the level of extracellular calcium cause a decrease (which is reversible) in the rate of PTH gene expression and decreased PTH mRNA levels in dispersed bovine parathyroid cells. More specific molecular studies on the control of parathyroid hormone gene expression by calcium have been performed by Okazaki et al (1992) and have identified a 15 base pair palindromic sequence (TGAGACAGGGTCTCA) approximately 3.5 Kilobase pairs upstream from the PTH gene, the negative calcium response element (nCaRE), which is required for the negative regulation of gene expression by extracellular calcium.

The effects of 1,25(OH)$_2$ vitamin D$_3$ levels upon PTH mRNA transcription have also been examined by Silver et al (1985) and Russell et al (1986), using the technique of "nuclear run-off" in order study the effect of the active vitamin D metabolite upon PTH mRNA transcription. Both studies demonstrated a specific effect of 1,25(OH)$_2$ vitamin D$_3$ in decreasing the rate of PTH mRNA transcription. Furthermore, experiments performed by Okazaki et al (1988), using plasmid constructs containing fragments of the PTH promoter fused to a neomycin reporter gene, have identified a 684 base pair fragment of the PTH promoter directly upstream from the startpoint of transcription that mediates the effect of 1,25(OH)$_2$ vitamin D$_3$ upon PTH mRNA expression. This finding would appear to indicate the likely presence of a vitamin D responsive element direct repeat sequence, or VDRE, as characterised for the osteocalcin gene (Morrison et al 1989, Demay et al 1990) in this fragment, but sequence analysis has revealed no such homologous element (Okazaki et al 1992).
1.1.3 Biosynthesis of Parathyroid Hormone

The parathyroid hormone gene, which consists of 3 exons and 2 introns, is transcribed in the nucleus as a large precursor mRNA molecule from which the intronic sequences are removed prior to translation of the mRNA. This process known as mRNA splicing requires the presence of consensus sequences (Breathnach et al 1978, Mount 1982) at the exon-intron boundaries, which are recognised by the splicing machinery of the cell. The mature spliced PTH mRNA is then transported to the cytoplasm and is translated to give the 115 amino acid preproPTH molecule. During translation, the 25 amino acid hydrophobic signal sequence of the preproPTH molecule binds with a particle known as the signal recognition particle (Walter et al 1981), which mediates entry of the nascent preproPTH peptide into the lumen of the rough endoplasmic reticulum. The 25 amino acid signal sequence is then removed by a signal peptidase to yield the intermediate pro-PTH precursor, which is transported to the Golgi apparatus of the cell. In the Golgi, the six amino acid prohormone sequence is enzymatically removed to yield the mature 84 amino acid PTH peptide. The parathyroid hormone is then packaged into secretory granules that fuse with the plasma membrane of the cell and release PTH into the bloodstream.

Experiments performed by Freeman et al (1987), transfecting mutant PTH gene constructs into rat GH4 pituitary cell lines have established the importance of the PTH signal sequence in parathyroid hormone biosynthesis and secretion. Retroviral transfection of mutant PTH genes, missing the first 10 or the first 13 amino acid residues of the PTH hydrophobic signal sequence, demonstrated abnormal processing of the PTH molecule, and revealed that the mutant precursor PTH proteins are degraded rapidly intracellularly without entering the secretory pathway.
1.1.4 Parathyroid Hormone Related Peptide

The possibility that malignant tumours could cause hypercalcaemia by producing a substance resembling parathyroid hormone was first suggested by Albright et al (1941), based upon the coexistence of hypophosphataemia and hypercalcaemia in a patient with renal carcinoma. This humoral hypercalcaemia of malignancy (HHM), which often occurs in patients with squamous cell carcinoma of the lung without bony metastases, appeared to be caused by tumour products acting on bone to promote resorption and on the kidney to restrict calcium excretion (Stewart et al 1983). Moseley et al (1987) purified a protein with similar biological activities to parathyroid hormone from the culture medium of a human lung cancer cell line, and demonstrated that the cancer cells contained no PTH mRNA indicating the presence of a protein distinct from PTH.

The complementary DNA (cDNA) for this parathyroid hormone-related peptide (PTHrp) has been cloned (Suva et al 1987, Mangin et al 1988) and localised (Mangin et al 1988) to the short arm of chromosome 12 (12p12.1-p11.2). The cDNA encodes a protein of 177 amino acids, containing a prepro-sequence of 36 amino acids followed by the mature peptide of 141 residues in length. Of the first 13 amino acids of the mature peptide, 8 are identical to parathyroid hormone, although the sequences of the two peptides diverge completely after this point. The genomic organisation of parathyroid hormone related peptide gene has been reported by Yasuda et al (1989); the gene consists of 7 exons and spans approximately 13 Kilobases of genomic DNA. Hammonds et al (1989) reported the presence of two forms of PTHrp, one of which is the full length 141 amino acids, and another form truncated at its carboxy-terminus of 108 amino acids, which arise due to alternative splicing of the PTHrp messenger RNA. Studies of the sequence homology and chromosomal organisation of the PTH and PTHrp genes (Yasuda et al 1989), together with evidence that chromosomes 11 and 12 are ancestral homologues (Comings 1972), have led to the proposal that the PTH and PTHrp genes
are derived from a common ancestral sequence.

The function of parathyroid hormone-related peptide in human physiology is under intense study at the present time. It has been postulated that the peptide plays a role in foetal and neonatal calcium metabolism (Burton et al 1990, Moniz et al 1990). In addition, it has been demonstrated that PTHrp is expressed in lactating mammary tissue (Thiede et al 1988), and that PTHrp may act to regulate maternal bone resorption and intestinal calcium absorption during lactation (Budayr et al 1989).
1.1.5 The PTH/PTHrp Receptor

Parathyroid hormone regulates calcium and phosphate metabolism by binding to specific receptors located on the plasma membrane in kidney and bone. Pharmacological and physicochemical evidence (Juppner et al 1988), from experiments performed with rat osteosarcoma cells, strongly indicates that both parathyroid hormone and parathyroid hormone related peptide bind to the same receptor.

The physiological effects of PTH on its target tissues are principally mediated by two distinct receptor-linked effector systems, adenylate cyclase and phospholipase C, which initiate a cascade of intracellular events upon binding of PTH. Binding of PTH to its receptor activates the stimulatory guanyl nucleotide regulatory subunit, $G_s$, (Nissenson et al 1981), leading to activation of adenylate cyclase (Chase et al 1967, Chase et al 1968, Keutmann et al 1985), accumulation of cyclic AMP (cAMP), and activation of cAMP-dependent protein kinases (Partridge et al 1981, Livesay et al 1982). Mutation of the $\alpha$ subunit of the stimulatory G protein ($G_\alpha$) of adenyl cyclase has been demonstrated (Patten et al 1990) to be associated with the condition of pseudohypoparathyroidism type IA (Albright's hereditary osteodystrophy), in which there is a resistance of target organs to parathyroid hormone and other hormones that act by stimulating adenyl cyclase activity.

In addition to the activation of adenyl cyclase, binding of PTH to its receptor has been demonstrated to stimulate the activity of phospholipase C (Hruska et al 1987), which hydrolyses phosphatidylinositol 4'5-bisphosphate to produce the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). Inositol 1,4,5-triphosphate acts to release calcium stores from the endoplasmic reticulum of the cell thereby raising intracellular calcium levels (Rasmussen 1986). Diacylglycerol functions as an activator of the enzyme protein kinase C, which is translocated from the cytosol to the plasma membrane of the cell upon PTH stimulation (Abou-Samra et al 1989).
The complementary DNA (cDNA) encoding the PTH / PTHrp receptor has recently been isolated by Juppner et al (1991) from an opossum kidney cDNA library using an expression cloning strategy. Nucleotide sequencing of the cDNA revealed an open reading frame encoding a 585 amino acid protein, which when expressed in COS 7 cells, binds PTH and PTHrp with equal affinity leading to activation of intracellular adenylate cyclase. Hydrophobicity plot analysis of the deduced PTH/PTHrp receptor peptide sequence (Juppner et al 1991) identified the presence of seven putative transmembrane domains (Figure 1.3), thereby proposing a similar topology for the PTH/PTHrp receptor as that observed for another G-protein coupled receptor, the β-adrenergic receptor (Strader et al 1989). Comparison of the amino acid sequence of the PTH/PTHrp receptor protein to those of other G-protein linked receptors revealed less than 10% conservation of 35 "signature" sequences that are conserved among these other receptors (Attwood et al 1991). The PTH/PTHrp receptor shows strong homology, however, to the recently characterised porcine calcitonin receptor (Gorn et al 1992), and would indicate that the receptors for these calcium regulating hormones represent a new family of G-protein linked receptors.
Figure 1.3: Schematic representation of the PTH/PTHrp receptor (NH₂ terminus at top, single letter amino acid code) showing the predicted membrane associated topology of the protein. The receptor protein is predicted (Juppner et al 1991) to have seven transmembrane domains. Potential N-glycosylation sites (Ψ); cysteine residues that are conserved in the calcitonin receptor (●). Reproduced from Juppner et al (1991).
1.2 VITAMIN D

The primary physiological function of vitamin D, as for parathyroid hormone, is the homeostatic control of calcium in the extracellular fluid. Whereas it is parathyroid hormone that is the principal regulator for the minute-to-minute homeostatic control of calcium, it is the physiological role of vitamin D to maintain the day-to-day overall balance of calcium in the body. The classification of vitamin D as a vitamin, an essential dietary requirement required only in trace amounts, is somewhat of an historical misnomer. Vitamin D conforms to a much greater extent to the definition of a hormone, a substance produced in one part of an organism which has an effect on another part of the organism.

The control of extracellular calcium levels by vitamin D is achieved through the concerted action of the hormone upon its target tissues of the intestine and bone, although the principal physiological effects of vitamin D in calcium homeostasis are mediated through the action of the hormone on the small intestine. The active form of vitamin D, \(1,25(OH)_2\text{D}_3\), functions to enhance the absorption of dietary calcium and phosphorus in the small intestine. For the transport of calcium in the duodenum, the major calcium transport protein, is the calbindin D9K (Wasserman et al 1983), or calbindin. It has been demonstrated in the rat that \(1,25(OH)_2\text{D}_3\) specifically induces an increase in the rate of calbindin gene transcription (Leonard et al 1984) and that the expression of calbindin correlates with calcium transport activity in the small intestine (Bruns et al 1987).

The second action of vitamin D is upon bone. \(1,25(OH)_2\text{D}_3\) acts to mobilise calcium stores from bone by inducing the dissolution of bone mineral and matrix. Under normal physiological conditions, \(1,25(OH)_2\text{D}_3\) acts in a concerted manner with parathyroid hormone in this process as a part of the bone remodelling process and to maintain the homeostatic control of serum calcium.
Vitamin D₃ (cholecalciferol) can be produced in sufficient quantities in man provided that the individual is adequately exposed to sunlight. In primitive man, living with very few clothes and exposed to sunlight for considerable periods of time, sufficient vitamin D₃ would have been synthesized in the skin, only when man began to live in colder climes and covered most of his body in clothes has he become dependent upon a dietary source of the vitamin. In the skin, Vitamin D₃ is synthesized from its precursor molecule, cholesterol, via the intermediate of 7-dehydrocholesterol, utilising ultraviolet light in the epidermis for the conversion of 7-dehydrocholesterol to vitamin D₃ (cholecalciferol; Figure 1.4).

Whether vitamin D₃ is produced in the skin, or is provided in the diet, it must be modified by two hydroxylations (Figure 1.4) which occur sequentially in the liver and in the kidney before it may function in the homeostatic control of calcium.

The first hydroxylation reaction occurs in the liver to produce 25(OH) vitamin D₃, which is released into the bloodstream and transported to the kidney, where the second hydroxylation reaction takes place to form the active vitamin D metabolite, 1,25(OH)₂ vitamin D₃. The activity of the renal mitochondrial 25(OH)D-1α-hydroxylase enzyme has been demonstrated to be strongly regulated by plasma inorganic phosphorus concentration, hypophosphataemia activating the enzyme (Tanaka et al. 1973, Gray et al. 1979). In addition, hypocalcaemia, by the stimulation of parathyroid hormone secretion, acts to increase the activity of the 1α-hydroxylase enzyme (Fukase et al. 1982).

Another hydroxylation of 25(OH) vitamin D₃ may also occur in the kidney; a 24-hydroxylation (Figure 1.4), which produces 24,25(OH)₂ vitamin D₃. Although it is generally accepted that the 1,25(OH)₂ vitamin D₃ is the active form of the hormone (DeLuca 1980), the role of 24,25(OH)₂ vitamin D₃ is unclear.
Figure 1.4: Photochemical and metabolic pathways of Vitamin D$_3$ (cholecalciferol). Underlined letters and numbers denote specific enzymes: 25: vitamin D-25-hydroxylase; 1α: 25(OH)D-1α-hydroxylase; 24R: 25(OH)D-24R-hydroxylase. (Reproduced with modifications from Newsholme and Leech 1985).
1.2.1 The Vitamin D Receptor (VDR)

1,25(OH)$_2$ vitamin D$_3$ mediates its biological effects by binding to intracellular receptors (DeLuca et al 1990) which belong to the steroid receptor superfamily (Baker et al 1988). Binding of 1,25(OH)$_2$ vitamin D$_3$ to its receptor induces an allosteric change that allows the receptor-hormone complex to bind to its DNA response element in the promoter region of a target gene. This interaction results in the transcription of specific genes the products of which affect the biological response to the hormone. Isolation of the complementary DNA for the vitamin D receptor (VDR) by Baker et al (1988), indicated that the VDR protein was highly homologous to other steroid receptors and the thyroid hormone receptor. The area of strongest homology is at the N-terminus of the VDR protein, a hydrophilic region which is rich in cysteine, lysine, and arginine residues, and constitute the two zinc finger DNA binding domain of the VDR molecule (Evans 1988).

The investigation of the promoter regions of the rat calbindin D9K (Darwish et al 1992), rat (Demay et al 1990) and human (Kerner et al 1989) osteocalcin genes, which have been demonstrated to be transcriptionally controlled by 1,25(OH)$_2$ vitamin D$_3$, has allowed the characterisation of the vitamin D responsive element, or VDRE, for these genes. The vitamin D receptor-ligand complex, as for the thyroid hormone and retinoic acid receptors (Umesono et al 1991), interacts with a direct repeat consensus sequence in the promoter of the gene. For example, the characterisation of the VDRE for the rat osteocalcin promoter (Demay et al 1990) revealed the presence of three AGGTCA-related sequences in close proximity 5'-TGGGTGAATGAGGACATTACTGACCG-3', which confer responsiveness to 1,25(OH)$_2$ vitamin D$_3$.

Recent findings (Carlberg et al 1993) have demonstrated that the vitamin D receptor may bind to DNA as either a homodimer, as seen for other steroid receptor molecules such as the oestrogen receptor, or may bind DNA as a heterodimer with the retinoid-X receptor $\alpha$ (RXR-$\alpha$) molecule. Carlberg et
al have reported that VDR could activate the human osteocalcin vitamin D₃ as a homodimer, but that in order for the VDR to activate the mouse osteopontin vitamin D₃ responsive element, VDR was required to form a heterodimer with the RXRα molecule. These findings therefore demonstrate that there are two nuclear signalling pathways for vitamin D, and indicate a previously unrealised complexity for the action of the hormone.
1.3 DISORDERS OF CALCIUM AND PHOSPHATE HOMEOSTASIS

1.3.1 Familial Idiopathic Hypoparathyroidism

Hypoparathyroidism is an endocrine disorder which is characterised by hypocalcaemia and hyperphosphataemia due to a lack of active circulating parathyroid hormone. Hypoparathyroidism may occur as an isolated endocrinopathy, termed idiopathic hypoparathyroidism, or may also arise due to a number of other causes, for instance as part of a polyglandular autoimmune disorder, or as a congenital defect, for example DiGeorge syndrome.

Studies performed by Fattorossi et al (1988) demonstrated that antibodies in sera from patients with autoimmune hypoparathyroidism were cytotoxic to cultured bovine parathyroid cells, as well as to endothelial cells from bovine adrenal medulla and pulmonary artery. The mechanism by which such auto-antibodies arise is, as yet, unclear, but the observation of their cytotoxicity to endothelial cells from several different tissue types indicates an explanation for the involvement of several organs in autoimmune hypoparathyroidism. Hypoparathyroidism may also occur as part of a congenital defect, for example the DiGeorge syndrome, where there is an abnormality in the development of the derivatives of the third and fourth pharyngeal pouches. Affected individuals are athymic, aparathyroid, have aortic arch abnormalities and facial dysplasia. De la Chapelle (1981) first mapped the gene causing DiGeorge syndrome to chromosome 22q11, and postulated that deletion or an abnormality at this locus caused the observed phenotype, but positional cloning strategies have yet to identify the gene or genes involved in this developmental disorder.

As an isolated endocrine disorder, or idiopathic hypoparathyroidism, familial occurrences have been reported and autosomal dominant (Barr et al 1971), autosomal recessive (Bronsky et al 1968) and X-linked recessive (Peden et al 1960, Whyte et al 1981, Thakker et al 1990) forms of inheritance.
have been established.

**Autosomal Idiopathic Hypoparathyroidism.**

For the autosomal inherited forms of idiopathic hypoparathyroidism, the parathyroid hormone gene located on chromosome 11p15 (Mayer *et al* 1983, Naylor *et al* 1983) has been investigated for abnormalities that may be associated with the endocrinopathy. Ahn *et al* (1986) performed segregation studies using the PstI and TaqI restriction fragment length polymorphisms (RFLP’s) at the PTH locus (Schmidtke *et al* 1984) in eight pedigrees with familial isolated hypoparathyroidism.

Linkage analysis in two of these families with autosomal dominant hypoparathyroidism (pedigrees C and D, Ahn *et al* 1986) demonstrated segregation of PTH alleles with hypoparathyroidism. The results of segregation analysis in the remaining 6 pedigrees excluded an association between hypoparathyroidism and the PTH gene in four of these families, whilst the other two pedigrees were uninformative. Arnold *et al* (1990) investigated the PTH gene from an affected individual from pedigree D (Ahn *et al* 1986) for sequence abnormalities, and identified a T to C point mutation in the signal peptide of the prepro-PTH coding sequence. This mutation resulted in a cysteine to arginine substitution at position 18 of the 31 amino acid prepro-PTH sequence, disrupting the hydrophobic core of the PTH signal sequence. Arnold *et al* (1990) demonstrated, by *in vitro* translation of the mutant PTH allele in the presence of canine pancreas microsomal membranes, that the mutation caused abnormal processing of the prepro-PTH to the pro-PTH peptide, although the mechanism by which this mutation acts in a dominant manner is as yet unclear.
X-Linked Recessive Idiopathic Hypoparathyroidism.

Idiopathic hypoparathyroidism has been reported to occur as an X-linked recessive disorder in two multi-generation kindreds designated P/60 and W/81 (Peden 1960, Whyte et al 1981) from Missouri, USA. Affected individuals, who are all males, suffer from infantile onset of epilepsy and hypocalcaemia due to parathyroid hormone deficiency. In addition, the autopsy of an affected male from pedigree P/60 (Whyte et al 1986) revealed the absence of parathyroid tissue, thereby indicating that there is an isolated congenital defect in parathyroid gland development.

Linkage Studies.

The number of crossover events (or recombinants) that occur between a disease gene and a DNA marker locus on a chromosome reflects the genetic distance between the two loci. For linkage analysis in a pedigree, the ratio of recombinants to the total number of offspring is the recombination fraction ($\theta$), whose values range from 0 to 0.5. A value of 0 indicates that the loci are closely linked, while a value of 0.5 indicates that the loci are far apart and segregating independently (not linked). The calculated probability that two loci are linked is expressed as a "LOD" score, which is $\log_{10}$ of the odds favouring linkage. A LOD score of +3, which indicates a probability in favour of linkage of 1000:1, establishes linkage between two loci, and a LOD score of -2, indicating a probability against linkage of 100:1, is taken to exclude linkage between two loci. The odds ratio favouring linkage is defined as the likelihood that the two loci are linked at a specified recombination ($\theta$) versus the likelihood that the loci are not linked (ie. $\theta$=0.5). LOD scores are evaluated over a range of $\theta$, thereby enabling the genetic distance and maximum (or peak) probability favouring linkage between the two loci to be ascertained. The recombination value ($\theta$) at which the peak LOD score ($Z$) is obtained yields
the best estimate of the genetic distance between the two loci.

Previous family linkage analysis using cloned human X-chromosome sequences identifying restriction fragment length polymorphisms (RFLPs) have mapped the gene causing X-linked recessive hypoparathyroidism (*HPT*) to the long arm of the X-chromosome at Xq26-27 (Thakker et al. 1990). This analysis established linkage between the X-linked recessive hypoparathyroid gene (*HPT*) and the locus DXS98 (4D.8) with a peak LOD score of 3.82 (θ=0.05). Multipoint analysis indicated that the *HPT* locus is proximal to the DXS98 locus but distal to the F9 (Factor IX) locus on the distal long arm of the X-chromosome establishing the locus order Xcen-F9-HPT-DXS98-Xqter. Additional markers are required however as the recombination rate between HPT and F9 is 4% and that between HPT and DXS98 is 3%. Further linkage studies were therefore undertaken using the DNA probes pCDR and CX55.7, defining the loci CDR and DXS105 respectively, which have been localised to Xq26-27 (Anson et al. 1988) in order to define a more detailed genetic map of the region containing the *HPT* gene. The probe pCDR corresponds to the cDNA for the cerebellar degeneration-related antigen, a target molecule recognized by autoantibodies in patients with paraneoplastic cerebellar degeneration (Dropcho et al. 1987, Chen et al. 1990). The probe CX55.7 consists of a single copy 2.4 Kbp EcoRI fragment (Willard et al. 1985). The probe pCDR reveals a restriction fragment length polymorphism (RFLP) with the enzyme HincII (Siniscalco et al. 1989), and the probe CX55.7 reveals RFLP’s with the enzymes TaqI (Hofker et al. 1987), and StuI (Rekila et al. 1988). The TaqI polymorphism for the probe CX55.7 was found to be uninformative in pedigrees P/60 and W/81; the data shown for linkage analysis for the probe CX55.7 (locus DXS105) is that obtained using the StuI polymorphism.

Analysis of the members of family W/81 using RFLPs revealed by the probes CDR and CX55.7 proved to be informative, demonstrating no recombinations between *HPT* and
the two loci in this kindred. The pedigree is also informative (Thakker et al 1990) for the X-linked RFLP loci DXS294, F9, DXS98 and DXS52 (Figure 1.5). Individual IV.4 is a carrier mother who is informative and phase known for DXS294, F9, DXS105 and DXS52, her affected son V.1 is recombinant for HPT and the proximal loci DXS294 and F9 but non-recombinant for DXS105, DXS98 and DXS52. Individual II.4 is a deceased carrier mother whose genotype was deduced from her eleven children. Her unaffected son III.6 is recombinant for HPT and the distal loci DXS98 and DXS52 but is non-recombinant for the proximal locus DXS105. Thus the members of pedigree W/81 do not show recombination between HPT and the loci DXS105 and CDR, and these results suggest the locus order Xcen-DXS294-F9-(DXS105, CDR, HPT)-DXS98-DXS52-Xqter. The combined results of two point linkage analysis from families W/81 and P/60 are shown in Table 1.

Linkage between the X-linked recessive hypoparathyroid gene (ie. a LOD score greater than 3.0), and the loci DXS105, CDR and DXS98 was established (Thakker et al 1991). For the marker DXS105, the peak LOD score was 7.62 at 0% recombination, with CDR the peak LOD score was 3.58 at 0% recombination, and with DXS98 the peak LOD score was 4.16 at 4.6% recombination. These results further support the location of the gene causing X-linked recessive idiopathic hypoparathyroidism to Xq26-Xq27.
Figure 1.5: Family W\81 (key individuals) segregating for X-linked recessive idiopathic hypoparathyroidism (HPT) with the distal X-chromosome loci DXS294, Factor IX, DXS105, CDR, DXS98 and DXS52. For some females, the inheritance of paternal and maternal alleles can be ascertained, and in these the paternal X chromosome is shown on the left. Recombinants between HPT and each allele are indicated by an asterisk. Deduced genotypes are shown in brackets. The members of family W/81 do not show recombination between HPT and the loci DXS105 and CDR, and these results suggest the locus order Xcen-DXS294-F9-(DXS105,CDR,HPT)-DXS98-DXS52-Xqter.
Table 1: LOD scores for X-linked markers and HPT.

<table>
<thead>
<tr>
<th>Locus</th>
<th>LOD Scores Z(θ)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>θ</td>
</tr>
<tr>
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</tr>
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<tr>
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</tr>
<tr>
<td>DXS52</td>
<td>0.133</td>
</tr>
</tbody>
</table>
Deletion Mapping Studies

In order to further characterise the region containing the X-linked hypoparathyroid gene, deletion mapping studies were examined by Dr Derek Blake and Professor Kay Davies at the Institute of Molecular Medicine, Oxford, in two male patients (Manchester 1 and Manchester 2) with haemophilia B due to deletions involving the Factor IX gene locus, but who did not have the manifestations of hypoparathyroidism. The results obtained for the deletion mapping are shown in Figure 1.6.

For the patient Manchester 1 (Anson et al 1988), DNA hybridisation analysis revealed the presence of the three loci DXS105, CDR and DXS98. However, for the haemophilia patient Manchester 2, hybridisation signals were detected with the probe DXS98, but were absent with the probes DXS105 and CDR. Assuming that the deletion in this patient is continuous, these results would therefore indicate that the X-linked recessive hypoparathyroid gene locus is not included in the deletion and is thus distal to the two loci CDR and DXS105, suggesting the locus order:

Xcen-DXS294-F9-(DXS105,CDR)-HPT-DXS98-DXS52-Xqter.
Figure 1.6: Schematic representation of deletion mapping data for the patients Manchester 1 and Manchester 2 with haemophilia B due to a Factor IX deletion (Anson et al 1988). The results for Southern blot hybridisation analysis with probes for the Factor IX, DXS105, CDR and DXS98 loci are shown. The presence of a hybridisation signal for a probe is denoted by a plus sign (+), and absence of a hybridisation signal is denoted by a minus (−) sign. For the patient Manchester 1, hybridisation signals were obtained for the loci DXS105, CDR and DXS98. For the patient Manchester 2, hybridisation signals were obtained only with the DXS98 locus, and were absent for the DXS105 and CDR loci. These results therefore indicate that HPT is distal to CDR and DXS105. (These studies were performed by Dr D. Blake and Professor K. Davies, and I am grateful to them for making these results available)
1.3.2 Inherited Forms of Rickets

There are a variety of causes of hereditary rickets, which may be classified according to the predominant metabolic abnormality into two main groups. For the first group, the observed hypophosphataemia is due to a renal tubular defect, which may consist of a single phosphate transport defect as occurs for X-linked hypophosphataemic rickets, or multiple transport defects in the handling of phosphate, amino acids, glucose, bicarbonate and potassium as occurs in the Fanconi syndrome. Such disorders are termed Vitamin D resistant rickets.

For the second group, vitamin D metabolism is abnormal, either because of an abnormality in the renal 1α-hydroxylase enzyme, or due to an abnormality in the 1,25(OH)₂ vitamin D₃ receptor leading to end organ resistance. Such disorders are termed vitamin D dependent rickets.
Vitamin D-resistant hypophosphataemic rickets was first described by Albright et al (1937) who recognized that some patients did not respond to therapy with normal doses of vitamin D, but would respond to large doses of vitamin D. Clinically, X-linked hypophosphataemic rickets (HYP) is characterised by short stature, hypophosphataemia, and inadequate mineralisation of both the cartilagenous growth plate and bone. The X-linked dominant mode of inheritance was first delineated by Winters et al (1958), who noted that in a large pedigree from North Carolina there were no instances of male to male transmission of either bone disease or hypophosphataemia, the bone disease was much less severe in females, and that all daughters of hypophosphataemic males were themselves hypophosphataemic.

The biochemical and physiological features of HYP are due to a decrease in the renal tubular reabsorption of phosphate in the kidney, although the primary defect causing this has yet to be elucidated.

**Hypophosphataemic Mouse Models: Hyp and Gyro**

The investigation of the etiology of X-linked hypophosphataemic rickets has been greatly facilitated by the characterisation of two hypophosphataemic mouse models: hyp and gyro. The hyp mouse was first described by Eicher et al (1976), with mutant male mice manifesting with hypophosphataemia, dwarfism, diminished body weight and bone deformities, therefore showing close phenotypic similarities to affected males with X-linked hypophosphataemic rickets. Genetic studies of hyp mice revealed that the mutation was dominant and mapped to the distal part of the mouse X chromosome (Eicher et al 1976), providing further evidence that the human and mouse diseases are caused by mutations affecting an homologous gene.

The second hypophosphataemic mouse model, gyro, was
described by Lyon et al (1986). The mutation in the gyro mouse, as with hyp, causes hypophosphataemia due to a defect in renal phosphate transport, but in addition the gyro mutation causes circling behaviour, inner ear abnormalities, and sterility in males. The human counterpart to the phenotype caused by the gyro mutation has not yet been identified. Davies et al (1984) reported individuals with X-linked hypophosphataemic rickets who in addition presented with sensorineural deafness and tinnitus, but it is not known whether this results from bony overgrowth or calcification of the ligaments in the inner ear, or as an independent inherited defect. The gyro gene (symbol gy) has been mapped close to hyp on the distal part of the mouse X chromosome, but the possibility of the two genes being allelic has been excluded by the observation of a crossover event between the two loci (Lyon et al 1986). The finding that the hyp and gyro gene loci are distinct indicates that there are two gene products from the distal part of the mouse X chromosome which are involved in a renal component of phosphate homeostasis.
Phosphate Transport Studies in Patients with X-linked Hypophosphataemic Rickets and in the Hyp Mouse

Dennis et al (1977) described the presence of two separate phosphate transport systems in mammalian kidney, one of which is PTH sensitive, located in the late proximal convoluted tubule and controlled by an X-linked gene, and another which is PTH insensitive, located in the early proximal tubule and controlled by an autosomal gene product. This finding is in agreement with the earlier studies of Glorieux and Scriver (1972), who described evidence that the hypophosphataemia in patients with X-linked hypophosphataemic rickets was due to a defect in the PTH sensitive component of phosphate transport, which is responsible for approximately two-thirds of the total net reabsorption of phosphate in the human kidney. Physiological experiments in the hyp mouse have defined that there is a defect in a sodium dependent phosphate transport molecule at the brush border (Tenenhouse et al 1978) of cells of the proximal convoluted tubule, and have demonstrated that in the hyp mouse, the defect is also independent of PTH (Cowgill et al 1979).

Abnormalities of Vitamin D Metabolism

In addition to the observed abnormalities in renal tubular phosphate transport, investigation of vitamin D metabolism in hypophosphataemic patients and in the hyp mouse have demonstrated an altered response of the 1-alpha-hydroxylase enzyme in the proximal convoluted tubule of the kidney to regulating factors such as phosphate and parathyroid hormone. A low or low normal serum concentration of 1,25(OH)₂ vitamin D₃ is observed in association with the hypophosphataemia in patients with X-Linked hypophosphataemic rickets and the hyp mouse. This observation is in marked contrast to the known stimulatory effect of a low serum phosphate concentration upon the activity of the renal mitochondrial 1-alpha-hydroxylase enzyme in both rat (Tanaka
et al 1973) and in healthy adults (Gray et al 1979). In addition, studies of the effects of parathyroid hormone upon synthesis of 1,25(OH)₂ vitamin D₃ in man (Lyles et al 1982) and the hyp mouse (Nesbitt et al 1986) have demonstrated that raising serum PTH levels fails to increase the 1-alpha-hydroxylase enzyme activity. Experiments performed by Nesbitt et al (1989) demonstrated that the lack of modulation of the 1-alpha-hydroxylase enzyme by parathyroid hormone in hyp mice was not due to an abnormality of intracellular cAMP production by the PTH receptor-linked adenyl cyclase enzyme. Nesbitt et al therefore proposed three possible explanations for the abnormal control of the 1-alpha-hydroxylase enzyme in the hyp mouse kidney: Firstly, a defect in the cAMP-dependent activation of intracellular protein kinases. Secondly abnormal protein phosphorylation by cAMP activated protein kinases. Thirdly, since the aberrant renal 1-alpha-hydroxylase activity is confined to the segment of the kidney tubule in which abnormal phosphate transport occurs (Tenenhouse et al 1978, Cowgill et al 1979), the defective modulation of the enzyme may result from a cAMP-independent alteration of the intracellular milieu (diminished phosphate flux) that may directly impair mitochondrial function or inhibit new protein synthesis.

Studies performed in the gyro mouse, however, have demonstrated (Davidai et al 1990) that there is normal control of the renal 1-alpha-hydroxylase enzyme and production of 1,25(OH)₂ vitamin D₃ in response to hypophosphataemia and parathyroid hormone. This finding therefore indicates that the defect of phosphate transport in the proximal tubule of gyro mice does not negatively influence the milieu of the cells containing the 1-alpha-hydroxylase enzyme in the kidney.
There is considerable controversy over whether the abnormalities observed in patients with X-linked hypophosphataemic rickets and in the hyp mouse occur due to an intrinsic renal defect or are due to the presence of a circulating humoral factor that alters proximal tubule brush border function and vitamin D metabolism. Bell et al (1988), using established primary cultures of renal epithelial cells from normal and hyp male mouse kidneys, observed that the cultured cells exhibited abnormal phosphate uptake and vitamin D metabolism after 5-6 days in culture, and therefore concluded that the defect was intrinsic to the hyp mouse kidney cells. In contrast, cross-transplantation experiments performed by Nesbitt et al (1992) obtained results that appeared to indicate unequivocally that the kidney is not the target organ for the genetic abnormality that underlies the hyp phenotype. The hyp mouse phenotype was neither transferred nor corrected by renal transplantation, and Nesbitt et al therefore postulated that the disorder in the hyp mouse, and by extrapolation in patients with HYP, is the result of the action of a humoral factor (of unknown source) rather than an intrinsic renal abnormality. Nesbitt et al suggested that a possible explanation for the disparate findings between the cross-transplantation and cell culture experiments may be that whilst the cultured renal cells from the hyp mice may have been removed from the influence of the humoral factor, they may not have been exposed to "essential factors" necessary to reinitiate the correct expression of the hyp gene product; thus the abnormalities of these cells in vivo would be perpetuated in culture.

The hypothesis of the role of a humoral factor in the control of renal phosphate handling is supported by findings in patients with oncogenic hypophosphataemic osteomalacia, in which a phosphaturic factor or factors are produced by tumour tissue that cause renal phosphate wasting. Removal of the tumour tissue has been demonstrated (Agus et al 1983) to restore renal phosphate handling to normal.
Mapping of the Human X-Linked Hypophosphataemic Rickets Gene

From the study of comparative mapping data of the human and mouse X chromosomes, and the known location of the hyp gene, Buckle et al (1985) predicted that the human X-linked hypophosphataemic rickets (HYP) gene may be either between the alpha-galactosidase gene and the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene or in the distal part of the short arm of the human X chromosome. Studies using X chromosome probes detecting restriction fragment length polymorphisms (Read et al 1986, Machler et al 1986) established linkage with the marker 99.6 (locus DXS41, Figure 1.7) with a peak LOD score of 4.82 at 10% recombination. Multilocus linkage analysis indicated that the X-linked hypophosphataemic rickets (HYP) gene mapped distal to the DXS41 locus on the short arm of the X chromosome at Xp22.31-p21.3. Thakker et al (1987), by a study of 16 families, established that the HYP gene lay distal to the DXS41 locus (probe 99.6) and proximal to the DXS43 locus (probe pD2), thereby defining bridging markers for the disease (Figure 1.7). Additional linkage analysis (Thakker et al 1990) using the markers DXS197 and DXS207 further defined the locus order: Xtel-DXS85-(DXS207, DXS43, DXS197)-HYP-DXS41-Xcen.
Figure 1.7: Diagram of the human X-chromosome showing the position of the X-linked hypophosphataemic rickets (HYP) gene at Xp22.31-p21.3. Reproduced from Thakker and O’Riordan (1988).
Econs et al (1992) performed further analysis in five families with hypophosphatemic rickets and demonstrated linkage between the markers DXS365 (peak LOD score 13.65 at 0% recombination) and DXS257 (peak LOD score 8.09 at 6% recombination) with the HYP locus. Multipoint analysis indicated the locus order Xtel-DXS43-DXS257-HYP-DXS41-Xcen. The marker DXS365 was localised to the DXS43 to DXS41 interval but could not be located with respect to HYP and DXS257 (Econs et al 1992). Rowe et al (1992) further investigated the genetic map around the HYP locus, and defined DXS274 as the closest proximal marker, giving the locus order: Xtel-DXS43-(DXS197,DXS257)-HYP-DXS274-DXS41-Xcen.

The identification of a microsatellite polymorphism at the DXS365 and DXS443 loci (Brown et al 1992) and use of the Genethon markers AFM234yf12 and AFM163yh2, which map to the HYP region, for linkage analysis in 14 families with hypophosphataemic rickets (Rowe et al 1993) allowed the further refinement of the genetic map around the HYP locus. The results of this linkage analysis, together with analysis of a yeast artificial chromosome clone isolated using the marker AFM163yh2 has allowed the proposal of the locus order:

<----------HYP---------->
Xptel-DXS43-DXS197-AFM234yf12-(DXS443,DXS365)-AFM163yh2-
DXS274-DXS41-Xcen
Mapping of the Murine Hypophosphataemic Rickets (hyp) Locus

Linkage studies performed by Eicher et al (1976) using the murine X-linked mutant genes Tabby (symbol Ta) and Bent-Tail (symbol Bn) localised the hyp gene to the distal end of the mouse X-chromosome. In order to facilitate the more precise localisation of the hyp gene, Kay et al (1991) utilised the approach of an interspecific backcross, segregating for hyp and Ta (the putative homologue of the human genetic disease hypohidrotic ectodermal dysplasia), in order to map the hyp locus within a framework of markers on the mouse X-chromosome. For this approach, crosses between the two evolutionary divergent mouse species, Mus spretus and Mus musculus domesticus, were established (Figure 1.8), facilitating the detection of restriction fragment length variants or RFLV’s for DNA probes in the backcross progeny.

Multipoint analysis of recombination events between loci in the backcross progeny was performed indicating the gene order Xcen-Pgkl-DXSmh43-hyp-Cbxrs1-Amg-Xter. The distance between DXSmh43 and hyp is 2.5 ± 1.4 centiMorgans, and the distance between Cbx-rs1 and hyp is 1.8 ± 1.3 centiMorgans (Figure 1.9).
Figure 1.8: Details of the interspecific *M. m. domesticus*/*M. spretus* cross. Ova from female *M. m. domesticus* mice carrying the mutations *Ta* and *hyp* were fertilised with sperm from a *M. spretus* male. Of the resultant F1 female progeny, two carried both the *Ta* and *hyp* mutations and two carried only the *hyp* mutation. These mice were backcrossed to *M. m. domesticus* male mice carrying the *hyp* mutation. The resultant backcross progeny were scored for *Ta* and *hyp* before use in molecular genetic mapping experiments. (Reproduced from Kay et al 1991).
Figure 1.9: Diagram of the mouse X chromosome indicating gene order and genetic distance, in centiMorgans ± standard error, between the loci. Loci defined by molecular markers are on the right-hand side and the mutant loci segregating in the backcross are on the left-hand side. Anchor loci are indicated by bars extending through the chromosome; other loci are indicated by bars extending to the left or right only; (reproduced from Kay et al 1991).
VITAMIN D DEPENDENT RICKETS

In addition to the forms of vitamin D resistant hypophosphataemic rickets, autosomal forms of vitamin D-dependent rickets (VDDR) have been described (McKusick 1988). Investigation of the physiological and molecular basis for the disorder has allowed the characterisation of two distinct forms of vitamin D-dependent rickets, type I and type II. Type I VDDR is characterised by a low serum 1,25(OH)₂ vitamin D₃ concentration, with only small doses of 1,25(OH)₂ vitamin D₃ required in order to correct the clinical and biochemical abnormalities observed in patients (Fraser et al 1973). Fraser et al proposed that the defect in these patients was due to an abnormality in the 25(OH)b-la-hydroxylase enzyme. For the porcine model of type I VDDR, renal homogenates from affected pigs were demonstrated to have no detectable 25(OH)D-1α-hydroxylase activity (Fox et al 1985), although owing to the inaccessibility of renal tissue from affected human individuals, this has not been demonstrated in man.

Type II vitamin D-dependent rickets is characterised by a markedly raised level of 1,25(OH)₂ vitamin D₃ in affected individuals, and was postulated (Brooks et al 1978, Liberman et al 1980) to occur due to abnormalities in the vitamin D receptor molecule, causing end organ resistance. The identification of the complementary DNA (cDNA) encoding the vitamin D receptor (Baker et al 1988) has allowed investigation of the gene for abnormalities that may be associated with type II VDDR. Sone et al (1990) investigated the vitamin D receptor gene in patients with type II VDDR and identified a mutation in exon 3 of the gene which results in an arginine to glutamine substitution. Expression of the mutant vitamin D receptor protein demonstrated that the receptor bound 1,25(OH)₂ vitamin D₃ with normal affinity but was unable to promote transcription of a reporter gene fused to a vitamin D responsive element, thereby defining the molecular pathology of type II VDDR in the affected individuals.
CHAPTER TWO:

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 Patients and Families Studied

2.1.1 Autosomal Idiopathic Hypoparathyroidism

Pedigree A/89: One multi-generation kindred, designated family A/89, of Bangladeshi origin in whom autosomal recessive idiopathic hypoparathyroidism had occurred following a consanguineous marriage was investigated. The patients (IV.1, IV.3 and IV.4, Figure 3.1) suffered in the neonatal period from hypocalcaemic seizures and further investigations revealed undetectable circulating immunoreactive PTH concentrations and a normal renal response to synthetic 1-38 human PTH (Roelen et al 1989). Clinical immunodeficiency or autoimmune related deficiencies were not observed in any of the patients. The clinical and biochemical findings for members of family A/89 are presented in table 2. Normocalcaemia was restored with oral calcitriol therapy. Unaffected relatives had no history of tetany or epilepsy and were normocalcaemic. Venous blood was obtained, after informed consent, from 3 affected and 6 unaffected members of family A/89, and from 10 unrelated normocalcaemic Bangladeshi individuals who were studied as controls.
Table 2: Clinical and Biochemical Findings in Members of Family A/89.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Age (years)</th>
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<th>Phosphate mmol/1</th>
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<td>28</td>
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Normal Adult: - - - 2.16-2.58 0.75-1.36 40-135 1.0-5.3 10-120

Normal Child: - - - 2.16-2.58 1.25-2.15 100-235 1.0-5.3 10-120

All members had normal renal function with no aminoaciduria or glycosuria. U.D. = undetectable.
Pedigree 2/89: The biochemical and clinical findings for individuals from pedigree 2/89 have previously been published by Shaw et al (1991).

Pedigree 7/90: In family 7/90, autosomal dominant inheritance of hypoparathyroidism was observed, and the patients (I.1, II.1 and II.4) suffered in late childhood or adulthood from hypocalcaemic symptoms due to hypoparathyroidism. Clinical immunodeficiency or autoimmune-related deficiencies were not observed in any of the patients. In addition, these patients had none of the dysmorphic features associated with pseudohypoparathyroidism and the renal cyclic adenosine monophosphate (cAMP) response to synthetic 1–38 human PTH (Roelen et al 1989) was normal. Normocalcaemia was restored with oral 1α-hydroxycholecalciferol therapy. Unaffected relatives had no history of hypocalcaemic symptoms and were demonstrated to be normocalcaemic with normal circulating immunoreactive PTH concentrations (Nussbaum et al 1987).

Pedigree 23/92: The clinical and biochemical findings for members of pedigree 23/92 have been previously been published by Bilous et al (1992).

2.1.2 X-Linked Recessive Idiopathic Hypoparathyroidism

Two families from the state of Missouri, designated P/60 and W/81, in whom idiopathic hypoparathyroidism had been inherited in an X-linked recessive manner in five or more generations (Peden et al 1960, Whyte et al 1981) were investigated. The clinical and biochemical findings for individuals from pedigrees P/60 and W/81 have previously been published by Thakker et al (1991).
2.2 Molecular Biology Protocols

The methods utilised for the work presented in this thesis were mostly adapted from those published by Sambrook, Frisch and Maniatis (1989) in Molecular Cloning/A Laboratory Manual (2nd Edition).

2.2.1 Extraction of Nucleic Acids

Extraction of Genomic DNA from Blood:

The protocol used for the extraction of genomic DNA is based upon the method published by Kunkel et al (1977).

a) 10 ml of blood collected in an EDTA (0.5M pH=8.0) tube was divided between two 50 ml polypropylene tubes.

b) 45 ml of lysis buffer (kept at 4°C) was added to each tube, and the tubes spun at 1000g for 10 minutes at 4°C.

c) The supernatant was immediately decanted (to prevent degradation of the DNA by nucleases in the lysis buffer), and each nuclear pellet resuspended in 4.5 ml of 0.075M NaCl, 0.024M EDTA pH=8.0. The nuclear pellets were then homogenised using a long glass Pasteur pipette.

d) 0.5 ml of a 5% SDS, 2mg/ml proteinase K solution was added to the homogenate and the mixture incubated overnight at 37°C.

e) The solution was extracted with an equal volume (5 ml) of phenol (Tris pH=8 saturated) and then spun at 1000g for 15 minutes. The viscous aqueous layer was then transferred to another 50 ml tube using a plastic Pasteur pipette.

f) The aqueous layer was then extracted with an equal volume (5 ml) of chloroform/isoamyl alcohol mixture (24:1 v/v), and then spun at 1000g for 15 minutes.

g) The viscous aqueous layer was removed and the chloroform/isoamyl alcohol extraction repeated.

h) The genomic DNA was then precipitated from the aqueous solution by adding 0.5 ml of 3M sodium acetate and 12.5 ml of ethanol.

i) The precipitated genomic DNA was then removed using a glass
hook and dissolved in 1 ml of TE pH=8.

j) The DNA was allowed to dissolve at 4°C for 2 days and the yield assessed by ultraviolet optical densitometry (OD).

Extraction of Genomic DNA from Cultured Cells:

a) Approximately $2 \times 10^8$ cultured lymphoblastoid cells were pelleted in a 50 ml polypropylene tube(s) at 1000g for 10 minutes.

b) The cell pellet was resuspended in 50 ml of 0.075M NaCl/0.024M EDTA pH=8.0 and respun at 1000g for 10 minutes.

c) The cell pellet was then resuspended in 9 ml of 0.075M NaCl/0.024M EDTA pH=8.0.

d) 1 ml of 5% SDS, 2 mg/ml proteinase K was added and the mixture incubated overnight at 37°C.

e) The aqueous mixture was then extracted once with phenol and twice with chloroform/isoamyl alcohol.

f) The genomic DNA was precipitated by adding 1 ml of 3M sodium acetate and 25 ml of ethanol.

g) The precipitated DNA was then removed using a glass hook and dissolved in 1 ml of TE pH=8.

h) The DNA was allowed to dissolve at 4°C for 2 days and the yield assessed by ultraviolet optical densitometry (OD).

Preparation of Agarose Blocks of DNA for Pulsed Field Gel Electrophoresis:

a) Approximately $10^8$ cultured lymphoblastoid cells were transferred to a 50 ml polypropylene tube and spun at 1000g for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 50 ml of phosphate buffered saline (PBS) and respun at 1000g for 10 minutes.

b) The cells were then resuspended in 5 ml of PBS and 50μl removed and added to 50μl of PBS for counting of the cells in an haemocytometer.

c) The remainder of the cultured cells were then respun and resuspended in a volume of PBS to give $1.25 \times 10^8$ cells/ml.
d) An equal volume of 1% low melting point (LMP) agarose (kept at 42°C) was added, and the cell suspension/agarose mix pipetted in 80μl aliquots into plastic block formers kept on ice. Each block therefore contains approximately 5 x 10^5 cells.

d) The agarose blocks were then incubated at 50°C for 48 hours in a 0.5M EDTA pH=8, 2 mg/ml proteinase K and 1% (w/v) sodium lauroyl sarcosinate solution.

e) The blocks were then washed three times in an excess volume of TE pH=8, and then incubated in TE pH=8 plus 0.04 mg/ml of phenyl methyl sulphonyl fluoride (PMSF) at 50°C for 30 minutes.

f) The incubation in TE/PMSF was repeated, and the blocks stored in 0.5M EDTA pH=8 at 4°C to prevent degradation of the DNA.

g) Prior to digestion of the DNA in the agarose blocks, the EDTA was removed by incubating the blocks in a large volume of TE pH=8 for 30 minutes at 50°C. This incubation was repeated prior to use of the blocks.
Extraction of RNA from Tissues/Cultured Cells:

Isolation of Total RNA from Tissues:

Total cellular RNA was extracted from tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987).

a) The frozen tissue sample was transferred directly from liquid nitrogen into 1ml of a solution of 4M guanidinium thiocyanate, 25mM sodium citrate pH=7, 0.5% sarcosyl (w/v) and 0.1M 2-mercaptoethanol (Solution D) in a small petri dish.
b) The tissue was then minced with scalpels and the homogenate divided between two 1.5ml Eppendorf tubes.
c) To each tube was sequentially added 50μl of 2M sodium acetate pH=4, 500μl of water saturated phenol and 100μl of chloroform, with thorough mixing between each addition. The mixture was then incubated on ice for 15 minutes.
d) The two phases were then separated by centrifugation at 13000g for 20 minutes at 4°C.
e) The aqueous phase was then removed and the RNA precipitated by the addition of an equal volume (500μl) of isopropanol.
f) The RNA was recovered by centrifugation at 13000g for 10 minutes at 4°C, and the pellet redissolved in 600μl of Solution D.
g) The RNA was then reprecipitated by the addition of an equal amount of isopropanol and incubation at -20°C overnight.
h) The precipitate was then pelleted by centrifugation at 13000g for 10 minutes at 4°C, washed in 80% (v/v) ethanol/water, dried under vacuum, resuspended in sterile distilled water, and stored at -70°C.
Isolation of Total RNA from Cultured Lymphoblastoid Cells:

Total RNA was isolated from cultured lymphoblastoid cells by the lithium chloride/urea method (Lovell-Badge, 1987). This method relies upon the selective precipitation of RNA by lithium chloride/urea whilst leaving protein and DNA in solution.

a) Approximately 10⁸ cells were pelleted by centrifugation at 2000g for 5 minutes, and the cells washed in PBS.
b) The cells were then recentrifuged at 2000g for 5 minutes, the supernatant removed, and the cells resuspended in 10ml of 6M urea/3M lithium chloride.
c) The cell suspension was then homogenised using a polytron for 1 minute (at full speed), in order to shear the cellular DNA. The suspension was then left to precipitate overnight at 4°C.
d) The RNA precipitate was then recovered by centrifugation at an average of 7691g for 20 minutes at 0°C. The supernatant was discarded, the pellet resuspended in 8ml of LiCl/urea, and recentrifuged as before.
e) The pellet was drained thoroughly and resuspended in 6ml of 10mM Tris.HCl pH=7.5, 0.5 (w/v) SDS containing proteinase K at 50μg/ml. The mixture was incubated for 2 hours at 37°C.
f) The solution was extracted 3 times with an equal volume of phenol (water saturated), and three times with chloroform. For each extraction, the aqueous and organic phases were separated by centrifugation at 930g for 5 minutes.
g) The RNA was then precipitated by the addition of 0.6ml of 2M sodium acetate pH=5.5, and 15ml of ethanol and incubated overnight at -20°C.
h) The precipitated RNA was recovered by centrifugation at an average of 7691g for 30 minutes at 0°C, the pellet washed in 80% (v/v) ethanol/water and then dried briefly under vacuum.
i) The RNA pellet was then redissolved in water and the yield quantified by optical densitometry.
j) In order to remove any contaminating DNA the RNA
preparation was then treated with RNase free DNase I enzyme (1 unit/μg of RNA) for 60 minutes. The RNA solution was then extracted with phenol/chloroform (1:1), ethanol precipitated and redissolved in sterile water. The yield of pure total RNA was then assessed by optical densitometry, and the RNA preparation stored at -70°C.
Plasmid DNA Extraction:


Mini-preparations:

a) 2.5ml of LB-broth containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated overnight at 37°C.
b) 1.5ml of the overnight culture was removed and the bacteria pelleted by centrifugation in a microfuge at 6500g for 5 minutes.
c) The supernatant was removed by aspiration and the pellet resuspended in 100μl of solution I.
d) The bacteria were lysed by adding 200μl of freshly prepared solution II (alkaline SDS), and the mixture incubated for 5 minutes at room temperature.
e) To the viscous bacterial lysate was added 150μl of solution III, the tube was vortexed and incubated for 5 minutes at room temperature.
f) The mixture was then centrifuged at 13000g for 10 minutes and the supernatant transferred to a fresh 1.5ml Eppendorf tube.
g) The plasmid DNA was then precipitated with 0.6 volumes of isopropanol (approximately 270μl), incubated for 5 minutes at room temperature and recovered by centrifugation at 13000g for 10 minutes.
h) The pellet was washed in 70% (v/v) ethanol/water, dried briefly under vacuum, and resuspended in 200μl of sterile distilled water containing DNase free pancreatic RNase A (20μg/ml), and incubated at room temperature for 5 minutes.
i) The aqueous plasmid DNA solution was then extracted with 200μl of equilibrated phenol:chloroform (1:1) and centrifuged at 13000g for 5 minutes.
j) The aqueous phase was removed to a clean tube and the plasmid DNA precipitated by the addition of 20μl of 3M sodium
acetate pH=5.2 and 500μl of ethanol.

k) The plasmid DNA was recovered by centrifugation at 13000g for 10 minutes. The pellet was then washed in 70% (v/v) ethanol/water, dried under vacuum, and resuspended in 50μl of sterile distilled water.

Plasmid DNA prepared by this protocol was used for restriction enzyme digestion and dideoxy DNA sequencing.

Maxi-preparation:

a) 200ml of LB-broth containing the appropriate antibiotic was inoculated from either a glycerol stock or with a bacterial colony from an LB-agar plate and incubated overnight in an environmental shaker at 37°C.

b) The bacteria were pelleted by centrifugation at an average of 2661g for 10 minutes, the supernatant discarded, and the pellet resuspended in 10ml of solution I containing lysozyme at 1mg/ml. The mixture was left at room temperature for 5 minutes.

c) 40ml of solution II was added and the mixture incubated on ice for 5 minutes.

d) 15ml of ice-cold solution III was added and the mixture left on ice for a further 15 minutes.

e) The bacterial lysate was centrifuged at an average of 7691g for 10 minutes and the supernatant carefully decanted to a fresh 250ml plastic bottle.

f) 0.6 volume of isopropanol was added, the bottle incubated at room temperature for 10 minutes, and the plasmid DNA recovered by centrifugation at an average of 7691g for 15 minutes.

g) The nucleic acid pellet was then redissolved in 4ml of sterile distilled water containing DNase free RNase A (20μg/ml) and incubated at room temperature for 30 minutes.

h) The aqueous plasmid DNA solution was then extracted 3 times with phenol(Tris/HCl, pH=8) and 3 times with chloroform. For each extraction, the aqueous phase was separated by centrifugation at 930g for 5 minutes.
i) The plasmid DNA was then precipitated by the addition of 1/10 volume of sodium acetate pH=5.2 plus 2.5 volumes of ethanol, and recovered by centrifugation at an average of 7691g for 15 minutes.

j) The DNA pellet was then washed in 70% (v/v) ethanol/water, dried and dissolved in 500μl of sterile distilled water. The concentration of plasmid DNA was then assayed by UV optical densitometry.

Preparation of Single-Stranded Phagemid DNA:

The Bluescript plasmid vector used for the cloning of PCR products contains a copy of the intergenic region of the M13 filamentous bacteriophage. Such vectors, which contain both the ColEl plasmid origin of replication and the intergenic region of a filamentous bacteriophage are known as phagemids. The infection of cells carrying the Bluescript plasmid with a helper virus (such as VCSM13) results in the production of bacteriophage particles containing single-stranded copies of the phagemid genome. The single-stranded DNA purified from these particles may then be used as a template for dideoxy DNA sequencing.

Single-stranded phagemid DNA was prepared using the following procedure:

a) A single colony from an agar plate was inoculated into 2ml of LB-broth (containing 50μg/ml ampicillin), and incubated at 37°C for 6-7 hours until the culture was turbid.

b) 20μl of the culture was then inoculated into 2ml of fresh LB-broth (no antibiotic) containing VCSM13 helper phage at 2×10⁷ pfu/ml, and the mixture incubated for 1 hour at 37°C with vigorous shaking.

c) Kanamycin antibiotic was then added to a final concentration of 70μg/ml, and the incubation continued for a further 14-18 hours at 37°C.

d) 1.5ml of the culture was transferred to a 1.5ml Eppendorf tube, and the bacterial cells pelleted by centrifugation at 13000g for 5 minutes.
e) 1.3ml of the supernatant was transferred to a fresh 1.5ml tube, and 200μl of a solution of 20% polyethylene glycol (PEG 8000) in 2.5M NaCl was added. The tube was vortexed briefly and incubated for 15 minutes at room temperature to allow precipitation of the bacteriophage particles.

f) The bacteriophage particles were recovered by centrifugation at 13000g for 5 minutes, and all the supernatant removed.

g) The bacteriophage pellet was then resuspended by vortexing in 100μl of water, and 50μl of phenol (Tris.HCl pH=8 saturated) was added. The tube was vortexed for 30 seconds, allowed to stand for 1 minute, and then vortexed for another 30 seconds.

h) The aqueous and organic phases were separated by centrifugation at 13000g for 5 minutes, and the supernatant transferred to a fresh 1.5ml tube.

i) The aqueous phase was then extracted with an equal volume of chloroform, and the two phases separated once more by centrifugation.

j) The upper aqueous phase was transferred to a fresh 1.5ml tube and the single-stranded bacteriophage DNA precipitated by the addition of 10μl of sodium acetate pH=5.2 and 250μl of ethanol.

k) The bacteriophage DNA precipitate was recovered by centrifugation at 13000g for 10 minutes, the pellet washed in 70% (v/v) ethanol/water, dried briefly under vacuum, and redissolved in 20μl of water.

Quantification of DNA and RNA by UV Spectrophotometry:

In order to quantify the concentration of DNA or RNA in aqueous solutions, their optical density (OD) was measured at 260nm and 280nm in an ultraviolet spectrophotometer. An optical density of 1 at 260nm corresponds to 50μg/ml for double stranded DNA, 40μg/ml for RNA, and 20μg/ml for oligonucleotides. The ratio of the readings for optical densities for 260nm/280nm provides an estimate of the nucleic
acid purity, with pure preparations of DNA and RNA having ratios of 1.8 and 2.0 respectively.

eg. For a sample of human genomic double stranded DNA, diluted 60 fold in distilled water for quantification:

\[
\begin{align*}
\text{OD}_{260} & : 0.6 \\
\text{OD}_{280} & : 0.3 \\
\end{align*}
\]

DNA Concentration (\(\mu g/ml\)): \(0.6 \times 50 \times 60 = 1800\).

2.2.2 Restriction Enzyme Digestion

DNA was incubated in the appropriate restriction enzyme buffer in the presence of a fourfold excess of the restriction endonuclease(s). These digests were incubated at the appropriate temperature as per manufacturer’s instructions from 1-14 hours. For genomic DNA, digests were carried out in the presence of 3.75mM spermidine unless the enzyme required a low salt buffer in which case 0.1875mg/ml of bovine serum albumin was used.

2.2.3 Agarose Gel Electrophoresis

Conventional Gel Electrophoresis:

For resolution of DNA fragments, samples were electrophoresed on 0.8-1.5% agarose gels containing ethidium bromide at 0.5\(\mu g/ml\) under voltage-limiting conditions. For small gels to analyse PCR products/plasmid DNA or to check the completion of genomic DNA digests, agarose gels were cast in 1xTBE buffer, and run at 100-150 Volts. For agarose gels to resolve DNA for Southern blot analysis, gels were cast in 1xTAE buffer and run at 37 Volts to improve resolution of the DNA fragments. In order to identify the size of DNA fragments of interest, size markers (lambda phage DNA digested with
HindIII and/or Φx174 phage DNA digested with HaeIII) were electrophoresed alongside the DNA samples.

**Pulsed Field Gel Electrophoresis:**

In order to resolve large DNA molecules on agarose gels, pulsed field gel electrophoresis was performed. DNA samples in agarose blocks were electrophoresed on 0.8%-1% TBE agarose gels in an LKB 2015 Pulsaphor electrophoresis unit using an hexagonal electrode apparatus. The temperature of the circulating TBE buffer was maintained at 10.5°C throughout the electrophoresis using an LKB Multitemp II thermostatic circulator. The pulse time and length of electrophoresis run was controlled using an LKB 2015 Pulsaphor plus control unit. Pulse times (between 40 and 100 seconds) and the total run times (20-31 hours) were varied depending upon the range of DNA fragment sizes to be resolved upon the agarose gel.

In order to accurately determine the size of the resolved DNA fragments (up to 1 Megabase in size), two types of size markers were used: Concatamers of lambda phage, and the intact chromosomes from the yeast *Saccharomyces cerevisiae*.

When the electrophoresis was complete, the DNA in the gel was stained with ethidium bromide, and the gel photographed.

**Photography of Agarose Gels:**

The ethidium bromide stained DNA on the agarose gel was visualised by ultraviolet (254 nm) transillumination and photographed onto Polaroid 667 instamatic film using a Kodak 22A Wrattan filter.
2.2.4 Transfer of DNA from Agarose Gels onto Nylon Filters
(Southern Blotting)

Southern Blotting of Conventional Gels:

The method used is based upon that published by Southern (1975).

a) The DNA fragments in the agarose gel were denatured by
immersing the gel in denaturing solution for 45 minutes. The
denaturing solution was then replaced and the DNA denatured
for a further 45 minutes.
b) The gel was then neutralised for 45 minutes in neutralising
solution. The neutralising buffer was then replaced and the
gel incubated for a further 45 minutes.
c) A piece of Whatman 3MM paper which is the same width but
approximately twice as long (prewetted in 3xSSC) was placed
over the gel to act as a wick, and the gel was then inverted
and placed on a support into a tray containing 20xSSC as the
transfer buffer. A plastic pipette was used to smooth out any
air bubbles which may prevent the efficient transfer of the
DNA from the gel to the membrane.
d) A piece of nylon membrane (Hybond-N) cut to the same size
as the agarose gel was wetted in 3xSSC and then placed onto
the gel. All air bubbles were removed using a plastic pipette.
e) Two pieces of Whatman 3MM paper, cut to the same size as
the agarose gel, were wetted in 3xSSC and placed on top of the
nylon membrane. Once more, all air bubbles were removed.
f) In order to draw the transfer buffer through the gel and
elute the DNA onto the nylon membrane, a stack of paper
tissues was placed onto the Whatman 3MM paper overlying the
gel. A sheet of perspex was placed over the tissues and a
weight of about 500g placed on top.
g) The assembled blot was then left to allow the transfer of
DNA to proceed (overnight for genomic DNA gels, 1-2 hours for
plasmid digests/PCR products).
h) After blotting was complete, the filter was then removed
from the gel, washed in 3xSSC, and the DNA crosslinked to the
nylon filter by exposure to ultraviolet light (120 Joules) in a Stratalinker apparatus (Stratagene).

**For Southern Blotting of Pulsed Field Gels:**

The above protocol was also used, but with the following modifications:

a) The DNA in the pulsed field agarose gel was "acid nicked", to facilitate transfer, by immersing the gel in 0.25M HCl for 25 minutes. The gel was then washed in deionised water for 5 minutes.

b) The DNA fragments in the gel were then denatured for 30 minutes in 0.4M NaOH.

c) The pulsed field gel was then Southern blotted overnight as above using 0.4M NaOH as the transfer solution onto an Hybond-N+ nylon membrane.

d) The nylon filter was then washed in 3xSSC and stored.

NB. No UV-crosslinking is required for Hybond-N+ membrane

**2.2.5 Radiolabelling of DNA with $^{32}$P**

**Random Prime Labelling of DNA fragments:**

DNA fragments were radiolabelled to high specific activity (approximately $10^8$ counts per minute/µg DNA) according to the random priming procedure of Feinberg and Vogelstein (1983) using a "Megaprime" kit purchased from Amersham.

a) Approximately 50ng of the DNA fragment to be labelled was suspended in 28µl of water and to this was added 5µl of the primer mix (random nonamers in aqueous solution).

b) The mixture was heated to 100°C for 5 minutes, and left at room temperature for the nonamer primers to anneal to their complementary sequences.

c) To the DNA mixture was added 10µl of the reaction buffer (containing dATP, dGTP and dTTP in a Tris-HCl pH=7.5 buffer), 5µl of [$\alpha-^{32}$P]-dCTP (50µCi), and 2µl (2 units) of Klenow DNA
polymerase.

d) The labelling reaction was incubated at 37°C for 15 minutes.
e) Once labelling is complete, unincorporated [α-\(^{32}\)P]-dCTP nucleoside was then removed by centrifuging the reaction mixture through a G-50 Sephadex gel filtration column, packed in a 1ml disposable plastic syringe.

Prior to use, the \(^{32}\)P-labelled probe was denatured for 10 minutes at 100°C, and then kept on ice before adding to the hybridisation fluid.

5’-End Labelling of Oligonucleotides using T4 Polynucleotide Kinase:

The following procedure was used to label oligonucleotide primers at their 5’ terminus for use in microsatellite detection or as probes:

a) 100ng of the primer to be labelled was suspended in 3μl of water, and to this was added 1μl of 10 x kinase buffer, 5μl of \([\gamma-^{32}\)P]-ATP and 1μl (4 units) of T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 minutes.
b) The 10μl reaction was made up to 100μl with water and unincorporated [\(\gamma-^{32}\)P]-ATP removed by centrifuging the reaction mixture through a G50 Sephadex gel filtration column.

2.2.6 Hybridisation of Nylon Membranes/cDNA Library Filters with \(^{32}\)P Labelled Probes

a) The nylon membranes/cDNA library filters were prehybridised in Church buffer (containing sonicated salmon sperm DNA at 100μg/ml) for 4 hours at 65°C.
b) Denatured \(^{32}\)P-labelled probe was then added and the membranes/filters hybridised overnight at 65°C.
c) Non-specific hybridised probe was then removed by washing the membranes/filters in SSC/SDS solutions of increasing stringency at 65°C.
d) Hybridising DNA fragments/phage colonies were then revealed by autoradiography onto photographic film at -20°C.

**Stripping of $^{32}$P-Probes from Nylon Membranes:**

Stripping of hybridised nylon membranes for reprobing was performed by immersing the membrane in boiling water for 10 minutes. The membrane was then reautoradiographed to check that all the probe had been removed.
2.2.7 Microbiological/Cloning Protocols

Preparation of Competent Cells:

a) A single colony from an agar plate was inoculated into 2ml of LB-broth and cultured overnight at 37°C.
b) 1ml of the overnight culture was used to inoculate 100ml of LB-broth and the culture incubated at 37°C with vigorous shaking until an optical density of 0.5 at 600nm was achieved (approximately 4 hours).
c) The culture was then chilled on ice for 10 minutes before pelleting the cells by centrifugation at 2000g for 5 minutes at 4°C.
d) The supernatant was discarded and the cells resuspended in 50ml of ice-cold 50mM CaCl₂, and the cell suspension incubated on ice for 15 minutes.
e) The cells were then pelleted by centrifugation at 2000g for 5 minutes at 4°C, the supernatant discarded, and the cells resuspended in 3ml of 50mM CaCl₂.

For each transformation, 100μl of the prepared competent cells suspension was used.

Construction of T-Vector for Direct Cloning of PCR Products:

The template-independent terminal transferase activity of Taq DNA polymerase to add a single nucleotide (almost exclusively an adenosine, due to the strong preference of the polymerase for dATP) may be exploited for the use of cloning PCR products into plasmid vectors by producing a modified plasmid with a complimentary 3′ thymidine overhang (Marchuk et al 1991). The plasmid vector modified for use as a T-vector was Bluescript, thereby allowing blue/white selection of recombinants.

The following protocol was used:
a) 1μg of Bluescript vector was blunt end digested with ECoRV restriction endonuclease for 1 hour at 37°C. The reaction mixture was then extracted with an equal volume of
phenol/chloroform (1:1), and the plasmid DNA ethanol precipitated.
b) The plasmid DNA was then resuspended in 50 µl of a buffer consisting of 50 mM KCl, 10 mM Tris.HCl pH=8.3, 1.5 mM MgCl₂, 2 mM dTTP and 5 units of Taq DNA polymerase, and incubated for 2 hours at 72°C.
c) The plasmid DNA was phenol/chloroform extracted, precipitated, and redissolved in water. 10 ng of this Bluescript T-vector plasmid was used for each ligation reaction.

Ligation of DNA:

Ligations were carried out in a total volume of 10 µl in 1x ligase buffer (containing 1 mM ATP) and 1 unit of T4 DNA ligase. The mixture was incubated for 1-4 hours at room temperature to allow ligation to proceed.

Transformation of Plasmid DNA:

a) The ligation reaction (10 µl) was diluted 5-fold with water and added to 100 µl of competent cells.
b) The mixture was incubated on ice for 30 minutes, and then the bacteria were "heat shocked" at 42°C for 90 seconds.
c) 1 ml of LB-broth (containing no antibiotic) was added and the cells incubated at 37°C for 30 minutes, before plating onto LB-agar plates containing the appropriate antibiotic.

Bacteriophage λ cDNA Library Screening

Preparation of Host Plating Bacteria:

a) A single colony (Escherichia coli strain XL1- Blue or NM514) from an agar plate was inoculated into 2 ml of LB-broth (0.2% maltose), and cultured overnight at 37°C.
b) 1 ml of the overnight culture was used to inoculate 100 ml of LB-broth (0.2% maltose), and the culture incubated at 37°C
with shaking until an optical density of 0.5 at 600nm was achieved.
c) The cells were then pelleted by centrifugation at 2000g for
5 minutes, resuspended in 5ml of 10mM MgSO$_4$, and stored at 4°C.

**Plating out of the cDNA Library:**

a) Serial dilutions of the cDNA library stock ($10^{-2}$ to $10^{-7}$)
were prepared in SM buffer.
b) 100µl of these dilutions was mixed with 100µl of the
appropriate plating bacteria, and the mixture incubated at
37°C for 15 minutes.
c) 10ml of LB-agarose (kept at 45°C) was added to the
phage/bacteria, and the mixture quickly poured on top of a
dried 140mm LB-agar plate.

The plate was incubated overnight at 37°C, and the number
of plaque forming units (pfu) per ml of library was
 calculated. The appropriate dilution was then made of the
library to give approximately 50,000 pfu per 100µl. Using this
dilution, 10 plates were prepared as above.

**Bacteriophage Plaque Lifts:**

a) The plates which had been incubated overnight at 37°C, were
placed at 4°C for 1 hour to allow the agarose to harden.
b) A labelled nitrocellulose filter was carefully placed using
forceps onto the top of the agarose, and orientation marks
made on both the filter and the plate.
c) The replica filter was then carefully lifted from the
plate, placed face upwards onto a piece of Whatman 3mm paper
soaked in denaturing solution, and left for 5 minutes.
d) The filter was then transferred onto Whatman paper soaked
in neutralising solution, and the filter neutralised for 5
minutes.
e) The nitrocellulose filter was then washed in 3xSSC, dried,
and then baked at 80°C for 1 hour under vacuum in order to fix
the bacteriophage DNA.
Two filters were prepared for each plate, and the filters were then hybridised, washed and autoradiographed as previously described.

**cDNA Library Used:**

The mouse cDNA library used was obtained from Clontech Laboratories (distributed by Cambridge Biosciences, Cambridge). The library was prepared from whole mouse embryo (17.5 days post coitum), was oligo d(T)-primed and cloned into the EcoRI site of lambda gt10 bacteriophage vector. The mouse embryo used was from a cross between ICR outbred females and outbred Swiss Webster males.

**2.2.8 Sequencing of DNA**

For the sequencing of single and double stranded DNA templates, the dideoxy chain termination protocol of Sanger et al (1977) was followed using a Sequenase version 2.0 DNA sequencing kit purchased from United States Biochemicals.

For single stranded M13 or phagemid DNA and double stranded plasmid DNA templates, annealing of the sequencing primer and the sequencing reactions were performed as per the manufacturer’s instructions. For the direct sequencing of double stranded PCR products, the following modifications were made to the Sequenase protocol:

a) The PCR amplified DNA fragment was purified using a Geneclean kit protocol to remove excess primers.
b) 100ng of the double-stranded PCR product was denatured at 100°C for 3 minutes and then snap cooled onto dry ice in the presence of 200ng of sequencing primer and 0.5% NP40/Tween20 detergent. The addition of detergent to the annealing reaction prevents secondary structure formation and reannealing of the PCR product (Bachmann et al 1990).
c) The Sequenase protocol was then followed, with the concentration of detergent being maintained throughout.
The sequencing reaction samples were then resolved by electrophoresis on 6% acrylamide/7M urea denaturing gels.

2.2.9 Polymerase Chain Reaction (PCR) Amplification of DNA

Amplification of target DNA sequences was performed according to the method described by Saiki et al (1988) using an automated DNA thermal cycler (Hybaid Omnigene). The PCR mixture consisted of 1 x PCR buffer (containing the appropriate MgCl₂ concentration), 0.05% W1 detergent, 100ng of each oligonucleotide primer, and 1 unit of thermostable Thermus Aquaticus (Tag) DNA polymerase (BRL) in a 25μl reaction volume. The reaction mixture was then overlaid with liquid paraffin oil to prevent evaporation. For PCR amplification from genomic DNA, 250ng of DNA was added to the reaction mixture.

After an initial denaturation of 5 minutes at 94°C, 30-35 cycles of PCR amplification were performed. Each cycle consisted of 30 seconds at 94°C to denature double-stranded DNA, 30 seconds at the appropriate temperature to allow the primers to anneal to their complementary sequences, and 1 minute (2 minutes for products of >1 kb) for the extension of the DNA strands. At the end of the 30-35 cycles, a period of 10 minutes at 72°C was allowed for final extension of the DNA strands.

In order to check for contamination by extraneous DNA of the PCR buffer, oligonucleotide primer and Taq DNA polymerase stocks, a negative control, to which no sample DNA was added, was always performed alongside the other samples.

PCR Detection of Microsatellite Polymorphisms:

For the detection of polymorphic microsatellite repeats, oligonucleotide primers flanking the repetitive sequence were synthesized and used in the polymerase chain reaction amplification of the repeat. In order to visualise the PCR products (polymorphic in size due to variation in tandem
repeat number), one of the primers was labelled at its 5' end with $^{32}$P using T4 polynucleotide kinase.

PCR amplification was carried out as described above, using 100ng of each unlabelled primer and 1ng of $^{32}$P end-labelled primer. Following amplification, the PCR products were then electrophoresed on 6% acrylamide/7M urea sequencing gels, and the microsatellite polymorphisms visualised by autoradiography.
2.2.10 cDNA Synthesis

a) 10µg of total RNA was heat denatured for 5 minutes at 95°C, cooled on ice, and added to 200ng of primer complementary to the 3' domain of the mRNA in a reaction mix containing 1xPCR buffer (2.5mM MgCl₂), 35 units of RNase inhibitor, and 50 units of Avian Myeloblastosis Virus (AMV) reverse transcriptase.

b) The reaction was incubated for 10 minutes at 22°C to allow annealing of the oligonucleotide primer, 60 minutes at 42°C to allow cDNA synthesis, and for 5 minutes at 95°C to terminate the reaction.

PCR amplification of cDNA's:

For amplification of the cDNA, 4µl of the 20µl reaction mix containing the reverse transcribed cDNA strands was used in a 25µl polymerase chain reaction. In order to check for contamination by extraneous cDNA, a duplicate reaction mix to which no AMV reverse transcriptase had been added was also used.
2.2.11 Isolation and Characterisation of Yeast Artificial Chromosome Clone DNA

Preparation of High Molecular Weight Yeast Artificial Chromosome (YAC) DNA in Agarose Blocks:

High molecular weight YAC DNA was prepared in agarose plugs for pulsed field gel electrophoresis analysis as well as other procedures using the following protocol (Modified from Anand et al (1990):

a) An individual yeast colony from an SD-agar plate was inoculated into 10ml of SD medium and cultured overnight in an environmental shaker at 30°C.
b) The yeast cells were pelleted by centrifugation at 2000g for 5 minutes, and resuspended in 500μl of yeast resuspension buffer.
c) 25 units of lyticase were added and the cells incubated at 37°C for 45 minutes. The spheroplasting of the yeast cells was monitored by mixing 3μl of cells with 3μl of 10% (w/v) SDS and examination of the cells under a phase contrast microscope. With the phase contrast microscope used, spheroplasts appeared dark purple, whereas the intact yeast cells were translucent.
d) When approximately 80% of the yeast cells are spheroplasts, 600μl of 1% low melting point agarose/YRB (no 2-mercaptoethanol) kept at 37°C was added and the mixture dispensed into 100μl plastic block formers.
e) The agarose plugs (approximately 12 in number) were then transferred to 5ml of yeast lysis buffer (YLB), and incubated for 1 hour at room temperature.
f) The YLB was then removed and replaced with 10ml of fresh YLB, and incubated overnight at 45°C.
g) The agarose plugs were then washed in 15ml of TE pH=8 for 15 minutes at 45°C, before storing the blocks in fresh YLB at room temperature.

For polymerase chain reaction (PCR) amplification of YAC clone DNA sequences a single agarose plug was melted in 500μl of water, and 5μl used for each reaction.
Isolation of Terminal Sequences from YAC Clones Using The Vectorette Procedure:

In order to isolate the terminal sequences of YAC clones, the vectorette procedure described by Riley et al (1990) was used. The YAC DNA was first blunt-end digested and then ligated to the vectorette, a pair of annealed oligonucleotide universal adaptors which have an internal region of non-complementarity or "bubble". PCR amplification was then performed using one primer that is specific for one arm (left or right) of the YAC, and one primer that is specific for the vectorette sequence. Using this approach, only terminal fragments containing vector sequences will be amplified. Figure 2.1 shows a schematic representation of the vectorette procedure.
Figure 2.1: Schematic representation of the vectorette procedure for the isolation of YAC clone terminal sequences. The YAC DNA is first digested with the restriction enzyme (X), and the annealed vectorette unit ligated. The YAC terminal sequence may then be amplified by the polymerase chain reaction using YAC vector (pYAC4) and vectorette specific oligonucleotides.
For the isolation of YAC clone terminal sequences the following protocol was used:

a) The YAC DNA agarose plug (100 μl in volume) was incubated in 50ml TE pH=8 overnight at 4°C.
b) The agarose plug was then removed from the TE buffer, and cut into 4 equal slices.
c) Each slice was then incubated in 500 μl of the appropriate 1x restriction endonuclease buffer for 1 hour at 4°C.
d) The buffer was then removed and replaced with 50 μl of fresh buffer containing 25 units of restriction endonuclease and incubated overnight at 37°C. Each of the four YAC DNA slices was digested with one of the following enzymes: EcoRV, PvuII, AluI or RsaI.
e) The buffer was then removed, replaced with 75 μl of 1x ligase buffer (containing no ATP), and the agarose block melted by heating the mixture at 65°C for 10 minutes.
f) 40 μl of the mixture was transferred to a fresh 1.5ml Eppendorf tube and 10 μl of a solution containing 0.5 pmol annealed vectorette and 4 units of T4 DNA ligase in 1x ligase buffer (4 mM ATP) added. The ligation reaction was incubated at 37°C for 2 hours to allow the ligation of the vectorette unit to the digested YAC DNA fragments.
g) The ligation mix was then diluted by adding 200 μl of water, mixed, and stored at -20°C. This solution is the "vectorette library". Vectorette libraries were thus prepared for EcoRV, PvuII, AluI and RsaI digested YAC clone DNA.

In order to isolate the terminal sequences from the vectorette library, PCR amplification was performed on an aliquot of the library as follows:

a) A 2 μl aliquot of the vectorette library was used as template in a 50 μl PCR amplification reaction with the vectorette primer (primer 224) and one oligonucleotide primer complementary to either the left arm (primer 1210), or the right arm (primer 1091) of the YAC vector sequence. After an
initial denaturation of 5 minutes at 96°C, 39 cycles of PCR amplification were performed, denaturing at 92°C for 2 minutes, annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes, followed by 10 minutes at 72°C for final extension of the DNA strands.

b) A secondary PCR reaction, using nested primers to enhance the specificity of the reaction, was then performed as follows: 1μl of the primary PCR reaction was used as template in a 100μl PCR reaction with the nested vectorette primer (primer 10108) and one nested primer either from the left arm (primer 1211) or the right arm (primer 20359) of the YAC vector sequence. The same conditions as described above for the primary PCR were used, with the exception that only 37 cycles of amplification were performed.

An aliquot of the secondary PCR reaction mixture was then analysed by agarose gel electrophoresis. Amplification products were then digested with EcoRI to remove the amplified vector sequence (81 base pairs in size), and the PCR product repurified by agarose gel electrophoresis, before use of the fragments as hybridisation probes. In addition the nucleotide sequence of the intact PCR product was determined by direct double stranded DNA sequencing utilising primer 368 (universal sequencing primer from the vectorette) as the sequencing primer.
2.3 Linkage Disequilibrium Analysis

The ASSOC (version 2.2) computer program (Ott 1985), available from the Medical Research Council at the Human Genome Mapping Resource Centre (HGMP), was utilised to detect linkage disequilibrium between the (AAAT)$_n$, TaqI and PstI polymorphisms of the PTH gene. Chi squared values ($\chi^2$) at 4 degrees of freedom, together with p-values and the standardized disequilibrium coefficients ($\Delta$) were ascertained. Linkage disequilibrium was established by a significant p-value and a $\Delta$ value greater than zero was indicative of the strength of the association.
2.4 Buffers and stock solutions

(All autoclaving carried out at 121°C, 15lbs/square inch.)

40% Acrylamide Stock: 38% (w/v) acrylamide,
2% (w/v) N,N’-methylenebisacrylamide

Church Buffer: per litre:
700ml 10%(w/v) SDS,
9.6g NaH₂PO₄,
59.6g Na₂HPO₄.
Make up to 1 litre with sterile distilled water.

Denaturing Solution: 1.5M NaCl, 0.4M NaOH.

Ligase Buffer 10x: 300mM Tris.HCl pH=7.8,
100mM MgCl₂,
100mM dithiothreitol,
10mM ATP

Kinase Buffer 10x: 500mM Tris.HCl pH=7.5,
100mM MgCl₂,
50mM dithiothreitol,
1mM spermidine.

LB-Broth (Luria-Bertani medium): Per litre:
Bacto-tryptone 10g
Bacto-yeast extract 5g
NaCl 10g.
Make up to 1 litre with deionised water and sterilise by autoclaving.
LB-Agar: LB-broth + 15g bacto-agar per litre. Autoclave and cool to 50°C before addition of antibiotic.

LB-Agarose: LB-broth + 7g agarose per litre. Autoclave and cool to 45°C before use.

Ligase Buffer 10x: 300mM Tris.HCl pH=7.8, 100mM MgCl₂, 100mM dithiothreitol, 10mM ATP.

Lysis Buffer: 10mM Tris.HCl pH=7.5 5mM MgCl₂, 0.32M Sucrose, 1% Triton X-100.

Maltose: 20% (w/v), sterilise by filtration through 0.2μm filter.

Neutralising Solution: 1M Tris.HCl pH=8, 1.5M NaCl.

Phosphate Buffered Saline (PBS): 130mM NaCl, 2mM KCl, 8mM Na₂HPO₄, 1mM KH₂PO₄ pH=7.4.

PCR buffer 10x: 500mM KCl, 100mM Tris.HCl pH=8.4, 25mM/20mM/15mM MgCl₂, 2mM dGTP, dATP, dTTP and dCTP.
RNase A (DNase free): Stock solution 10mg/ml, boil for 10 minutes, snap cool and store at 20°C.

Restriction Endonuclease Digestion Buffers (10x):

Low-Salt Buffer: 100mM Tris.HCl pH=7.5, 100mM MgCl₂, 10mM dithiothreitol.

Medium Salt Buffer: 500mM NaCl, 100mM Tris.HCl pH=7.5, 100mM MgCl₂, 10mM dithiothreitol.

High Salt Buffer: 1M NaCl, 100mM Tris.HCl pH=7.5, 100mM MgCl₂, 10mM dithiothreitol.

SmaI Buffer: 200mM KCl, 100mM Tris.HCl pH=8, 100mM MgCl₂, 10mM dithiothreitol.

Sequencing Buffer: 95% (v/v) Formamide, 20mM EDTA, 0.05% (w/v) Bromophenol Blue, 0.05% (w/v) Xylene Cyanol.

Solution I (for alkaline lysis): 50mM Glucose, 25mM Tris.HCl pH=8, 10mM EDTA pH=8; autoclaved and stored at 4°C.
Solution II: 0.2M NaOH, 1% SDS.

Solution III: Per 100ml:
60ml 5M Potassium acetate, 11.5ml Glacial acetic acid, 28.5ml Sterile water.

Sodium Saline Citrate (SSC) 20x: 3M NaCl, 0.3M Sodium citrate.

SD Medium: 0.7% (w/v) Yeast Nitrogen Base (minus amino acids), 2% Glucose, 55mg/l Adenine, 55mg/l tyrosine. After autoclaving, add 28ml of 20% (w/v) filter sterilised casamino acids per 500ml before use.

SM Buffer: 100mM NaCl, 10mM MgSO₄, 50mM Tris.HCl pH=7.5, 0.05% (w/v) gelatin; sterilise by autoclaving.

Tris-Borate (TBE) 10x: 0.89M Tris Base, 0.89M Boric Acid, 20mM EDTA; (final pH=8).

Tris-Acetate (TAE) 10x: 0.4M Tris.Acetate pH=8, 10mM EDTA.

Yeast Lysis Buffer (YLB): 0.1M EDTA, 10mM Tris.HCl pH=8, 1% (w/v) Lithium dodecyl sulphate.
Yeast Resuspension Buffer (YRB): 1.2M Sorbitol,  
20mM EDTA, 
10mM Tris.HCl pH=7.5, 
14mM 2-mercaptoethanol.
RESULTS AND DISCUSSION.
CHAPTER THREE:
AUTOSOMAL IDIOPATHIC HYPOPARATHYROIDISM.
3. AUTOSOMAL IDIOPATHIC HYPOPARATHYROIDISM

For autosomal idiopathic hypoparathyroidism, it has been postulated that mutations either within or in the regulatory regions of the parathyroid hormone gene may be associated with the endocrinopathy. Human parathyroid hormone is an 84 amino acid peptide encoded by a single gene on chromosome 11p15 (Mayer et al 1983, Naylor et al 1983). The parathyroid hormone locus was therefore investigated in four families with autosomal idiopathic hypoparathyroidism. Pedigrees A/89 and 2/89 demonstrate an autosomal recessive mode of inheritance, and pedigrees 7/90 and 23/92 demonstrate an autosomal dominant mode of inheritance.

In order to determine whether mutations at the PTH locus were associated with autosomal idiopathic hypoparathyroidism in these pedigrees, segregation analysis was first performed on the families using 2 restriction fragment length polymorphisms at the PTH locus (Schmidtke et al 1984) to investigate for cosegregation of PTH alleles with the disease. If there were no recombination events observed between the PTH locus and hypoparathyroidism in the pedigree, then the PTH gene was further investigated for DNA sequence abnormalities.

The results obtained for the investigation of the PTH locus for each pedigree are presented.
3.1 Pedigree A/89

A multi-generation kindred of Bangladeshi origin in whom autosomal recessive idiopathic hypoparathyroidism had occurred following a consanguineous marriage was investigated (Parkinson and Thakker 1992a).

Segregation Studies at the Parathyroid Hormone (PTH) Locus:

The PTH genomic probe (p20.36) reveals 2 restriction length polymorphisms (RFLP’s) detected by the restriction endonucleases TaqI and PstI (Figure 3.1). An analysis of family A/89 revealed similar results with TaqI-derived and PstI-derived RFLP’s. The mother (III.2, Figure 3.1) was heterozygous and the father (III.1) was homozygous and the four children (IV.1 to IV.4) were homozygous. This preliminary study therefore revealed no recombinants between hypoparathyroidism and the PTH locus. Thus an association between hypoparathyroidism and the PTH locus was not excluded in family A/89 and the PTH gene was investigated further for abnormalities.
Parathyroid Hormone (PTH) RFLP's

Family A/89

III

IV

Enzyme

Pst I

Taq I

2.8 kb

2.2 kb

2.5 kb

2.4 kb

Figure 3.1: Segregation analysis at the PTH locus in pedigree A/89 with autosomal recessive idiopathic hypoparathyroidism using PstI and TaqI restriction fragment length polymorphisms (RFLPs). For members of the family, affected males are indicated by solid squares, unaffected males by open squares, affected females by solid circles and unaffected females by open circles. The family is drawn so that each individual appears above his or her RFLP alleles. For the PstI RFLP, the father (III.1) is homozygous for the 2.8 Kb allele, and the mother (III.2) is heterozygous having both 2.8 and 2.2 Kb alleles, and the four children (IV.1 to IV.4) are all homozygous for the 2.8 Kb allele. The 2.3 Kb constant band observed with PstI (Schmidtke et al 1984) is indicated by a closed triangle. For the TaqI RFLP, the father (III.1) is homozygous for the 2.5 Kb allele, the mother (III.2) is heterozygous having both 2.5 and 2.4 Kb alleles, and the four children are all homozygous for the 2.5 Kb allele. Segregation analysis at the PTH locus using PstI and TaqI RFLPs therefore demonstrates no recombinants between hypoparathyroidism and the PTH gene in this pedigree.
Investigation of the PTH Gene for DNA Sequence Abnormalities:

The polymerase chain reaction (PCR) was utilised to amplify the 3 exons and 4 exon-intron boundaries of the PTH gene using 3 pairs of oligonucleotide primers (PTH1L+PTH1R, PTH2L+PTH2R, and PTH3L+PTH3R (Appendix I); Figure 3.2). The DNA sequence of these amplified segments was determined by direct double-stranded DNA sequencing and a total sequence of 930 nucleotides of the PTH gene was determined for patient IV.1 from family A/89 and for an unrelated normal individual. A comparison of the results revealed that the DNA sequence obtained from patient IV.1 differed from the normal individual and the published sequence (Vasicek et al. 1983, Reis et al. 1990) by a single nucleotide at 2 separate sites. The first of these was a g→a transition at position +53 of intron 2 of the PTH gene and this represents the previously observed polymorphism (Schmidtke et al. 1984) in the TaqI restriction endonuclease site (ticga). This finding demonstrated that the individual IV.1 was homozygous for the absence of the TaqI cleavage site, and thus the results of the DNA sequence analysis confirm the findings using RFLP analysis at the PTH locus.

The second DNA sequence alteration was associated with a g→c substitution at position +1 of intron 2 of the PTH gene (Figure 3.3), and this mutation is of importance as it disrupts the invariant gt dinucleotide (Breathnach et al. 1978) of the donor splice site sequence. The g→c mutation in the donor splice site sequence (gtaagt) resulted in the occurrence of a recognition site (c↓taag) for cleavage by the restriction endonuclease DdeI, and this facilitated the detection of this donor splice site mutation in other members of pedigree A/89. For this study, blood samples from the maternal grandmother and grandfather as well as the paternal grandfather were obtained and leukocyte DNA extracted, allowing the analysis of the PTH gene for this mutation to be carried out on an extended pedigree comprising of 3 generations (Figure 3.4).

DdeI restriction enzyme analysis in family A/89 revealed
inheritance of the mutant PTH donor splice site with autosomal recessive hypoparathyroidism (Figure 3.4). Incubation of the PCR-amplified 474 base pair PTH gene segment (amplified from genomic DNA using primers 2L and 2R, Figure 3.2) spanning the exon 2-intron 2 boundary with DdeI enzyme demonstrated that the affected individuals (IV.1, IV.3 and IV.4, Figure 3.4) were homozygous for the mutant allele (c↓taag) and that a panel of 10 unrelated normal individuals (N1 to N3 shown) were homozygous for the wild-type donor splice site sequence (gtaag). The unaffected members of family A/89, for example the parents (III.1 and III.2), and the grandfathers (II.2 and II.4) and the sibling (IV.2) were heterozygous for the mutant and wild-type alleles.

Thus, a donor splice site mutation, which is detected by DdeI cleavage, within the PTH gene is cosegregating with hypoparathyroidism in this family. The donor splice site sequence (gtaagt) is found to be highly conserved at the 5' end of introns (Lewin 1980), and the integrity of this consensus sequence has been demonstrated to be necessary for the correct splicing of mRNA molecules to produce the mature mRNA (Wieringa et al 1983). The donor splice site mutation detected in the PTH gene of patients from family A/89 would therefore be likely to result in abnormal splicing of PTH mRNA.

In order to investigate the abnormalities in PTH mRNA splicing that may occur due to the donor splice site mutation observed to be segregating with hypoparathyroidism in pedigree A/89, it was necessary to detect and analyse parathyroid hormone mRNA from individuals with the mutation. Parathyroid tissue was unavailable from these patients and therefore a novel approach was used. I have used the sensitivity of the polymerase chain reaction to detect the "illegitimate" or "non-tissue specific" (Sarkar et al 1989, Chelly et al 1989, Berg et al 1990) transcription of the PTH gene in lymphocyte cell lines from normal individuals and individuals with the donor splice site mutation.
Figure 3.2: Schematic representation of the human parathyroid hormone (PTH) gene, which consists of 3 exons (hatched) and 2 introns (stippled), (Vasicek et al 1983). Three pairs of PCR primers were designed to amplify the 3 exons and 4 exon-intron boundaries: primers PTH1L and PTH1R (shown as 1L and 1R in figure) amplify a 228 bp segment which consists of exon 1 and the exon 1-intron 1 boundary; primers PTH2L and PTH2R amplify a 474 bp segment which consists of the intron 1-exon 2 boundary, exon 2, the exon 2-intron 2 boundary, intron 2, the intron 2-exon 3 boundary and part of exon 3; primers PTH3L and PTH3R amplify a 565 bp segment of exon 3. An additional 2 pairs of PCR primers, designated PTH4L and PTH4R, and PTH5L and PTH5R were designed to specifically amplify PTH cDNA, which in normals consists of exons 1,2 and 3. The TaqI polymorphic site (closed triangle) within intron 2 and the PstI polymorphic site (open triangle) which is 1.7 Kbp downstream are indicated; (from Parkinson and Thakker 1992a).
Figure 3.3: Autoradiograph showing the nucleotide sequences of the PTH gene exon 2-intron 2 boundary obtained from a normal individual and patient IV.1 from pedigree A/89. The exon sequence is indicated by upper case letters, the intron sequence is indicated by lower case letters and the exon-intron boundary is shown (broken line). The patient has a single base substitution (g to c), and this has altered the normal consensus 5' donor splice site sequence (gtaagt). This mutation has resulted in the occurrence of a DdeI restriction enzyme site (ctaag) and this has facilitated the detection of the mutation in other members of the family (Figure 3.4). (From Parkinson and Thakker 1992a).
Figure 3.4: DdeI restriction enzyme analysis in members of family A/89 (upper panel) together with 10 unrelated normal (N_1 to N_3) individuals. The 474 bp PTH gene segment spanning the exon 2-intron 2 region was amplified by PCR using primers PTH2L and PTH2R (Figure 3.2) and incubated with DdeI enzyme. In the 10 normal individuals (N_1 to N_3 shown) the 474 bp PCR product was not cleaved with DdeI, thereby revealing homozygosity for the wild type (W) alleles. However, in the patients (IV.1, IV.2 and IV.4) DdeI cleavage resulted in 2 fragments of 298 bp and 176 bp, demonstrating homozygosity for the mutant (m) alleles. In the unaffected members (II.2, II.4, III.1 and III.2 and IV.2) of the family both the wild type and mutant alleles were detected, indicating heterozygosity (Wm). These results demonstrate that a g to c donor splice site mutation, which is detected by DdeI, in the PTH gene segregates with hypoparathyroidism in this pedigree. (From Parkinson and Thakker 1992a)
Detection of Correctly Spliced PTH mRNA in Lymphocyte Cell Lines from Normal Individuals:

Total RNA isolated from Epstein Barr Virus (EBV)-transformed lymphocytes was used for the specific reverse transcription of PTH mRNA using the oligonucleotide PTH4R (Figure 3.2), which was specific for the 3' end of exon 3, as the primer for AMV reverse transcriptase. The resulting specific first strand PTH cDNA was amplified by 2 rounds of PCR using the primers PTH4L and PTH4R for the first round of amplification and the nested primers PTH5L and PTH5R (Figure 3.2) for the second.

The amplified PTH cDNA was then analysed by agarose gel electrophoresis, and the expected 258 base pair cDNA derived fragment observed (Figure 3.5). This amplified cDNA fragment was not observed either when genomic DNA (G) was used as the amplification template, or when the reverse transcriptase enzyme was omitted from the cDNA synthesis reaction (-), (Figure 3.6). Thus, this PTH cDNA arose from the non-tissue specific expression of PTH mRNA and not from amplification of a genomic sequence. Sequence analysis of the 258 bp PCR-amplified fragment using oligonucleotide PTH5R as the sequencing primer and comparison to the previously published sequence (Vasicek et al 1983) confirmed that this cDNA was derived from a correctly spliced PTH mRNA transcript in which exon 1 was spliced to exon 2 and exon 2 was spliced to exon 3 (Figure 3.5).

These results, which have subsequently been confirmed by Handt et al (1992), therefore demonstrate that parathyroid hormone mRNA expression is not confined to parathyroid cells, but that a low level of correctly spliced PTH mRNA expression can be detected in a non-tissue specific manner by utilising the sensitivity of the polymerase chain reaction.
Figure 3.5: Autoradiograph showing a 123 bp segment from the total 258 bp DNA sequence of the normal PTH cDNA synthesized from illegitimately transcribed PTH mRNA. A comparison of the published sequence (Vasicek et al, 1983) to the sequence shown: 5'GTTCTAAGACATTTGTATGTGAAGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTTTCTTACAAAATCGGATGGGAAATCTGTTAAGAA GAGATCTGTGA 3', reveals correct splicing in the order exon 1-exon 2-exon 3. Thus illegitimate transcription of the PTH gene in cultured lymphocytes from normal individuals is associated with correct splicing of PTH mRNA. (From Parkinson and Thakker 1992b)
Characterisation of Mutant PTH cDNA in Lymphocyte Cell Lines from Individuals with the Donor Splice Site Mutation:

The finding of a donor splice site mutation at the exon 2-intron 2 boundary of the PTH gene in affected individuals of family A/89 (Figure 3.4) indicated that hypoparathyroidism in this family may result from abnormal PTH mRNA splicing. Parathyroid cells were not available for studies from these patients, and PTH mRNA processing abnormalities were therefore investigated by the detection of non-tissue specific transcription of the PTH gene in EBV-transformed lymphocytes from members of family A/89 (Figure 3.6).

The results revealed an abnormal PTH cDNA of 168 bp from the patients IV.1, IV.3 and IV.4, who were previously observed to be homozygous for the g->c donor splice site mutation. The mutant cDNA therefore differed from the normal cDNA by 90 bp in size, which corresponds to the size of exon 2 of the PTH gene. Both the normal (258 bp) and mutant (168 bp) PTH cDNA fragments were obtained from the parents (III.1 and III.2, Figure 3.6) and from the sibling (IV.2) who were heterozygous for the donor splice site mutation. Thus, the g->c base substitution at the donor splice site segregating with hypoparathyroidism in this family, is associated with a smaller PTH cDNA which reflects the altered size of the PTH mRNA transcript and the abnormal splicing of PTH mRNA.
Figure 3.6: The illegitimate transcription of the PTH gene in EBV-transformed lymphocytes was detected by PCR amplification of PTH cDNA which had been synthesized by addition (+) of the AMV reverse transcriptase to extracts of RNA obtained from EBV-transformed lymphocytes of normal and affected individuals. In 10 normal individuals (N1 to N3 shown), correctly spliced PTH cDNA was observed at the expected size of 258 bp. This product was not present when reverse transcriptase was omitted from the reaction (−) or when only genomic DNA (G) or a water blank (B) were used, thereby demonstrating that this product is not due to amplification of a genomic sequence but is RNA specific. In family A/89, the affected individuals (IV.1, IV.3 and IV.4) who were homozygous for the donor splice site mutation (Figure 3.4) were found to differ from the normals in having an abnormal PTH cDNA of 168 bp in size. The mutant (m) PTH cDNA differed from the normal or wild type (W) by 90 bp, which corresponds to the size of exon 2 (Figure 3.2). The parents (III.1 and III.2) and the unaffected sibling (IV.2) who are heterozygous for the mutation (Figure 3.4) have both the mutant and wild type PTH cDNA. (From Parkinson and Thakker 1992a).
In order to further characterise the abnormally spliced PTH mRNA, the nucleotide sequence of the mutant 168 bp cDNA obtained from patient IV.1 was determined by direct DNA sequencing (Figure 3.7). This revealed that the loss of the 90 bp in the size of the mutant PTH cDNA was indeed the result of a loss of exon 2, and that the mutant PTH cDNA consisted of exon 1 spliced to exon 3. The loss of exon 2 removed the initiation codon (ATG) and the nucleotide sequence encoding the 25 amino acid signal sequence of the PTH transcript (Figure 3.2). Thus the loss of exon 2 will prevent the commencement of PTH mRNA translation (Kozak 1989) at the correct point to the pre-pro-PTH protein and the translocation (Emr et al 1980) of any PTH peptide through the endoplasmic reticulum prior to secretion. The possible alternative use of the ATG codons at the 3' terminus of exon 1 (Figure 3.7), and at codon 20 of exon 3 for the initiation of translation would yield a protein lacking the signal peptide, which would prevent its translocation.

Thus, autosomal recessive hypoparathyroidism in family A/89 has resulted from a g→c donor splice site mutation, which has caused abnormal splicing (exon skipping) of the PTH mRNA and lead to a loss of exon 2 from the mRNA transcript.
Figure 3.7: Autoradiograph showing an 18 bp segment from the total 168 bp DNA sequence of the mutant PTH cDNA from patient IV.1. A comparison of this mutant PTH cDNA sequence to that of the normal (Figure 3.5), and the published sequence (Vasicek et al 1983) reveals that, in the mutant PTH cDNA, exon 1 has been spliced to exon 3. Thus, exon skipping with a loss of the 90 bp of exon 2, which contains the initiation codon (ATG) and the 25 amino acid signal peptide sequence, has occurred. (From Parkinson and Thakker 1992a).
3.2 Pedigree 7/90

For pedigree 7/90, autosomal dominant inheritance of idiopathic hypoparathyroidism was observed (Figure 3.8).

**Segregation Studies at the PTH Locus:**

Segregation analysis using the PstI and TaqI restriction fragment length polymorphisms at the PTH locus was performed for individuals from family 7/90 (Figure 3.8). An examination of the results for the PstI RFLP revealed that the father (I.1) was heterozygous and the mother (I.2) was homozygous for allele 1. Individuals II.1 and II.4 who are both affected, have therefore inherited allele 1 from their mother but have inherited different PTH alleles from their father. In addition, the unaffected daughter (II.2) has inherited the same PTH allele from her father as the affected son (II.4), demonstrating a second recombination event between hypoparathyroidism and the PTH locus in this pedigree. Analysis of TaqI RFLP's at the PTH locus in this family proved uninformative.

The polymorphic PstI restriction endonuclease site is located approximately 1.7 Kb downstream from the 3' end of the PTH gene, and it is therefore very unlikely that this result could arise from meiotic chromosomal recombination between the PTH allele and the PstI polymorphic site of the father (I.1).

This result therefore demonstrates recombination between the PTH locus and hypoparathyroidism in this pedigree, and would thus exclude an association between the PTH gene and autosomal dominant hypoparathyroidism in this family.
Figure 3.8: Segregation analysis at the PTH locus in pedigree 7/90 with autosomal dominant idiopathic hypoparathyroidism using PstI (P) and TaqI (T) restriction fragment length polymorphisms (RFLPs). For members of the family, affected males are indicated by solid squares, unaffected males by open squares and unaffected females as open circles. Recombinants between idiopathic hypoparathyroidism and the PTH locus are indicated by an asterisk. Analysis of the results for the PstI RFLP demonstrate that individuals II.1 and II.2 show recombination between hypoparathyroidism and the PTH locus, thereby excluding an association between the PTH gene and hypoparathyroidism in this family.
3.3 Pedigree 2/89

For pedigree 2/89, autosomal recessive hypoparathyroidism together with renal insufficiency and developmental delay was observed in affected individuals (Shaw et al., 1991) following consanguineous marriage.

Segregation Studies at the PTH Locus:

Segregation analysis using PstI and TaqI restriction fragment length polymorphisms at the PTH locus was performed for individuals from family 2/89 (Figure 3.9). An examination of the results obtained revealed no recombination events between the PTH locus and hypoparathyroidism in this family and the PTH locus was further investigated for DNA sequence abnormalities.

Investigation of PTH Gene for DNA Sequence Abnormalities:

The polymerase chain reaction was utilised to amplify the 3 exons and 4 exon/intron boundaries of the PTH gene from an affected individual from family 2/89 (III.9, Figure 3.9). The nucleotide sequence of the resulting DNA fragments was determined by direct DNA sequencing. Comparison of the sequence obtained from individual III.9 to the normal PTH gene sequence (Vasicek et al., 1983, Reis et al., 1990) revealed no abnormalities. In addition to the investigation of the coding sequence of the PTH gene, the 5' promoter region of the PTH gene was also examined for DNA sequence abnormalities that may be associated with hypoparathyroidism in this pedigree. Oligonucleotides PTHIR and PTHPROM were utilised to amplify an 807 base pair fragment encompassing the characterised region of the parathyroid gene promoter (Reis et al., 1990) from individual III.9. The resulting PCR product was ligated into Bluescript T-vector as described in Materials and Methods, and the DNA sequence of 10 individual clones determined. Comparison of the sequence obtained from individual III.9 to
the published sequence revealed no DNA sequence abnormalities associated with the proposed TATA box sequence (at -30 in the human gene) CAT box sequences (CCAAT, at -175 and -140) and a GC box (putative SP1 transcription factor binding site) at position -146 of the PTH promoter (Reis et al 1990). Thus PTH gene abnormalities were excluded as a cause of hypoparathyroidism in this family.
Figure 3.9: Segregation analysis at the PTH locus in pedigree 2/89 with autosomal recessive idiopathic hypoparathyroidism using PstI (P) and TaqI (T) RFLPs. For members of the family, affected males are shown as solid squares, unaffected males as open squares, affected females by solid circles and unaffected females as open circles. Analysis of the results for the PstI and TaqI RFLPs revealed no recombination events between the PTH locus and hypoparathyroidism. An association between the PTH gene and hypoparathyroidism cannot therefore be excluded.
3.4 Pedigree 23/92

For pedigree 23/92, autosomal dominant familial hypoparathyroidism together with renal dysplasia and sensorineural deafness was observed in affected individuals (Bilous et al 1992), as shown in Figure 3.10. I have investigated the PTH gene for abnormalities that may be associated with this disorder.
Figure 3.10: Pedigree 23/92 with hypoparathyroidism, sensorineural deafness, and renal dysplasia. For members of the family, affected males are indicated by solid squares, unaffected males as open squares, affected females as solid circles and unaffected females as open circles. Individuals who are shown as striped symbols denote that they are possibly or partially affected. The arrow denotes the index patient (individual III.3). MI denotes myocardial infarction, and SIDS sudden infant death syndrome. Segregation analysis using PstI and TaqI RFLPs demonstrated that individual III.2 was homozygous for both polymorphisms and therefore proved uninformative. (From Bilous et al 1992).
Segregation Studies at the PTH Locus:

Segregation analysis in pedigree 23/92 using PstI and TaqI RFLPs proved uninformative (data not shown) as individual III.2 (Figure 3.10) was homozygous for both polymorphisms. An association between hypoparathyroidism and the PTH gene in this kindred could therefore not be excluded, and the locus was therefore further investigated.

Investigation of PTH Gene for DNA Sequence Abnormalities:

An analysis of the PTH gene sequence together with its associated promoter was performed (as for pedigree 2/89) on individual III.2 from pedigree 23/92. Comparison of the DNA sequence to the normal published sequence (Vasicek et al 1983, Reis et al 1990) revealed no abnormalities.
3.5 Discussion:

Pedigree A/89:

The detection by PCR of a low level of transcription of a tissue-specific gene in cells that do not exhibit a physiological expression of the gene has been referred to as either "non-tissue specific" or "ectopic" or "illegitimate" transcription (Sarkar et al 1989, Chelly et al 1989, Berg et al 1990). For example, such illegitimate transcription of the Duchenne muscular dystrophy (DMD) gene encoding dystrophin, which is physiologically expressed only in muscle, has been observed to occur in fibroblasts, lymphoblastoid cells, HepG2 hepatoma cell lines and peripheral blood lymphocytes (Chelly et al 1988, Scolesser et al 1990). Additional studies have demonstrated that such illegitimate transcription with correct splicing of the mRNA also occurs for other highly tissue-specific genes which encode clotting factor VIIc, β-globin, anti-Mullerian hormone and aldolase A (Chelly et al 1989). The extent of the non-tissue specific expression of these genes has been estimated (Chelly et al 1988, Berg et al 1990, Chelly et al 1989) to be approximately one molecule of correctly spliced mRNA per 1000 cells and the physiological relevance and mechanisms involved in this low level of non-tissue specific transcription are not known. It has been postulated that the promoter regions of the gene may be activated by the ubiquitous transcriptional factors in the nucleus, for example TATA box binding factors, and CAAT box binding proteins in the absence of the respective tissue-specific transcription factors (Chelly et al 1989). The binding of these ubiquitous transcriptional factors would be facilitated by the chromatin disruption that occurs during DNA replication, and non-tissue specific transcription has been observed to be greater in actively proliferating lymphoblasts than in confluent fibroblasts (Chelly et al 1989). The demonstration of this non-tissue specific transcription is of medical importance as it enables the use of easily accessible peripheral blood
lymphocytes for the detection of abnormalities in mRNA processing and thereby avoids the requirement for expressing tissue that may only be obtainable by biopsy. These results demonstrated that the PTH gene was illegitimately transcribed and this facilitated the investigation of the donor splice site mutation.

The investigation of the PTH locus in individuals from family A/89 with autosomal idiopathic hypoparathyroidism has identified a mutation involving a single base substitution (g->c) at position +1 of intron 2 of the gene, and this has altered the invariant gt dinucleotide of the 5’ donor splice site consensus sequence gtaagt (Mount 1982). This consensus sequence is complementary to the RNA component of the U1 small nuclear ribonucleoprotein complex (U1-snRNP), which anneals to the 5’ end of the intron during the process of mRNA splicing (Lerner et al 1980, Rogers et al 1980, Lewin 1980). Thus an alteration of the 5’ donor splice site sequence of the intron will affect the annealing of the U1-snRNP, and previous studies have demonstrated that such mutations are associated with abnormalities of mRNA processing (Wieringa et al 1983, Weatherall et al 1982, Treisman et al 1982). For example, an analysis of patients suffering from β-Thalassaemia (Treisman et al 1982, Orkin et al 1982, Treisman et al 1983), Tay Sachs disease (Ohno et al 1988), and the Ehlers–Danlos type IV (Kontusaari et al 1990) and type VII (Nicholls et al 1991) syndromes have revealed that mutations in the 5’ donor splice site regions were associated with: either an accumulation of unspliced precursor mRNA, a retention of incompletely spliced precursors, or to the appearance of aberrantly processed mRNA which had resulted from the utilisation of alternative normally occurring 5’ splice sites or from the use of cryptic splice sites. In addition, in vitro studies utilising the human adenovirus late transcription unit (Talerico and Berget 1990), and the rat preprotachykinin gene (Kuo et al 1991) have demonstrated that alterations at an internal exon-intron boundary result in the splicing out of the exon together with its adjacent intron sequences.
This form of abnormal splicing out of the exon has been referred to as "exon skipping" and has been reported to occur in patients with Tay Sachs disease (Ohno et al 1988), Ehlers-Danlos type VII syndrome (Nicholls et al 1991) and hereditary elliptocytosis (Garbarz et al 1991). The in vitro mRNA splicing studies performed by Talerico and Berget (1990), together with analysis of the phenotypes of 18 published naturally occurring mutations in the donor splice site consensus sequence of internal exons, indicated that exon skipping was the preferred (15 out of 18) in vivo phenotype for such mutations. Talerico and Berget therefore postulated that mutation at the 5′ donor splice site sequence at the 3′ end of an internal exon inhibits the ability of splicing factors to detect this exon despite the presence of valid splicing signals within the upstream intron, indicating that the exon is the basic unit of spliceosome assembly in multi-exon precursor mRNA's.

I have investigated the abnormalities in parathyroid hormone mRNA splicing in these hypoparathyroid patients who had inherited a 5′ donor splice site mutation of the PTH gene. The results demonstrate that this mutation, which is at the exon 2-intron 2 boundary, lead to an abnormality of mRNA processing in which the normal 5′ donor splice site sequence at the exon 1-intron 1 boundary of the gene was utilised, and exon 1 was spliced to exon 3. Thus, mutation of the donor splice site sequence at the 3′ end of exon 2 has resulted in the failure of the exon to be recognized by the splicing apparatus of the cell, and exon skipping has occurred in these hypoparathyroid patients. The resulting PTH mRNA transcript lacked exon 2 and this lead to a loss of the initiation codon and the signal peptide which are required respectively for the commencement of PTH mRNA translation and for the translocation of the PTH peptide. These findings therefore have defined the molecular pathology of the PTH gene that causes autosomal recessive hypoparathyroidism in this family.
Pedigree 7/90:

The finding of recombination between the PTH locus and hypoparathyroidism in pedigree 7/90 demonstrates that there is genetic heterogeneity for familial idiopathic hypoparathyroidism. This premise is in agreement with previous reports which have used either PstI and TaqI RFLPs or polymorphic PTH gene mutations to exclude an association between idiopathic hypoparathyroidism and the PTH locus (Ahn et al 1986, Schmidtke et al 1986, Miric et al 1992).

However in the study published by Ahn et al, although the PTH gene was excluded as the cause of idiopathic hypoparathyroidism in 4 of the 8 pedigrees investigated, in 2 of the families with autosomal dominant hypoparathyroidism, cosegregation of a PTH allele with the condition was observed. For one of these families (pedigree D, Ahn et al 1986) the PTH gene from an affected individual (II.1) was cloned and the DNA sequence investigated for abnormalities that may be associated with the endocrinopathy (Arnold et al 1990). Comparison of the DNA sequence obtained from the patient to the normal sequence demonstrated the presence of a T to C transition in exon 2 of the PTH gene. This single base change resulted in a cysteine to arginine substitution at position 18 in the 25 amino acid signal sequence. The introduction of a charged amino acid into the midst of the hydrophobic PTH signal sequence was demonstrated, by in vitro translation of the mutant cDNA in the presence of canine microsomal membranes, to dramatically interfere with the processing of the preproPTH peptide to the proPTH peptide (signal peptide recognition and cleavage), thereby impairing the release of mature PTH peptide from the parathyroid gland.

The finding of this mutation acting in a dominant manner, when the DNA sequence for the other PTH allele of the patient appeared to be normal was proposed to be due to the mutant preproPTH protein obstructing the translocatory pathway of the parathyroid cell, thereby blocking processing and secretion of PTH protein encoded by the wild-type allele; indeed PTH
secretion in affected individuals was not completely abolished and levels of the hormone, while low, were still detectable (Arnold et al 1990).

The investigation of the PTH gene in pedigree 7/90 has excluded such a mutation at this locus as the cause of autosomal dominant hypoparathyroism in this kindred, and therefore another locus or loci must be investigated. The identification of such genes which may be involved in the development, regulation or function of the parathyroid glands will be of tremendous interest for the future investigation of the molecular basis of idiopathic hypoparathyroidism.
Pedigrees 2/89 and 23/92:

For both pedigrees 2/89 and 23/92, the presence of familial idiopathic hypoparathyroidism has been shown to be associated with other congenital abnormalities such as renal insufficiency and developmental delay in family 2/89, and renal dysplasia and sensorineural deafness in family 23/92. For both families, the investigation of the parathyroid hormone gene has identified no abnormalities in either the coding sequence of the gene or in the characterised elements of the PTH gene promoter. Whilst it may be possible that in either of these families, there may be abnormalities in as yet uncharacterised elements of the PTH gene promoter, which reduces the level of PTH mRNA transcription, it would appear more likely that another, as yet unidentified, locus or loci are involved in the pathophysiology of the observed phenotypes in affected individuals.

Idiopathic hypoparathyroidism has been reported to occur in association with a diverse range of developmental abnormalities, such as in the DiGeorge syndrome where a congenital anomaly in development of derivatives of the third and fourth pharyngeal pouches causes affected individuals to be athymic, aparathyroid, have reduced thyroid tissue and have craniofacial abnormalities. In addition, hypoparathyroidism may be associated with the presence of growth retardation, ophthalmologic abnormalities, delayed closure of the anterior fontanelle, anaemia and radiological skeletal abnormalities as in the Kenny-Caffey syndrome (Kenny et al 1966, Bergada et al 1988). Other reports have described idiopathic hypoparathyroidism in association with distinct dysmorphic facial features as in the Sanjad-Sakati syndrome (Sanjad et al 1991).

The clinical heterogeneity of syndromes in which idiopathic hypoparathyroidism plays a part indicates an underlying genetic heterogeneity that is the cause of the observed endocrinopathy. It may be postulated that the syndrome may arise due to a deletion or rearrangement
involving a number of genetic loci, one of which may affect the embryological development, cellular composition or homeostatic regulation of the parathyroid glands and that the disease represents a contiguous gene syndrome. Alternatively, these syndromes may arise due to an abnormality at a single locus such as a homeobox gene, which are thought to act as "master switches" in specifying regional information and development in the early embryo (Nusslein-Volhard et al 1980).

For the example of the DiGeorge syndrome, the cytogenetic investigation of affected individuals (De la Chapelle 1981) has revealed an unbalanced translocation and deletion of part of chromosome 22 (band 22q11) as a possible cause of the abnormality. However, in experiments carried out by Chisaka and Capecchi (1991), targeted disruption of the mouse homeobox gene \textit{hox-1.5} in embryonic stem cells and the production of mice that are homozygous for the mutated gene has revealed that the loss of \textit{hox-1.5} gene function results in developmental defects remarkably similar to those of the DiGeorge syndrome. There are important differences however between the human and the mouse syndrome in that DiGeorge syndrome is autosomal dominant whereas the \textit{hox-1.5} phenotype is autosomal recessive (mice heterozygous for the mutated gene are normal). In addition, whereas the DiGeorge syndrome has been associated with deletions and translocations involving chromosome 22, the human homologue to \textit{hox-1.5} maps to chromosome 7. It may be possible, however, that the human \textit{Hox-1.5} gene and the gene (or genes) involved in deletions on chromosome 22 in DiGeorge syndrome may be involved in a common developmental pathway.

Thus the analysis of the molecular basis of DiGeorge syndrome with the observed differing Mendelian modes of inheritance and genetic heterogeneity is likely to act as an excellent model for the investigation of other syndromes of which idiopathic hypoparathyroidism plays a part.
CHAPTER FOUR:

A NOVEL TETRANUCLEOTIDE (AAAT)$_n$ POLYMORPHISM IN THE PARATHYROID HORMONE GENE.
4. A NOVEL TETRANUCLEOTIDE (AAAT)$_n$ POLYMORPHISM IN THE PARATHYROID HORMONE GENE.

The parathyroid hormone gene locus is associated with 2 restriction fragment length polymorphisms detected by the enzymes PstI and TaqI (Schmidtke et al 1984) which have previously been utilised for segregation analysis in pedigrees with autosomal idiopathic hypoparathyroidism (Ahn et al 1986, Schmidtke et al 1986). The use of the PstI and TaqI bi-allelic polymorphic systems is hampered both by the difficulties in resolving the PTH alleles, and the need for good quality high molecular weight genomic DNA in order to perform the analysis.

In order to overcome these difficulties, I have investigated the parathyroid hormone gene locus for a microsatellite tandem repeat polymorphism. Such polymorphisms, which occur due to length variations in microsatellite repeats may be detected using the polymerase chain reaction, and are inherited in a Mendelian manner (Litt et al 1989, Weber et al 1989). The results of this study revealed that an (AAAT)$_n$ polymorphism is associated with the PTH gene (Parkinson et al 1993).

4.1 (AAAT)$_n$ Tetranucleotide Polymorphism

An examination of the published PTH genomic sequence (Reis et al, 1990) revealed a tandem repetitive sequence consisting of the tetranucleotide (AAAT)$_n$, at position 2859 to 2874 of intron 1. Oligonucleotide primers PTHVNTRL (position 2729-2751) and PTHVNTRR (position 3005-2982) flanking this microsatellite were synthesized to enable PCR amplification of the tandem repeat. An investigation of the tandem repeat in a panel of 39 normal unrelated individuals (25 European and 14 Asian) demonstrated the presence of 2 alleles at this locus (Figure 4.1) with a frequency for allele 1 of 0.49 and for allele 2 of 0.51, therefore yielding a polymorphism information content (PIC) value (Botstein et al, 1980) of 0.375. Segregation analysis in 4 multi-generation kindreds
(Pedigree 18/92 shown, Figure 4.2) demonstrated that the alleles for this microsatellite polymorphism were inherited in a Mendelian manner.
Tetranucleotide polymorphism associated with the PTH gene

Figure 4.1: Tetranucleotide (AAAT)$_n$ polymorphism associated with the PTH gene. The autoradiograph shows some of the nucleotide sequence obtained from the amplified segment containing the microsatellite in intron 1 of the PTH gene. The individual, II.2 from family A/89, (Figure 4.3) was found to have the sequence (AAAT) tandemly repeated 4 times on one chromosome and 5 times on the other. The occurrence of (AAAT)$_5$ is designated allele 1 and that of (AAAT)$_4$ is designated allele 2. Thus, this individual is heterozygous (allele 1, 2) for the tetranucleotide polymorphism in the PTH gene.
Figure 4.2: Mendelian inheritance of the tetranucleotide (AAAT)$_n$ polymorphism associated with the PTH gene. The presence of the tandem repeat (AAAT)$_3$ was revealed by the 281 bp fragment (allele 1) and that of (AAAT)$_4$ was revealed by the 277 bp fragment (allele 2). An analysis of the inheritance of these alleles is shown for family 18/92, which is of Northern European origin and is not affected with hypoparathyroidism. The family is drawn so that each individual appears above his or her alleles and the genotype is indicated for each individual as in Figure 4.1. The grandmother (I.2) is heterozygous (allele 1,2) and the grandfather (I.1) is homozygous (allele 1,1). An examination of their children and grandchildren reveals Mendelian inheritance of the alleles.
4.2 Segregation Studies in Families with Idiopathic Hypoparathyroidism

The (AAAT)$_n$ tetranucleotide intragenic tandem repeat polymorphism at the PTH locus was utilised for segregation analysis in pedigrees 7/90, A/89 and 2/89 with autosomal idiopathic hypoparathyroidism (Figure 4.3). For pedigree 7/90 with autosomal dominant hypoparathyroidism, recombination was observed between the PTH locus and hypoparathyroidism, thereby excluding an association between the PTH gene and hypoparathyroidism in this family. This result confirms the finding of recombination observed in this pedigree using the PstI restriction fragment length polymorphism (see Results and Discussion, Chapter 3).

For pedigrees A/89 and 2/89, with autosomal recessive idiopathic hypoparathyroidism, segregation analysis using the tetranucleotide polymorphism proved uninformative.
Figure 4.3: Segregation studies in families A/89, 2/89 and 7/90 with idiopathic hypoparathyroidism. For the three families designated A/89, 2/89 and 7/90, affected males are indicated by solid squares, unaffected males by open squares, affected females by solid circles and unaffected females by open circles. The genotypes at the PTH locus detected by the polymorphic (AAAT)$_n$ microsatellite are represented by alleles 1 and 2, and are indicated below the symbol for each family member with the paternal allele on the left and the maternal allele on the right. In family 7/90, recombination, which is indicated by an asterisk, is observed between the PTH locus and hypoparathyroidism, thereby excluding an association. In families A/89 and 2/89 an association between the PTH locus and hypoparathyroidism cannot similarly be excluded. (From Parkinson et al 1993).
4.3 Linkage Disequilibrium Analysis for the Tetranucleotide (AAAT)<sub>n</sub>, PstI and TaqI Polymorphisms at the PTH Locus

The genotypes obtained with the (AAAT)<sub>n</sub> polymorphism in 36 unrelated individuals were compared to those obtained with PstI and TaqI RFLPs. The genotypes obtained with each of the three polymorphisms were the same in 21 individuals, but in the remaining 15 individuals the (AAAT)<sub>n</sub> and TaqI genotypes differed in 13 individuals, the (AAAT)<sub>n</sub> and PstI genotypes differed in one individual and the (AAAT)<sub>n</sub> genotype differed from both the PstI and TaqI genotypes in one other individual. The combined use of these three polymorphisms of the PTH gene enabled the detection of heterozygosity for segregation studies in 66% of individuals. Linkage disequilibrium analysis of the (AAAT)<sub>n</sub> polymorphism and the TaqI and PstI RFLPs yielded similar results from the European and Asian populations (Table 3), and significant linkage disequilibrium between these three polymorphisms was observed.

Linkage disequilibrium is usually observed between markers that have a low mutation rate and are closely linked (Snell et al 1989). The (AAAT)<sub>n</sub> polymorphism in intron 1 of the PTH gene is 383 bp upstream from the polymorphic TaqI site in intron 2, and 2.4 Kbp upstream from the polymorphic PstI site (Figure 4.4). Thus the finding of linkage disequilibrium is in keeping with the close proximity of these three polymorphic sites and provides indirect evidence for a low mutation rate at these sites.

However, linkage disequilibrium has not been previously observed (Miric et al 1992) between two internal polymorphic mutations of the PTH gene designated Mir1 and Mir2 (Figure 4.4). Both Mir1 and Mir2 polymorphisms consist of neutral point mutations within the PTH gene which may be detected using the technique of denaturing gradient gel electrophoresis (DGGE). This analysis relies on the detection of the difference in electrophoretic mobility that the mutation confers upon the amplified PTH gene fragment under denaturing conditions. The Mir1 polymorphism occurs in intron 1, and

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consists of an A to G transition 10 base pairs upstream of exon 2. The Mir2 polymorphism occurs 364 bp downstream in exon 3, and consists of a neutral polymorphism (CGA to AGA) that conserves the arginine residue at codon 52 of the PTH gene. The absence of linkage disequilibrium between two such physically close sites suggests that these sites may be associated with high mutation rates. This complex pattern of linkage disequilibrium at the PTH locus in which some polymorphisms display a random association and are interspersed among those which display significant linkage disequilibrium is analogous to that observed at the Huntingdon disease locus (MacDonald et al 1991). The absence of linkage disequilibrium of the Mir1 and Mir2 polymorphisms does not necessarily imply that it does not exist, but rather that it was not detected. Further combined studies utilising the Mir1, Mir2, (AAAT)\textsubscript{n}, TaqI and PstI polymorphisms associated with the PTH gene will help to elucidate the degree of linkage disequilibrium and mutation rates among these PTH gene polymorphisms.
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Table 3: Linkage disequilibrium for PTH polymorphisms. Chi squared (χ²) values with corresponding P and Δ values obtained for each population (E, European; A, Asian) separately, and for the combined populations, are shown. (From Parkinson et al 1993)
Figure 4.4: Schematic representation of the PTH gene. The 3 exons of the PTH gene are shown as solid boxes and the introns as lines. The polymorphic sites associated with the PTH gene are indicated. The distance between the tetranucleotide \((AAAT)_n\) polymorphism and the polymorphic MIR1 mutation is 231 bp, that between the MIR1 mutation and the TaqI RFLP site is 152 bp, that between the TaqI RFLP site and the polymorphic MIR2 mutation is 212 bp, and that between the MIR2 mutation and the PstI RFLP site is 1821 bp. Linkage disequilibrium between the \((AAAT)_n\), TaqI and PstI polymorphic sites was established.
4.4 Discussion

This study, which has demonstrated an intragenic tetranucleotide polymorphism for the PTH gene, provides an additional useful marker for investigating the molecular pathology of the PTH gene in familial parathyroid disorders. In addition, the detection of linkage disequilibrium between this (AAAT)n polymorphism and the previously reported TaqI and PstI polymorphic sites of the PTH locus will facilitate haplotype analysis at this locus. The identification of a microsatellite polymorphism in the PTH gene which may be detected using the polymerase chain reaction is also of medical importance as it enables investigation of the limited quantities of DNA that are available either from neonates or from post-mortem tissue.
CHAPTER FIVE:

X-LINKED RECESSIVE IDIOPATHIC HYPOPARATHYROIDISM.
5. X-LINKED RECESSIVE IDIOPATHIC HYPOPARATHYROIDISM.

Idiopathic hypoparathyroidism has been reported to occur as an X-linked recessive disorder in two multi-generation kindreds designated P/60 and W/81 (Peden 1960, Whyte et al 1981) from Missouri, USA. Affected individuals, who are all males, suffer from infantile onset of epilepsy and hypocalcaemia due to parathyroid hormone deficiency. In addition, the autopsy of an affected male from pedigree P/60 (Whyte et al 1986) revealed the absence of parathyroid tissue, thereby indicating that there is an isolated congenital defect in parathyroid gland development. The gene causing X-linked recessive hypoparathyroidism has been previously mapped by linkage studies to the long arm of the X-chromosome at Xq26-27 (Thakker et al 1990, Thakker et al 1991), defining the locus order: Xcen-DXS294-F9-(DXS105, CDR)-HPT-DXS98-DXS52-Xqter.

I have combined the use of pulsed field gel electrophoresis physical mapping together with the isolation and investigation of yeast artificial chromosome (YAC) clones for the markers CDR and DXS98 in order to further characterise the region of interest and identify this gene may be involved in parathyroid gland genesis.
5.1 Physical Mapping Studies Using Pulsed Field Gel Electrophoresis (PFGE)

Physical mapping studies (Patterson et al 1987) in the Xq26-Xq27 region using pulsed field gel electrophoresis (PFGE) have indicated physical linkage for the markers DXS105 (CX55.7) and DXS98 (4D.8) on a 400 Kilobase pair DNA fragment generated by partial digestion of human genomic DNA with the rare cutter enzyme SfiI (Patterson et al 1987). From both the genetic linkage analysis and the deletion mapping performed, the order of the two loci DXS105 and CDR with respect to DXS98 cannot be ascertained. Due to the fact that both markers are deleted in the patient Manchester 2, it is therefore of importance to establish whether CDR maps proximal or distal to DXS105, and to further define the physical map of this region.

In order to attempt to determine the locus order, double digests of human genomic DNA from a normal individual were performed with SfiI and one of the following rare cutter restriction enzymes: SalI, SstII (SacII), or BssHII. The resulting fragments were resolved on an 0.8% agarose gel by pulsed field electrophoresis, and transferred to a Hybond-N+ membrane by Southern blotting. The Hybond-N+ filter was then probed with DXS98 (4D.8) and autoradiographed. The hybridised probe was then stripped off the filter, and the filter reautoradiographed to ensure that all the probe had been removed. The filter was then hybridised with CDR in order to identify DNA fragments that hybridise to both probes and establish physical linkage between the 2 loci. Figure 5.1 shows the results obtained for this analysis.

For the probe pCDR1 (CDR locus), hybridisation to the SfiI+ SalI digested DNA revealed bands at approximately 275, 330 and 400 Kbp, and the SfiI+ SstII digested DNA revealed bands at 250 and 300 Kbp. When the filter was rehybridised with the probe 4D.8 (locus DXS98), for the SfiI+ SstII digested DNA, a band of 300 Kbp in size was observed, thus providing preliminary evidence that the CDR locus and the
DXS98 locus are physically linked on a 300 Kbp SfiI/SstII fragment. Hybridisation of the probe CX55.7 (locus DXS105) to this filter was unsuccessful and it can therefore not be determined whether this probe also localises to the same fragment.

This analysis therefore suggests that the maximum physical distance between the flanking loci CDR and DXS98 is 300 Kbp. In order to further investigate the physical distance between these two markers, and to characterise this region of interest, the markers CDR and DXS98 were used to isolate yeast artificial chromosome (YAC) clones.
Figure 5.1: Autoradiographs of pulse field blot hybridised with the probes pCDR1 (locus CDR) and 4D.8 (locus DXS98). Pulsed field gel electrophoresis was performed using a pulse time of 70 seconds at 170 Volts for 31 hours on a 0.8% agarose gel. Both pCDR1 and 4D.8 probes hybridise to an SfiI/SstII fragment of 300 Kbp indicating physical linkage between these two loci at a maximum of 300 Kbp. Previous physical mapping data in this region (Patterson et al 1987) has demonstrated physical linkage of the markers CX55.7 (locus DXS105) and 4D.8 (locus DXS98) on a 400 Kbp SfiI fragment. This data may therefore indicate that the marker CDR is distal to CX55.7, and suggests a 300 Kbp region containing the X-linked recessive hypoparathyroid gene.
5.2 Isolation of Yeast Artificial Chromosome (YAC) Clones for CDR and DXS98

In order to further characterise the region containing the X-linked recessive hypoparathyroid gene, yeast artificial chromosome (YAC) clones for the CDR and DXS98 loci from the ICI human genomic library (Anand et al 1990) were isolated. This library has been constructed from a 48XXXX human lymphoblastoid cell line in the artificial chromosome cloning vector pYAC4, and is thought to represent approximately 3.5 times coverage of the human genome with an average insert size of 350 kilobase pairs. The library consists of approximately 35000 clones which are arranged and stored as glycerol stocks in 96 well microtitre plates. The ICI library has been organized so that it may be screened by using a polymerase chain reaction based strategy. YAC clone DNA from each of the 96 clones on a single microtitre plate is pooled to make a subpool. Nine such subpools are themselves pooled to make a masterpool; each masterpool therefore contains YAC DNA from 864 individual clones.

In order to screen the library, locus specific oligonucleotide primers are used in a polymerase chain reaction assay on DNA from each of the 40 masterpools which constitute the YAC library. Masterpool(s) which are positive may then be identified, and the relevant subpools selected. For the final stage of screening, once the positive subpool has been identified, YAC clone DNA is prepared from the rows (1-12) and the columns (A-H) of the corresponding 96 well microtitre plate. PCR screening of these DNA samples gives the coordinate position on the microtitre plate for the positive YAC clone of interest.

For the CDR locus, primers CDRLEFT and CDRRIGHT were chosen from the published sequence (Dropcho et al 1987) which amplified a 345 base pair fragment from genomic DNA. For the DXS98 locus, the probe used (4D.8) consists of a single copy 1.5 Kbp HindIII fragment for which there is no DNA sequence available. In order to design sequence specific PCR primers to
screen the YAC library, the DNA sequence of approximately 400 base pairs of the 1.5 Kbp probe was determined. Figure 5.2 shows the results of the DNA sequence analysis, together with the sequence of the oligonucleotide primers used (DXS98LEFT and DXS98RIGHT, shown in capital letters) which amplify a 266 base pair fragment from human genomic DNA.

Screening of the ICI library for YAC clones for the loci CDR and DXS98 was performed at the Human Genome Mapping Resource Centre at the Clinical Research Centre in Harrow, UK.
Figure 5.2: Nucleotide sequence of 392 base pairs from the single copy 1.5 Kb HindIII fragment 4D.8 (locus DXS98). The oligonucleotide primers chosen for PCR screening of the ICI yeast artificial chromosome library are shown in capital letters; DXS98LEFT corresponds to nucleotides 11 to 35 of the sequence shown and DXS98RIGHT corresponds to nucleotides 277 to 252. The primers DXS98LEFT and DXS98RIGHT amplify a single 266 base pair PCR product from human genomic DNA.
Screening of the ICI library with the primers CDRLEFT and CDRRIGHT identified a single positive YAC clone (CDRYAC1). Screening of the library with the primers DXS98LEFT and DXS98RIGHT identified five positive YAC clones (DXS98YAC1-5).

The strategy used for the investigation and characterisation of the CDR and DXS98 positive YAC clones is shown in Figure 5.3. Firstly, the YAC clones were investigated using pulsed field gel electrophoresis. Hybridisation of the YAC DNA pulsed field Southern blot with the probes pCDR and 4D.8 was performed in order to confirm that the YAC clones were positive for the CDR and DXS98 loci respectively. In addition, hybridisation with pBR322 plasmid DNA (which anneals to pBR322 sequences in the YAC vector arms, Burke et al 1987) was performed in order to confirm the size of the YAC clones, their stability, and to investigate the possibility of the cotransformation of two distinct YAC clones into the same yeast cell.

Secondly, the technique of Alu-PCR was utilised to fingerprint the YAC clones (Butler et al 1992) in order to investigate the overlap between the CDR and DXS98 positive YAC clones. Thirdly, the vectorette procedure (Riley et al 1990) was used to isolate the terminal sequences from the YAC clones. Establishment of sequence tagged sites or STS’s (Olson et al 1989), and the assignment of their chromosomal location, allows investigation of the YAC clone for chimaerism. The establishment of STS’s from the ends of the YAC clones and their use in rescreening of the YAC library for overlapping clones in order to establish contiguous maps or "contigs" across the region of interest.
Figure 5.3: Strategy used for the investigation of the CDR and DXS98 positive YAC clones.
Yeast cells containing each of the YAC clones were streaked onto SD-agar plates and incubated at 30°C for 2 days. Four individual yeast colonies for each clone were cultured in SD medium (ie. under selection), and agarose plugs containing high molecular weight yeast DNA prepared as described in Materials and Methods. The agarose plugs for each of positive YAC clones were then analysed by pulsed field gel electrophoresis in order to determine their size and stability. The DNA from the pulsed field gel was Southern blotted onto a Hybond-N+ membrane, and hybridised with either the probe 4D.8 (locus DXS98) or pCDR1 (locus CDR); Figure 5.4 shows the results of this analysis.

Hybridisation of the probe 4D.8 to the CDR and DXS98 positive YAC clones demonstrated that the DXS98 locus is not present on the CDRYAC1 clone but confirms that the 5 clones DXS98YAC1 to DXS98YAC5 are all positive for the locus DXS98 and range in size from 125 to 550 Kilobase pairs. For the clone DXS98YAC1, the major band observed is at 550 Kbp but there is also a minor band at 450 Kbp which may indicate a degree of instability in the human DNA insert sequence in this clone. Hybridisation of pCDR1 to the CDR and DXS98 positive YAC clones demonstrated conversely that the CDR locus is not present on any of the DXS98 positive YAC clones, but confirms that the clone CDRYAC1 is positive for the locus CDR. For the clone CDRYAC1, two bands are observed at approximately 400 and 450 Kbp in size, although the intensity of these bands appears to be lower in comparison to that seen with the other YAC clones. In order to investigate this, an identical pulse field blot was prepared using two different preparations of CDRYAC1 agarose blocks and probed with radiolabelled intact pBR322 plasmid which hybridizes to the YAC vector arm sequences (Burke et al 1987). This analysis (Figure 5.5) demonstrated the presence of a much more abundant YAC species of approximately 175 Kbp in size, thereby indicating that the clone CDRYAC1 is unstable and that the most abundant deleted form would appear to arise due to a loss of sequences which include the CDR locus.
Figure 5.4: Autoradiograph of pulsed field blot of CDR and DSX98 positive yeast artificial chromosome clones hybridised with the probes pCDR1 and 4D.8. Pulsed field gel electrophoresis was performed using a pulse time of 40 seconds at 170 Volts for 21 hours on a 1% agarose gel. For both autoradiographs, DNA samples were loaded in the following order: Lane 1: CDRYAC1. Lanes 2 to 6: DXS98YAC1-DXS98YAC5.

A) Autoradiograph of hybridisation of the probe 4D.8 to CDR and DSX98 positive YAC clones reveals no hybridisation to CDRYAC1 (Lane 1) but gives strong hybridisation signals with DXS98YAC1-5 (Lanes 2-6). This analysis allows an estimation of the approximate size for each YAC clone: DXS98YAC1 is 550 Kbp (plus minor band at 450 Kbp), DXS98YAC2 is 125 Kbp, DXS98YAC3 is 350 Kbp, DXS98YAC4 is 250 Kbp and DXS98YAC5 is 200 Kbp in size.

B) Autoradiograph of hybridisation of the probe pCDR1 to CDR and DSX98 positive YAC clones reveals hybridisation to CDRYAC1 (Lane 1) but no hybridisation to DXS98YAC1 to DXS98YAC5 (Lanes 2-6). For CDRYAC1, two bands are observed at approximately 400 and 450 Kbp in size.
Figure 5.5: Autoradiograph of pulsed field blot of duplicate samples of CDRYAC1 yeast artificial chromosome clone DNA hybridised with pBR322 plasmid DNA. Pulsed field gel electrophoresis was performed using a pulse time of 40 seconds at 170 Volts for 21 hours on a 1% agarose gel. For both samples of CDRYAC1 clone DNA feint bands are observed at approximately 400 and 450 Kilobase pairs (Kbp). In addition to these two bands, hybridisation with pBR322 has also revealed the presence of a much more abundant YAC species of 175 Kbp in size, thereby indicating that the clone CDRYAC1 is unstable.
5.3 Alu-PCR Fingerprinting of CDR and DXS98 Positive YAC Clones

The technique of Alu-PCR allows the amplification of regions flanked by Alu repeats in human genomic DNA. Using oligonucleotide primers that correspond to consensus sequences within the Alu repeat, this method may be utilised to "fingerprint" YAC clones and thereby be used to provide an indication of the overlap between YACs without the need to use restriction mapping techniques (Butler et al 1992). The degenerate Alu primers PDJ33 and PDJ34 (Butler et al 1992) were used to Alu-PCR fingerprint the YAC clones CDRYAC1 and DXS98YAC1-5; the results of this analysis are shown in Figure 5.6. Using primer PDJ33 no amplification products are observed for the clone CDRYAC1. This may be due to the absence of Alu repeat sequences within this clone or alternatively the Alu sequences may be too far apart to allow PCR amplification of the intervening sequence. For the 5 DXS98 positive YAC clones (DXS98YAC1-5) complex patterns of PCR amplification products were observed, with multiple common bands between the clones indicating, as would be expected, a considerable degree of overlap. This finding was confirmed using the primer PDJ34, with once more multiple common bands observed for the DXS98 positive YAC clones.

In addition, alu-PCR analysis on the clone CDRYAC1 using primer PDJ34 reveals a band at approximately 600 base pairs which appears to be shared with DXS98YAC1, and would therefore imply that these two YAC clones overlap one another. This finding, however, of an alu-PCR product of the same size for both YAC clones may be coincidental and the possibility of these two clones overlapping to form a contiguous segment needs to be confirmed by another method. In order to investigate this further, the terminal sequences from the CDRYAC1 and DXS98YAC1 clones were isolated using the vectorette system devised by Riley et al (1990), and mapped by somatic cell hybrid analysis.
Figure 5.6: Alu-PCR fingerprint analysis of CDR and DXS98 positive YAC clones using the degenerate Alu consensus primers PDJ33 (lanes 1-6) and PDJ34 (lanes 7-12).

M: Lambda/HindIII,phiX174/HaeIII DNA size markers; C: control. Lane 1: CDRYAC1; Lanes 2-6: DXS98YAC1 to DXS98YAC5; Lane 7: CDRYAC1; Lanes 8-12: DXS98YAC1 to DXS98YAC5.

For both Alu primers PDJ33 and PDJ34, the DXS98 positive YAC clones demonstrate multiple common bands indicating considerable overlap between these clones. For example, with the Alu primer PDJ33, a band of approximately 500 base pairs (indicated by closed triangle) is observed with DXS98YAC1 to DXS98YAC4 (lanes 2 to 5). For the CDRYAC1 clone, PCR amplification with the PDJ34 Alu consensus primer (Lane 7) produces several products, one of which (approximately 600 base pairs in size) appears to be shared with DXS98YAC1 (Lane 8) suggesting possible overlap between these two YAC clones.
5.4 Isolation of Terminal Sequences from YAC clones CDRYAC1 and DXS98YAC1 using the Vectorette Procedure

The vectorette procedure (Riley et al 1990), as described in Materials and Methods, was used to isolate the terminal sequences from the CDR positive YAC (CDRYAC1) and the DXS98 positive YAC (DXS98YAC1). Isolation of the human DNA insert sequences adjacent to the left and right arms of the YAC vector from these clones will allow the establishment of novel sequence tagged sites (Olsen et al 1989), or STS's, which may then be used to rescreen the YAC library for overlapping clones. In addition, the chromosomal localisation of these STS's using somatic cell hybrid cell lines may be used to establish whether a YAC clone is chimaeric due to the coligation of human DNA fragments from different chromosomal locations in the preparation of the library. For the localisation of the sequence tagged sites derived from the YAC clones, three somatic cell hybrids were used: X3000.11 (Nussbaum et al 1986), which contains the Xq24-Xqter region as its only human component on a hamster background, and the cell lines SIN176 (Ingle et al 1985) and CF37 (Mohandas et al 1980) which both contain the long arm of the X-chromosome. Analysis of these somatic cell hybrids (data not shown) by PCR using locus specific oligonucleotides confirmed the presence of the CDR, DXS98 and Factor IX loci in all three cell lines.

The results of the isolation of the terminal sequences from the CDRYAC1 clone using the vectorette procedure are shown in Figure 5.7. For this YAC clone, the PvuII vectorette product from the left arm and the Rsal product from the right arm were investigated further. In order to verify that these PCR products result from amplification of the terminal sequences, the PvuII left and Rsal right products were digested with EcoRI restriction enzyme, which cleaves off the amplified pYAC4 vector sequences (approximately 80 bp), and analysed by agarose gel electrophoresis (data not shown). Both products demonstrated the expected reduction in size of approximately 80 base pairs as compared to undigested product, confirming that PCR amplification
of vectorette products had occurred across the EcoRI cloning site at both ends of the YAC insert. In order to establish sequence tagged sites (STS’s) for each end of this clone, the DNA sequence of the PvuII left and RsaI right PCR products was determined by direct double stranded sequencing using the oligonucleotide 368 (complementary to vectorette sequence) as the sequencing primer. From the DNA sequence derived from the PvuII left vectorette product (approximately 800 base pairs in size), the primers CDRVL1 and CDRVL2 were designed and synthesized which amplify a 175 base pair product from human genomic DNA (data not shown). PCR amplification on genomic DNA extracted from the hybrid cell line X3000.11 (Nussbaum et al 1986), which contains the Xq24-Xqter region as its only human component on a hamster background, localised this sequence tagged site to this part of the X-chromosome (data not shown). In addition, this STS was confirmed to be present in the CDRYAC1 clone, but to not be present in any of the DXS98 positive YAC clones (DXS98YAC1-DXS98YAC5).

For the terminal sequence from the right arm of the CDRYAC1 clone, owing to the proximity of the RsaI restriction enzyme site to the EcoRI cloning site of the human DNA insert, a relatively small vectorette product (approximately 200 base pairs, Figure 5.7) was produced. Direct DNA sequence analysis of the RsaI right vectorette PCR product (of which approximately 80 base pairs is derived from amplification of YAC vector sequences) allowed the design and synthesis of the oligonucleotide primers CDRVR1 and CDRVR2, which amplify a 63 base pair fragment from human genomic DNA. In order to visualise the resulting PCR product the CDRVR1 primer was labelled at its 5’ end with 32P using T4 polynucleotide kinase, the resulting labelled PCR product separated by polyacrylamide gel electrophoresis and visualised by autoradiography.

PCR amplification using the primers CDRVR1 and CDRVR2 on DNA extracted from the X3000.11 hybrid cell line revealed no product, indicating that the sequence tagged site defined by these primers does not map to the Xq24-Xqter region of the X-chromosome (Figure 5.8). In order to confirm this result, the hybrid cell lines SIN176 (Ingle et al 1985) and CF37 (Mohandas et al 1980) which
both contain the long arm of the X chromosome were investigated; PCR analysis using the primers CDRVR1 and CDRVR2 on DNA extracted from these hybrid cell lines also revealed no product. This data would therefore indicate that the YAC clone CDRYAC1 is chimaeric.

In addition, although this STS was found to be present in the CDRYAC1 clone, it was not present in any of the DXS98 positive YAC clones (DXS98YAC1 to DXS98YAC5).
Figure 5.7: Polymerase chain reaction amplification of vectorette libraries constructed from CD2YAC1 using the restriction enzymes Alul, EcoRV, PvuII and Rsal. Amplification products for terminal sequences from both the left (L) and right (R) arm of the YAC clone are shown for each of the four libraries.
Figure 5.8: Localisation by PCR of sequence tagged site derived from right arm terminal sequence of CDRYAC1 clone using oligonucleotide primers CDRVR1 and CDRVR2.

Lanes 1-4: DNA sequence reactions from M13 filamentous phage using universal (-40) sequencing primer, run as a size marker. Lane 5: Control. Lane 6: Mouse genomic DNA; Lane 7: Hamster genomic DNA; Lane 8: Human genomic DNA; Lane 9: X3000.11 Xq24-Xqter hybrid cell line; Lane 10: SIN176 hybrid cell line; Lane 11: CF37 hybrid cell line; Lane 12: CDRYAC1 clone; Lanes 13-17: DXS98YAC1 to DXS98YAC5.

PCR amplification using primers CDRVR1 and CDRVR2, which define a sequence tagged site from the right arm of the YAC clone CDRYAC1 demonstrates that this sequence is not present in the X3000.11, SIN176 or CF37 hybrid cell lines and would therefore indicate that it does not localise to the long arm of the X chromosome.
For the DXS98 positive YAC (DXS98YAC1) the vectorette procedure was used to isolate the terminal sequences from this YAC clone (data not shown). The Rsal vectorette product (approximately 800 bp in size) from the left arm and the AluI product (approximately 350 bp in size) from the right arm of this clone were further investigated. The DNA sequence of the Rsal left and AluI right PCR products was determined by direct double stranded sequencing. From the DNA sequence derived from the Rsal left PCR product, the oligonucleotide primers D98VL1 and D98VL2 were designed and synthesized, which amplify a product of approximately 550 base pairs from human genomic DNA (Figure 5.9). PCR amplification using these primers on DNA extracted from the hybrid cell line X3000.11 (Nussbaum et al 1986), which contains the Xq24-Xqter region as its only human component on a hamster background, localized this STS to this region of the X chromosome (Figure 5.9). In addition, this STS was found to be present in two other of the DXS98 positive YACs (DXS98YAC3 and DXS98YAC4).
Figure 5.9: Genomic localisation by PCR of sequence tagged site derived from left arm terminal sequence from DXS98YAC1 clone using oligonucleotide primers D98VL1 and D98VL2.

**M:** PhiX174/HaeIII DNA size markers; **C:** Control; Lane 1: Hamster DNA; Lane 2: Human genomic DNA; Lane 3: X3000.11 Xq24-Xqter hybrid cell line; Lane 4: CDRYAC1 clone; Lanes 5-9: DXS98YAC1 to DXS98YAC5.

PCR amplification using primers D98VL1 and D98VL2, which define a sequence tagged site (STS) from the left arm of the YAC clone DXS98YAC1, demonstrates the localisation of this STS to the Xq24-Xqter region of the X-chromosome. This STS is also demonstrated to be present on the DXS98 positive YAC clones DXS98YAC3 (Lane 7) and DXS98YAC4 (Lane 8).
For the AluI right vectorette product, the oligonucleotide primers D98VR1 and D98VR2 were designed and synthesized, which amplify a product of 150 base pairs from human genomic DNA. When the primers D98VR1 and D98VR2 were used in a PCR reaction with DNA from the X3000.11 hybrid cell line no amplification was observed, indicating that the STS defined by these primers does not map to the Xq24-Xqter region of the X chromosome. PCR analysis of DNA extracted from the SIN176 and CF37 cell lines also demonstrated no product (data not shown). In addition, although this STS could be mapped back to the YAC clone from which it originated (DXS98YAC1) it is not present in any of the other four DXS98 positive YAC clones (DXS98YAC2 to DXS98YAC5).

This data would therefore suggest that the clone DXS98YAC1 is chimaeric, owing to a coligation event in which sequences from a chromosomal location other than Xq24-Xqter have been juxtaposed with sequences from the region surrounding the locus DXS98.
5.5 Discussion

The results of previous linkage analysis (Thakker et al 1990) have localised the gene causing X-linked recessive idiopathic hypoparathyroidism (HPT) to the long arm of the X chromosome at Xq26-Xq27 and have defined the bridging markers of DXS98 and Factor IX that flank the disease locus. Analysis of the linkage data using the LINKMAP program demonstrated that the odds ratio for a location of the HPT locus distal as opposed to proximal to Factor IX were approximately 17:1, and the likelihood of a proximal as opposed to distal location of HPT to DXS98 as approximately 32:1. All the other odds ratios for possible locations of HPT within the framework of loci examined were less than 1000:1, thus demonstrating that the most likely order of genetic loci was Xcen-DXS37-FactorIX-HPT-DXS98-DXS52-DXS15-Xqter (Thakker et al 1990).

The linkage studies performed with the markers CDR and DXS105 (Thakker et al 1991) proved to be informative and established peak LOD scores of 3.58 for CDR and 7.62 for DXS105 with neither marker showing meiotic recombination with the HPT disease locus, indicating the locus order: Xcen-Factor IX-(CDR,DXS105,HPT)-DXS98-Xqter. These results indicate that the CDR and DXS105 loci map in the Factor IX-DXS98 interval and therefore are valuable markers close to the HPT gene.

Deletion mapping studies in two patients with haemophilia B due to deletions involving the Factor IX locus, Manchester 1 and Manchester 2 (Anson et al 1988), but who did not have the clinical manifestations of hypoparathyroidism, have further helped to characterise the region containing the HPT gene. The finding that for the patient Manchester 2, the DXS105 and CDR loci appeared to have deleted in addition to the Factor IX locus suggests that the HPT gene maps distal to these two markers, indicating the locus order Xcen-FactorIX-(DXS105,CDR)-HPT-DXS98-Xqter. The definition of this order, however, is based upon the assumption that the deletion in the patient Manchester 2 involving Factor IX, DXS105 and CDR is continuous. It is possible, although unlikely, that
whilst the DNA segments comprising the Factor IX, DXS105 and CDR loci may have been lost in this patient, the deletion may have been non-continuous and the HPT gene may have been retained. The physical distance between the markers DXS105 and DXS98 has previously been established by pulsed field gel electrophoresis (Patterson et al. 1987) to be approximately 400 Kilobase pairs (Kbp), and with the assumption that the deletion mapping data is correct, this suggests a 400 Kbp region containing the HPT disease locus. I have performed physical mapping studies using pulsed field gel electrophoresis, and have identified preliminary evidence of physical linkage for the markers CDR and DXS98 on a 300 Kbp DNA fragment. This data may therefore indicate that the marker CDR is proximal to DXS105, which is in agreement with the ordering of the 2 markers on a YAC contig map generated by Schlessinger et al. (1991), and may therefore specify a 300 Kbp region containing the HPT gene.

In order to further characterise the region containing the X-linked recessive hypoparathyroid gene, yeast artificial chromosome clones for the CDR and DXS98 loci were isolated from the ICI human genomic library (Anand et al. 1990). A single clone (CDRYAC1) was isolated for the locus CDR, and five clones (DXS98YAC1 to DXS98YAC5) were isolated for the locus DXS98. Investigation of the CDR positive clone (CDRYAC1) has demonstrated the presence of three distinct YAC species of approximately 450, 400 and 175 Kbp, of which only the 450 and 400 Kbp species appear to contain CDR. For the DXS98 positive YAC clones, these clones ranged in size from 125 to 550 Kilobase pairs, the largest of which (DXS98YAC1) also appears to demonstrate instability with two species of 450 and 550 Kbp being present.

Instability of the human DNA insert in yeast artificial chromosome clones may be identified in several ways, the most obvious of which, as observed here, is the occurrence of several different YAC species in a sample expected to contain a single YAC. This phenomenon could be due to the presence of a mixture of several YAC clones, or the cotransformation of
several YAC clones into the same yeast cell. For each of the CDR and DXS98 positive YAC clones, cultures of yeast cells were prepared from 4 independent colonies, all of which for CDRYAC1 and DXS98YAC1 demonstrated the observed instability, thereby ruling out the possibility of contamination with another YAC clone. The second possibility, that of cotransformation of several YAC clones into the same yeast cell, may be excluded for the DXS98YAC1 clone, as both of the YAC species are DXS98 positive. For the CDRYAC1 clone, of the three species observed, both the 400 and 450 Kbp species are positive for the CDR locus. The 175 Kbp species is negative for CDR and also appears to be present in much greater abundance than the two larger YAC species. Whilst it is possible that there has been a cotransfection of two YACs one of 450 Kbp which is unstable and is deleting a 50 Kbp fragment, and another stable YAC of 175 Kbp which is not CDR positive, it is more likely, given the relative intensity of the YAC species, that the 175 Kbp species is a derivative of the two larger forms of the same YAC clone. Subsequent preparations of the CDRYAC1 clone (D.Trump, personal communication), resolved by pulsed field gel electrophoresis and hybridised with pBR322, have demonstrated the presence of only the 175 Kbp (CDR negative) species, which would appear to confirm this postulate.

The proportion of YAC clones which are unstable may be estimated from two studies carried out by Abidi et al (1990) and Wada et al (1990) which have investigated YAC clones from the Xq24-Xq28 region. In the study performed by Wada et al, out of a total of 127 YACs investigated, only 1 of the clones was found to be unstable. For the study carried out by Abidi et al, two YAC clones out of a total of 467 were found to be unstable (ie. showed multiple bands upon pulsed field gel electrophoresis). The most common cause of the instability of YAC clones appears to be the presence of repetitive DNA sequences in the human insert. For the unstable YACs isolated by Abidi et al (1990), both contained DNA sequences from the colour vision gene region at Xq28, where the genes are
arranged in an approximately 39 Kbp tandemly repeated array. Other studies performed by Neil et al (1990) have analysed YAC clones containing tandem repeat sequences from the Y chromosome (DYZ1 to DYZ5), and have found these YAC clones to be highly unstable. The mechanism by which this instability is thought to occur is by homologous recombination carried out by the yeast cell. If the process of recombination depends upon homology, then the presence of homologous repetitive DNA sequences within a YAC clone would be likely to cause recombination events and instability. Two of the yeast genes that play a role in intrachromosomal recombination are RAD1 and RAD52 (Klein et al 1988), and the transformation of YAC clones into RAD52 deficient yeast strains has been demonstrated to improve the stability of clones containing tandemly repeated DNA sequences (Neil et al 1990).

The library from which the YAC clones for the markers CDR and DXS98 were isolated (Anand et al 1990) has been constructed in the Saccharomyces cerevisiae yeast strain AB1380, in which the RAD1 and RAD52 gene products are both functional; the use of this yeast strain may therefore have contributed to the observed instability of these YAC clones. In addition for the CDR positive YAC clone, the CDR gene sequence, which is 1.15 Kbp in length, contains an 18 nucleotide tandemly repeat sequence which is repeated 34 times in the cDNA sequence (Dropcho et al 1987); the presence of this repeat may therefore have resulted in the instability of this clone and deletion of the CDR sequence.

The physical proximity of the markers CDR and DXS98 (approximately 300 Kilobase pairs), and the finding of YAC clones for each marker both of which are unstable may imply that the same sequences are responsible in both YAC clones for the observed instability.

The method of Alu-PCR has been previously used for the amplification and isolation of human DNA fragments from somatic cell hybrids containing human chromosomes on rodent backgrounds using oligonucleotide primers that are specific for the human Alu repeat sequence (Nelson et al 1989). The use
of Alu-PCR as a means of "fingerprinting" YAC clones was investigated by Butler et al (1992), and found to be a valuable tool in the assessment of overlap between clones. I have used the Alu consensus oligonucleotide primers described by Butler et al (1992) and have derived Alu-PCR "fingerprints" for the CDR and DXS98 positive YAC clones. Use of the PDJ34 (Butler et al 1992) Alu primer appeared to identify a common band shared by the CDR positive YAC clone (CDRYAC1) and the DXS98 positive YAC clone (DXS98YAC1), thereby indicating that there may be a degree of overlap between these two clones.

This possibility was investigated by the use of the vectorette system devised by Riley et al (1990) to isolate the terminal sequences from these two YAC clones. For the CDRYAC1 clone, although a terminal insert fragment of approximately 900 base pairs was isolated from the left arm of the YAC clone, from the right arm a fragment of less than 200 base pairs (of which 81 base pairs is vector arm sequence) was isolated. The use of the vectorette system relies upon the presence of appropriate restriction enzyme sites close to the cloning site of the human DNA insert. The close proximity of the Rsal restriction enzyme site to the end of the human DNA insert results in the isolation of a small amount of insert DNA sequence from this arm of the YAC clone, from which it is difficult to design oligonucleotide primers for localisation of the sequence tagged site and YAC library rescreening.

As an alternative to using a blunt ended vectorette, the annealed oligonucleotides comprising the vectorette may be designed as to give a "sticky end", which would ligate onto YAC insert DNA cleaved with an appropriate restriction enzyme; for instance the enzymes BamHI, BglII, MboI (or its isoschizomer Sau3A) and BclI have different recognition sequences but will all give an identical cohesive "sticky" end for which a vectorette oligonucleotide may be designed. Thus, the combined use of blunt and sticky ended vectorettes may overcome the difficulty of isolating sufficient terminal sequence from YAC clones of interest.

A number of other methods have been developed to isolate
YAC ends such as subcloning the yeast DNA into bacteriophage lambda and screening using vector sequences, or the use of the pBR322-derived sequences in the arms of the YAC vector (Burke et al 1987) for the "plasmid rescue" of the human DNA insert terminal sequences. As an alternative to these cloning approaches, which are time consuming, the techniques of inverse PCR (Ochman et al 1988) or Alu-PCR (using an Alu primer with a primer derived from the vector sequence) may also be used.

The terminal sequence obtained from the right arm of the CDRYAC1 clone, allowed the design of oligonucleotide primers which amplify a 63 base pair product from human genomic DNA. Amplification of such a small PCR product may perhaps be unreliable, and for the purpose of rescreening the YAC library for overlapping clones the isolation of more sequence from the right arm of this YAC clone would be required.

Localisation of the sequence tagged sites derived from the left and right arm terminal sequences of the YACs CDRYAC1 and DXS98YAC1, indicated that both these YAC clones appear to be "chimaeric", due to coligation events that have occurred. The frequency of chimaeric clones in the ICI human genomic library has been estimated to be less than 10% (D.Ogilvie personal communication), which has been largely achieved by the procedure used to construct the library (Anand et al 1990). The partially digested human DNA was first size fractionated using pulsed field gel electrophoresis in order to remove fragments of a size less than 200 Kbp, before being ligated to the dephosphorylated pYAC4 vector which was present in a 20 fold molar excess. This procedure appears to have reduced the frequency of coligation events which for some libraries (for instance that constructed by Brownstein et al 1989) has been estimated (Green et al 1990, Bronson et al 1991) to be between 40 and 60% of the clones analysed. In addition, studies performed by Green et al (1991), which have sought to characterise a coligated clone which contains DNA fragments from chromosomes 7 and 10 have identified an Alu repeat sequence motif at the chimaeric junction between the
chromosome 7 and 10 DNA sequences. This finding indicates that this chimaeric clone has arisen due to a recombination event between two YAC clones transformed into the same yeast cell, and represents another way (although probably much less common than simple coligation) in which chimaeric clones may arise.

The finding that both the CDRYAC1 and DXS98YAC1 clones are seemingly chimaeric, with a comparatively low frequency of chimaeric clones having previously been demonstrated in the ICI library, would appear to be particularly unfortunate. For both of the sequence tagged sites derived from these clones which cannot be localised to the Xq24-Xqter region, the oligonucleotide primers chosen give a strong amplification product from human genomic DNA and yet no product is obtained from three somatic cell hybrid lines, all of which I have characterised as being CDR and DXS98 positive (data not shown). The chromosomal localisation of these sequence tagged sites, by the use of a somatic cell hybrid panel, for these YAC clones will facilitate the characterisation of the origins of the coligated human DNA fragments. In addition, characterisation of the chimaeric junctions for these clones may elucidate whether they have arisen due to a simple coligation or by an homologous recombination event as postulated by Green et al (1991).

Use of the sequence tagged sites derived from both arms of the CDRYAC1 and DXS98YAC1 clones has not identified any overlap between the two YAC clones as was indicated by the Alu-PCR fingerprinting studies. It may be possible that the 2 clones actually do overlap but that due to the coligation event that I propose has occurred for both of these clones, using the vectorette system I cannot isolate sequences that are common to both clones in order to confirm this overlap. The alternative explanation is that the result obtained for the Alu-PCR fingerprinting, with both clones giving a PCR product of the apparent same size, was coincidental and that there is no overlap between these clones.

The studies performed by Schlessinger et al (1991) to assemble YAC contigs in the Xq24-Xq28 region identified a 1.2
Megabase contig comprising 31 YACs containing the markers DXS105 and CDR, and a 2.4 Megabase contig comprising 29 YACs containing the DXS98 locus, and although two separate YAC libraries (Brownstein et al 1989, Little et al 1989) were used for this study the two contigs have not yet been joined together (Schlessinger et al 1991, Schlessinger et al 1993).

The other DXS98 positive YAC clones DXS98YAC2 to DXS98YAC5, which all appear to be stable, should be investigated and sequence tagged sites derived from each end for genomic localisation and library rescreening. In addition to this, Schlessinger et al (1991) have mapped the markers DXS119 and DXS259 in the interval between CDR and DXS98, and these markers should be used to isolate corresponding YAC clones from the ICI library.

Upon the establishment of a YAC contig across the area containing the X-linked recessive hypoparathyroid gene or, if this is not possible, the assembly of YAC clones which map to the correct region, then these YAC clones should be investigated for the presence of coding sequences. A variety of procedures have been used in order to identify coding regions within yeast artificial chromosome clones. The most widely used method is the identification of CpG islands, regions of hypomethylated DNA that are associated with either the 5', or 3' end of genes, or within transcribed regions of DNA (Bird 1987, Gardiner-Garden et al 1987). CpG dinucleotides are under-represented in the genome, and it has been demonstrated (Larsen et al 1992a) that all housekeeping and widely expressed genes investigated have a CpG island that covers at least the start of the transcription unit. For genes which demonstrate tissue specific or limited expression, it has been estimated (Larsen et al 1992a) that approximately 40% are associated with a CpG island. The identification of CpG islands within YAC clones is facilitated by the construction of restriction maps of the YAC clone using rare cutter restriction enzymes which contain one or more CpG residues in their cleavage recognition sequence. The clustering of two or more restriction enzyme sites for rare cutter enzymes such as
ToI, AsCl, BssHII, RsrII, EagI or SacII is highly indicative of the presence of a CpG island (Larsen et al 1992b). The potential use of the presence of CpG islands in order to identify novel genes was demonstrated in the human major histocompatibility complex (MHC) class III region (Sargent et al 1989). Rare cutter restriction mapping of cosmid clones from this region, using the enzymes BssHII, EagI and SacII, identified the presence of 18 putative CpG islands. Isolation of the DNA sequences flanking these CpG islands, and their use for hybridisation to Northern blots of RNA isolated from various cell lines, identified the transcripts from 12 novel single copy genes.

Therefore, if a putative CpG island has been identified in a YAC clone, the YAC DNA may then be digested with the appropriate rare cutter (eg BssHII) plus a frequent cutter such as Sau3A and the DNA fragment(s) surrounding the CpG island subcloned and investigated, either by DNA sequence analysis to look for the presence of open reading frames, or used as a probe for screening appropriate complementary DNA (cDNA) libraries.

As an alternative to this approach, which does not rely upon the gene sequence being associated with a CpG island, the whole YAC (after gel purification by pulsed field gel electrophoresis) may be used as a probe for hybridising to cDNA libraries to identify expressed transcripts within the YAC clone. This may be achieved in two ways, either by conventional hybridisation to cDNA library clones on nitrocellulose filters (Elvin et al 1990), or by using the PCR based technique of "cDNA selection" (Lovett et al 1991, Parimoo et al 1991) which has recently been used by Vetrie et al (1993) to identify the transcript involved in X-linked agammaglobulinaemia. In addition, the YAC clone may be used to screen a cosmid library in order to try and establish a cosmid contig over the region encompassed by the YAC clone. For YAC clones which are chimaeric, as it is unlikely that the coligated human insert DNA fragments are from the same chromosome, the use of a chromosome specific cosmid library
(eg. X chromosome) would allow the isolation of cosmid clones from the region of interest. These cosmid clones may then be used to screen, by hybridisation, cDNA libraries.

Hybridisation based techniques, however, rely upon the expression of the desired cDNA in the library which is being investigated, and several libraries may therefore need to be examined. In addition, it may well be the case that for the gene causing X-linked recessive hypoparathyroidism, expression of the cDNA may not only be highly tissue specific but it may also only have a very narrow window of expression during which the gene product is involved in the development of the parathyroid glands. In order to overcome this problem, if coding sequences within the YAC cannot be identified by the presence of CpG islands or recovered from cDNA libraries, then the YAC may be subcloned and techniques such as exon trapping (Duyk et al 1990) or species conservation of DNA sequences (zoo blots) used to identify candidate cDNA sequences. Once candidate cDNA sequences have been identified, they should then be examined for their pattern of expression and in pedigrees P/60 and W/81 for abnormalities (insertions, deletions or point mutations) that may be associated with this endocrine disorder.
CHAPTER SIX

MOLECULAR MAPPING OF THE MOUSE CALBINDIN D9K GENE USING AN INTERSPECIFIC BACKCROSS.
6. MOLECULAR MAPPING OF THE MOUSE CALBINDIN D9K GENE USING AN INTERSPECIFIC BACKCROSS.

The vitamin D-dependent calcium-binding protein, calbindin D9K, is a cytosolic protein of molecular weight 9 KiloDaltons. Calbindin D9K belongs to a family of calcium binding proteins which includes such proteins as calmodulin, parvalbumin, troponin C, and S100 protein (Wasserman et al 1983, Jeung et al 1992). Analysis of the calbindin protein (Szebenyi et al 1981) has demonstrated the presence of two helix-loop-helix domains or "EF"-hands (Tufty et al 1975) which constitute the two high affinity calcium binding sites of the protein.

The calbindin protein is expressed in the mammalian intestine (Davie 1981, Leonard et al 1984), kidney (Delorme et al 1983a) placenta (MacManus et al 1986) and uterus (Delorme et al 1983b). The calbindin D9K gene has been demonstrated to be under the transcriptional control of 1,25(OH)₂ vitamin D₃ in mammalian intestine (Leonard et al 1984), and expression of the calbindin has been shown to correlate with calcium transport activity in the small intestine (Bruns et al 1987). Investigation of the promoter region of the rat calbindin gene (Darwish et al 1992) has identified a vitamin D responsive element approximately 450 base pairs upstream from the startpoint of transcription of the gene.

The amino acid sequence for the rat (MacManus et al 1986) and murine (Hunt et al 1989) calbindin D9K proteins have been determined, and the two proteins demonstrate 95% homology to one another (Hunt et al 1989). In addition, the rat cDNA (Darwish et al 1987) and genomic sequence (Krisinger et al 1988) have been determined. The human calbindin D9K cDNA sequence has recently been isolated by Howard et al (1992), and the cDNA localised, by somatic cell hybrid analysis, to the short arm of the X chromosome.

Due to the high homology between the mouse and rat calbindin D9K proteins (Hunt et al 1989), and availability of the rat cDNA (Darwish et al 1987) and genomic (Krisinger et al
1988) nucleotide sequences, the rat calbindin D9K cDNA has been used to map the homologous gene on the mouse X chromosome using an interspecific backcross segregating for hyp (Kay et al 1991), the putative murine homologue of HYP (Eicher et al 1976). These molecular mapping studies of the calbindin D9K gene demonstrated that it localises to the region of the hyp locus on the mouse X-chromosome, and the calbindin D9K gene has therefore been investigated for abnormalities that may be associated with the hyp phenotype.
Preliminary mapping studies (Howard et al 1992) have localised the human calbindin D9K gene to the short arm of the human X chromosome. In order to establish a more precise localisation, a panel of human X chromosome somatic cell hybrids was utilised (Figure 6.1). The oligonucleotide primers HCALGL (cDNA nucleotide position 13 to 37, Howard et al 1992), and HCALGR (cDNA nucleotide position 139 to 115) were synthesized, which amplify a single product of approximately 900 base pairs from human genomic DNA (data not shown). The results for mapping of the human calbindin gene by somatic cell hybrid analysis are shown in Figure 6.1.

Analysis of the results obtained demonstrate that the human calbindin D9K gene maps to the region of the X-chromosome (Xp) containing the HYP locus.
Figure 6.1: Localisation of the human calbindin D9K gene by analysis of the somatic cell hybrids 1W1LA4.9, 835, Sin176 and CF37. PCR amplification of human calbindin D9K nucleotide sequence revealed the presence (+) or absence (−) of the gene sequence in the somatic cell hybrids shown. Analysis of the results obtained demonstrates that the human calbindin D9K gene maps to the region of the human X chromosome containing the HYP locus.
6.2 Isolation of the Rat Calbindin D9K cDNA

Total RNA was extracted from rat kidney by the guanidinium thiocyanate method (Chomczynski et al 1987) as described in Materials and Methods. The oligonucleotide, CALRATR, which is complementary to the 3' domain of the rat calbindin D9K cDNA (nucleotide position 383 to 359, Darwish et al 1987) was utilised to synthesize a specific first strand cDNA copy of the rat cDNA as described in Materials and Methods. The yield of reverse transcribed rat calbindin D9K cDNA was amplified by the polymerase chain reaction using the oligonucleotide primers CALRATL (nucleotide position 12 to 34, Darwish et al 1987) and CALRATR, generating a product of 347 base pairs. This product was not present when reverse transcriptase was omitted from the cDNA synthesis reaction or when only rat genomic DNA or a water blank were used (data not shown), therefore demonstrating that this product was not due to amplification of a genomic sequence but is RNA specific.

The 347 base pair product was gel purified and ligated into a Bluescript vector modified for use as a T-vector (Marchuk et al 1991). Sequence analysis, and comparison to the published sequence (Darwish et al 1987, Krisinger et al 1988), confirmed that the amplified PCR product was derived from the rat calbindin D9K mRNA.
6.3 Localisation of Calbindin D9K on the Mouse X-Chromosome using an Interspecific Backcross Segregating for hyp

The interspecific backcross established by Kay et al (1991) was utilised in order to determine the position of the calbindin D9K gene on the mouse X chromosome with respect to the hyp locus. The backcross was established using 2 evolutionary divergent mouse species, *Mus musculus domesticus* and *Mus spretus*, and in order to investigate recombination events and determine the molecular map position for a marker, a restriction fragment length variant or RFLV between the two mouse species for the calbindin cDNA probe was sought.

*Mus musculus domesticus* and *Mus spretus* mouse genomic DNA was digested with the following enzymes: TaqI, MspI, PstI, PvuII, EcoRI, EcoRV, HindIII, BglII and BamHI. The digested DNA fragments were resolved by agarose gel electrophoresis and transferred to a nylon membrane by Southern blotting. The "polyblot" was then hybridised with the rat calbindin cDNA probe, and autoradiographed. Analysis of the results obtained revealed an RFLV between *Mus musculus domesticus* and *Mus spretus* with the enzyme TaqI, with allele sizes of 2.2 and 1.9 Kilobases respectively (Figure 6.2).

Genomic DNA for male and female progeny from the interspecific backcross was digested with TaqI and Southern blots of the digested DNA prepared. The blots were hybridised with the rat calbindin cDNA, as described in Materials and Methods, and autoradiographed. An example of the results obtained for this analysis of the male progeny from the interspecific backcross is shown in Figure 6.2.
Figure 6.2: Autoradiograph of Southern blot of TaqI digested male backcross progeny DNA hybridised with rat calbindin cDNA to reveal *Mus musculus domesticus* and *Mus spretus* RFLV alleles. The mouse backcross number is shown above the revealed *domesticus* (size 2.2 Kb) or *spretus* (1.9 Kb) allele. D: Control *Mus musculus domesticus* DNA; S: Control *Mus spretus* DNA. The presence of a *domesticus* or *spretus* allele for each animal from the backcross panel was therefore ascertained for segregation analysis and localisation of the calbindin gene on the mouse X chromosome with respect to the hyp locus.
The results obtained for the scoring of the key male and female backcross progeny are shown in Figures 6.3 and 6.4 respectively. For the male mice, there are recombination events between calbindin and the pgk-1 and DXSmh43 loci thereby indicating a distal location of calbindin to these two markers. In addition, recombination is observed between the Amg locus and calbindin, demonstrating that calbindin is proximal with respect to the Amg locus. For calbindin and the marker Cbx-rsl, there are no recombination events between these two markers and thus they cannot be ordered. The localisation of the calbindin gene with respect to the hyp locus, however, demonstrates that two mice (backcross numbers 22 and 24) are recombinant between the two markers.

Analysis of the female progeny (Figure 6.4) confirms the localisation of the calbindin gene distal to DXSmh43 and proximal to Amg. A recombination event between calbindin and Cbx-rsl (mouse backcross number 187) allows the localisation of calbindin proximal to Cbx-rsl.

Thus, the combined male and female results of the mapping of the calbindin gene using the interspecific backcross segregating for hyp indicate the locus order:

pgkl - DXSmh43 - hyp - Calbindin - Cbxrs1 - Amg.
Figure 6.3: Molecular mapping of the calbindin D9K gene on the mouse X chromosome in male progeny of the interspecific backcross segregating for hyp. The results obtained for haplotype analysis with the calbindin cDNA probe for key male mice, denoted as their backcross mouse number, together with the loci pgk-1, DXSmh43, hyp, Cbx-rs1 and Amg (Kay et al 1991) are shown. Analysis of recombination events allows the localisation of calbindin distal to DXSmh43 and proximal to Amg. There are no recombination events between calbindin and Cbx-rs1, and thus the order of the two markers cannot be ascertained. Localisation of the calbindin gene with respect to hyp demonstrates two recombinants (backcross mouse numbers 22 and 24).
Figure 6.4: Molecular mapping of the calbindin D9K gene on the mouse X chromosome in female progeny of the interspecific backcross. The results obtained for haplotype analysis using the calbindin cDNA probe for key female mice, denoted as their backcross mouse number, together with the loci pgk-1, DXSmh43, Cbx-rs1 and Amg (Kay et al 1991) are shown. Analysis of recombination events confirms the localisation of calbindin distal to DXSmh43 and proximal to Amg. Recombination between calbindin and Cbx-rs1 (backcross mouse number 187) allows the order of the two loci to be ascertained, with calbindin mapping proximal to Cbx-rs1. The hyp phenotype could not be ascertained unequivocally in female mice, and this is therefore shown as blank (-).
The use of the interspecific backcross, mapping the calbindin D9K gene proximal to the Cbx-rsl locus, demonstrates that calbindin is the closest identified distal marker to the hyp locus on the mouse X chromosome.

The finding of recombination between the calbindin gene and the hyp locus does not necessarily exclude an association between the two loci. Although the biochemical analysis of control domesticus mice demonstrated (Eicher et al 1976, Kay et al 1991) that the hyp mutation resulted in significant hypophosphataemia (and raised alkaline phosphatase activity) in both males and females, it may still be possible for diagnostic confusion in the progeny from the backcross. Thus the calbindin D9K gene may represent a candidate locus for the hyp mutation and the sequence of the calbindin D9K gene in the hyp mouse therefore investigated.

6.4 Isolation of the Normal Mouse Calbindin D9K cDNA Sequence

The 347 base pair rat calbindin D9K cDNA fragment was used to screen, by hybridisation, a mouse 17.5 day (post coitum) embryonic cDNA library constructed in the vector λgt10 (see Materials and Methods for details of library). Approximately 1 million bacteriophage plaques were screened and a single positive clone identified. The cDNA insert was cloned into the plasmid vector Bluescript, and the DNA sequence determined.

Figure 6.5 shows the nucleotide sequence of the mouse calbindin D9K complementary DNA clone. The cDNA is 428 base pairs in length, and contains an open reading frame of 237 nucleotides encoding a peptide of 79 amino acids. The initiation codon (ATG) is at nucleotide 60 and the opel termination codon (TGA) is at nucleotide 297. In the coding region, the mouse calbindin D9K demonstrates 92% (220/237) nucleotide sequence homology to the published rat cDNA sequence (Darwish et al 1987). The mouse calbindin D9K peptide sequence is 94% (73/78) homologous to the rat peptide (Darwish
Figure 6.5: Nucleotide sequence of the 428 base pair mouse calbindin D9K cDNA. The derived amino acid sequence, in single letter code, is shown below the nucleotide sequence. The cDNA clone isolated contained a 59 base pair 5' untranslated region, a 237 base pair open reading frame encoding the 79 amino acid calbindin protein, and a 128 base pair 3' untranslated region. A putative polyadenylation signal (AATAAA) is present at nucleotides 395-400. A poly(A) tract of 35 adenosine residues was present at the 3' end of the sequence shown. Alternative splicing of the calbindin D9K mRNA (see section 6.5) results in the insertion of an additional CAG triplet, encoding the amino acid glutamine, between residues 194 and 195 of the sequence shown.
6.5 Investigation of the Calbindin D9K Gene in the hyp Mouse

Although Southern blot analysis of hyp mice in the interspecific backcross revealed no abnormalities, for example rearrangements, at the calbindin gene locus, the coding sequence of the gene should still be investigated for mutations that may be associated with the hyp phenotype.

Total kidney RNA was extracted from both a hyp and a normal control Mus musculus domesticus mouse by the guanidinium thiocyanate method (Chomczynski et al 1987). The oligonucleotide primer, MCALCR, complementary to the 3' end of the mouse calbindin D9K cDNA sequence (nucleotide position 341 to 318, Figure 6.5), was used to synthesize a specific first strand cDNA copy of the mouse calbindin mRNA. The yield of the cDNA was amplified by the polymerase chain reaction using the oligonucleotide primers MCALCL (nucleotide position 13 to 38, Figure 6.5) and MCALCR, as described in Materials and Methods.

Agarose gel electrophoresis of the resulting PCR product derived from both the normal and hyp mouse revealed the expected product of approximately 305 base pairs (data not shown), indicating that there was no gross abnormality in splicing of the calbindin D9K mRNA which may be associated with the hyp phenotype.

The nucleotide sequence of the amplified calbindin cDNA derived from the normal and hyp mice was determined by direct double stranded sequencing, using the oligonucleotides MCALCL and MCALCR as sequencing primers. Analysis of the results revealed no differences between the normal and hyp calbindin cDNA sequences.

However, for direct sequence analysis of both the normal and hyp calbindin cDNA's, using the oligonucleotide MCALCL, in the 3' direction from nucleotide 194 (Figure 6.5) onwards, a sequence pattern was observed indicating the presence of 2 cDNA species which differed by 3 base pairs in size. Conversely, direct sequence analysis of both the normal and hyp calbindin cDNA's, using the oligonucleotide MCALCR, in the 5' direction from nucleotide 195 (Figure 6.5) onwards, the
same pattern was observed. These findings indicated that there were two forms of the calbindin cDNA, and thus mRNA, present in both the normal and hyp mouse kidney which differed by 3 base pairs in size.

In order to investigate whether this was the case, and to determine whether both forms of the cDNA were present in equal amounts in the normal and hyp mouse kidney RNA, the oligonucleotide MCALCR was labelled at its 5’ end using T4 polynucleotide kinase, the calbindin cDNA species amplified from both normal and the hyp mouse, and the PCR products resolved by polyacrylamide gel electrophoresis. The results of this analysis, (Figure 6.6) demonstrates, as postulated, that there are two species of calbindin D9K mRNA, giving PCR products of 305 (cDNA1) and 308 (cDNA2) base pairs in size.
Figure 6.6: Identification of two forms of calbindin D9K cDNA in kidney RNA from normal and hyp Mus musculus domesticus mice. The autoradiograph shows PCR amplification of the two species of calbindin D9K cDNA synthesized by the addition (+) of reverse transcriptase to RNA extracted from normal (N) and hyp (H) mouse kidney. The two PCR products were not present when reverse transcriptase was omitted from the reaction (−), or when mouse genomic DNA (G) or a water blank (C) were used, thereby demonstrating that this product is not due to amplification of a genomic sequence and is therefore RNA specific. Analysis of the two amplified calbindin cDNA species demonstrates that they are 305 (cDNA1) and 308 (cDNA2) base pairs in length and thus differ by 3 base pairs in size.
In order to characterise the sequences of the two forms of calbindin D9K mRNA, the amplified calbindin cDNA PCR product from the normal mouse kidney RNA was ligated into a Bluescript T-vector, and the sequence of the cDNA insert from four independent clones determined. Figure 6.7 shows the DNA sequence obtained for two clones, cDNA1 and cDNA2 corresponding to nucleotides 186 to 203 for the calbindin cDNA clone isolated from the mouse embryonic library. Analysis of the sequence for the two clones demonstrates that there is an additional CAG codon, which encodes the amino acid glutamine (cDNA2, Figure 6.7), between nucleotides 194 and 195 (Figure 6.5) of the mouse calbindin cDNA sequence, thereby demonstrating the nucleotide sequence basis for the two observed calbindin D9K cDNA species.
Figure 6.7: Autoradiograph of sequencing gel showing the region of nucleotide sequence difference between the two forms of mouse calbindin D9K cDNA. The nucleotide sequence together with the predicted amino acid sequence are shown. Analysis of the sequence of the 2 cDNA clones reveals an additional CAG triplet (which encodes glutamine) between nucleotides 194 and 195 (Figure 6.5) as the nucleotide sequence basis for the two observed forms of mouse calbindin D9K cDNA.
Hunt et al (1989) previously reported, using the technique of tandem mass spectrometry, the identification of two calbindin D9K isoforms from mouse intestine, which differed by one amino acid in size. Amino acid sequence analysis of the two isoforms indicated, as I have demonstrated for the mouse kidney calbindin D9K cDNA, that the two protein species arose due to the insertion of a glutamine residue between Lys-45 and Ala-46 (Figure 6.5). In addition reverse-phase high performance liquid chromatography analysis of the two calbindin protein isoforms demonstrated that the ratio of the smaller form to the larger form was approximately 2:1, which may appear to correlate with the ratio of the two cDNA molecules that I have observed (Figure 6.6), although autodensitometry of the autoradiograph would clearly be required in order to substantiate this proposal.

There are two possible mechanisms by which the presence of two calbindin D9K mRNA species could arise. The first possibility is that there are two mouse calbindin D9K genes in the mouse genome. However, Southern blot analysis that I have performed on the mouse backcross, and data from Krisinger et al (1988) for the rat gene, strongly indicates that the mouse and rat calbindin D9K genes are represented as a single copy.

The second mechanism by which the two species of calbindin D9K mRNA species could arise would be by alternative splicing of a single gene mRNA product.

Hunt et al (1989) postulated that due to the fact that the inserted glutamine was located at the position of an intron (intron 2) of the rat gene (Krisinger et al 1988), the two isoforms of calbindin D9K arose by alternative splicing. The presence of two forms of rat calbindin D9K protein have been reported (MacManus et al 1986) but there is however no published direct evidence for alternative splicing of the rat calbindin D9K mRNA. Analysis of the rat intron 2/ exon 3 boundary of the calbindin D9K gene demonstrates the sequence shown in Figure 6.8.

Hunt et al proposed that the two forms of calbindin mRNA, and thus protein, arose due to the presence of two putative
splice acceptor (nucleotide sequence AG, shown in bold, Figure 6.8) sites (Mount et al 1982) at the intron 2/exon 3 boundary of the calbindin gene.

In order to investigate this possibility in the mouse calbindin gene, the oligonucleotide primers HLEFT (nucleotide position 153 to 176, Figure 6.5) and HRIGHT (nucleotide position 247 to 224) which by comparison to the rat genomic sequence (Krisinger et al 1988) flank intron 2 of the mouse calbindin D9K gene, were synthesized. HLEFT and HRIGHT were used to amplify, by the polymerase chain reaction, a product of approximately 2 Kilobases encompassing the exon 2/intron 2 boundary, intron 2 and the intron 2/exon 3 boundary sequences from a normal control Mus musculus domesticus mouse (data not shown).

Analysis of the intron 2/exon 3 boundary nucleotide sequence (Figure 6.8) demonstrates, as for the rat sequence, the presence of two putative splice acceptor dinucleotide consensus sequences at the intron/exon boundary. It is therefore reasonable to conclude that the two forms of mouse calbindin D9K mRNA and protein arise due to the utilisation of both donor acceptor sites in the post-transcriptional splicing of the calbindin mRNA precursor.

MOUSE 5' g t c t a a a a g C A G G C T T C A 3'

RAT 5' g t c t g g a a g C A G G C T T C A 3'

Figure 6.8: Intron 2/exon 3 nucleotide sequence of mouse and rat calbindin D9K genes demonstrating the presence of two putative splice acceptor "ag" dinucleotide consensus sequences (shown in bold).
6.6 Discussion

The use of an interspecific backcross segregating for hyp has allowed the localisation of the mouse calbindin D9K gene to the distal region of the hyp locus on the mouse X chromosome. Investigation of the mouse calbindin D9K gene for mutations that may be associated with the hyp phenotype has not identified any abnormalities in the coding sequence of the gene. However, mapping of the second X-linked dominant mutation (Lyon et al 1986), gyro (gene symbol gy), which causes hypophosphataemia, demonstrated a single recombination event between the hyp and gy genes indicating that they are separate, but apparently tightly linked (crossover value 0.4-0.8%) loci. The role of the calbindin D9K gene in the gyro mouse therefore requires investigation. Preliminary investigation of the calbindin D9K gene in the gyro mouse by Southern blot analysis (data not shown) has revealed no gross rearrangement or deletion, although the gene should be investigated further for abnormalities that may be associated with the gyro phenotype.

Isolation of the mouse calbindin D9K cDNA and the localisation of the gene as the closest distal marker to the hyp locus has allowed the further definition of the molecular genetic map of this region surrounding the hyp gene on the mouse X chromosome. The mouse calbindin D9K cDNA sequence may now be utilised for screening mouse genomic DNA yeast artificial chromosome libraries, such as the library constructed by Chartier et al (1992), in order to attempt to further characterise this region.

In addition, characterisation of the calbindin D9K cDNA from both the normal and hyp mice has demonstrated that there is alternative splicing of the mRNA transcript in which two forms of calbindin D9K mRNA, differing by 3 base pairs in size, are produced. Investigation of the cause of this alternative splicing has indicated that it is due to the presence of two putative splice acceptor consensus sequences, which are conserved in both rat and mouse, at the intron
2/exon 3 boundary of the calbindin gene. Analysis of the consequences of this alternative splicing shows that it results, for one of the two isoforms of the protein, in the insertion of a glutamine amino acid residue between the two helix-loop-helix calcium binding domains of the protein. The functional significance of this insertion between the two calcium binding domains of the protein remains to be elucidated.
7. SUMMARY.

This thesis presented the results obtained for the molecular genetic analysis of the disorders of familial hypoparathyroidism and X-linked hypophosphataemic rickets. The investigation of the parathyroid hormone gene in four pedigrees with autosomal idiopathic hypoparathyroidism has identified a donor splice site mutation as the cause of hypoparathyroidism in one of these pedigrees (A/89), whilst segregation analysis and DNA sequence analysis has excluded the PTH locus in the remaining three pedigrees. The identification of other loci, which may be involved in the embryological development, cellular composition or homeostatic regulation of the parathyroid glands, and their investigation in these, and other, pedigrees with idiopathic hypoparathyroidism will facilitate the understanding of the molecular pathology of this endocrine disorder.

For the X-linked recessive form of idiopathic hypoparathyroidism, linkage analysis, together with deletion mapping data, has localised the gene to the long arm of the X-chromosome at Xq26-27, and flanking markers for the gene have been established. The attempt to construct a contiguous segment, or contig, between these flanking markers using yeast artificial chromosomes (YAC's) has, as yet, proved unsuccessful, although the identification of more markers in this region, together with the screening of other YAC libraries, should accomplish this objective.

The mapping of the mouse calbindin D9K gene using an interspecific backcross to the region of the hyp locus on the mouse X-chromosome has provided a new marker in this area. Recombination between the hyp and the calbindin D9K loci, together with sequence analysis of the calbindin D9K gene in the hyp mouse, would appear to exclude an association between this gene and the hyp phenotype.
For both X-linked recessive hypoparathyroidism and X-linked hypophosphataemic rickets genes, the application of positional cloning strategies have not yet led to the identification of the genes involved in these disorders. However, the success of positional cloning approaches in identifying many of the genes that are the cause of inherited human disease provides encouragement, if encouragement is needed, that these genes will be identified.
8. REFERENCES


APPENDIX I: Oligonucleotide Sequences

The nucleotide sequence of the oligonucleotide primers used is given below in 5' to 3' orientation. Where appropriate, nucleotide sequence degeneracy is shown. For primers that have been used in the polymerase chain reaction, the annealing temperature ($T_m$ °C) and the magnesium concentration ([Mg]) of the buffer used in the PCR reaction is given.

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APPENDIX II: Abbreviations Used.

Nucleotides.
A : adenine  ATP : adenosine triphosphate
C : cytosine  CTP : cytidine triphosphate
G : guanine  GTP : guanosine triphosphate
T : thymine  TTP : thymidine triphosphate
NTP  : any nucleotide
dNTP : dideoxynucleotide
ddNTP: dideoxynucleotide

Amino acids.

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APPENDIX III: Units of Measurement.

Prefix:
K : kilo (10³)
m : milli (10⁻³)
µ : micro (10⁻⁶)
n : nanno (10⁻⁹)
p : pico (10⁻¹²)

g : gram
M : Mole
°C : Degrees Centigrade
V : Volt
Ci : Curie
m : metre.
A donor splice site mutation in the parathyroid hormone gene is associated with autosomal recessive hypoparathyroidism

David B. Parkinson & Rajesh V. Thakker

Investigation of one kindred with autosomal recessive isolated hypoparathyroidism, which had resulted from a consanguineous marriage, has identified a g to c substitution in the first nucleotide of intron 2 of the parathyroid hormone (PTH) gene. This donor splice mutation could be detected by restriction enzyme cleavage with Ddel, and this revealed that the patients were homozygous for the mutant alleles, the unaffected relatives were heterozygous, and unrelated normals were homozygous for the wild type alleles. Defects in messenger RNA splicing were investigated by the detection of illegitimate transcription of the PTH gene in lymphoblastoid cells. The mutation resulted in exon skipping with a loss of exon 2, which encodes the initiation codon and the signal peptide, thereby causing parathyroid hormone deficiency.
the invariant gt dinucleotide of the donor splice site sequence\(^9\) (gtaa) and produced a recognition site (ctaa) for the restriction endonuclease Ddel. This facilitated the detection of this donor splice site mutation in the other members of family A/89 (Fig. 1). The patients (IV.1, IV.3 and IV.4) were homozygous for the mutant allele (ctaa) and ten unrelated normal individuals were homozygous for the wild type donor splice site sequence (gtaa). The unaffected members of family A/89 were heterozygous for the mutant and wild type alleles. Thus, a donor splice site mutation detected by Ddel, within the PTH gene is segregating with hypoparathyroidism.

**PTH mRNA splicing defects**

This donor splice site mutation at the exon 2-intron 2 boundary of the PTH gene indicated that hypoparathyroidism may result from abnormal PTH mRNA splicing. Parathyroid cells were not available for studies from these patients, hence we investigated PTH mRNA processing by detection of illegitimate transcription of the PTH gene in EBV-transformed lymphocytes (Fig. 2). The results revealed a PTH cDNA of 258 base pairs (bp) from the normals and DNA sequence analysis of this revealed correct splicing with the order exon 1-exon 2-exon 3. However, an abnormal PTH cDNA of 168 bp was detected from the patients IV.1, IV.3 and IV.4, all of whom are homozygous for the g to c donor splice site mutation. The mutant PTH cDNA differed from the normal by 90 bp, which corresponds to the size of exon 2. Both the normal and mutant PTH cDNA fragments were obtained from the heterozygous parents (III.1 and III.2) and sibling (IV.2).

To further characterize these abnormalities the nucleotide sequence of the PTH cDNA from patient IV.1 was determined (Fig. 5). This revealed exon skipping in which exon 2 was lost and exon 1 was spliced to exon 3. The loss of exon 2 removed the initiation codon (ATG) and the region encoding the 25 amino acid signal peptide. These losses will respectively prevent the commencement of PTH mRNA translation\(^9\) to the pre-pro-PTH protein and the translocation\(^9\) of any PTH peptide through the endoplasmic reticulum prior to secretion. The possible alternative use of the ATG codons at the 3' terminus of exon 1 (Fig. 5) and at codon 20 of exon 3 (data not shown) for initiation of translation\(^9\) would yield a protein lacking the signal peptide, which would prevent its translocation.

**Discussion**

Demonstration of illegitimate transcription is medically important as it allows the use of easily accessible peripheral blood lymphocytes for the detection of abnormalities in mRNA processing and thereby avoids the requirement for tissue that may only be obtainable by biopsy. Parathyroid hormone gene expression is usually confined to the parathyroid glands and our results, which reveal transcription of the PTH gene together with correct splicing of the PTH mRNA in cultured lymphocytes from normal individuals, represent the first demonstration of our knowledge of illegitimate transcription of the PTH gene.

Our investigation of patients with autosomal recessive hypoparathyroidism has identified a single base substitution (g to c) in the 5' donor splice site\(^9\) consensus sequence, gtaa. Previous studies\(^9\) have revealed that mutations in the 5' donor splice site regions are associated with an accumulation of unspliced precursor mRNA, or retention of incompletely spliced precursors; or complete
absence of transcripts; or the appearance of aberrantly processed mRNA (from the use of alternative normally occurring splicing sites or cryptic splice sites). In vitro studies using the human adenovirus late transcription unit\(^2\) and the rat preprotachykinin gene\(^3\) have demonstrated that alterations at an internal exon–intron boundary result in exon skipping. Our investigation of these possibilities in hypoparathyroid patients who had inherited a 5' donor splice site mutation of the PTH gene demonstrated that this mutation was associated with exon skipping. The loss of exon 2 in the abnormal mRNA transcript removed the initiation codon and the signal peptide sequence that are required respectively for the commencement of PTH mRNA translation\(^4\) and translocation\(^5\) of the PTH peptide. Thus, our findings have defined the molecular pathology of the PTH gene that causes autosomal recessive isolated hypoparathyroidism in this family. Our demonstration of illegitimate transcription of the PTH gene in cultured lymphocytes opens the way to elucidate further defects in the processing of PTH mRNA that may cause disorders of parathyroid activity.

**Methodology**

**Family and subjects.** One family (A/89 in Fig.1), of Bangladeshi-Asian origin with autosomal recessive isolated hypoparathyroidism (following a consanguineous marriage) was investigated. The patients (IV.1, IV.3 and IV.4) suffered in the neonatal period from hypocalcaemic seizures and investigations revealed undetectable circulating immunoreactive PTH\(^6\) concentrations and a normal renal response to synthetic 1-38 human PTH\(^7\). Clinical immunodeficiency or autoimmune related deficiencies were not observed in any of the patients. Normocalcaemia was restored with oral calcitriol therapy. Six unaffected relatives, who had no history of tetany or epilepsy, were demonstrated to be normocalcaemic with circulating immunoreactive PTH concentrations in the range 3.0 to 4.6 pmol l\(^{-1}\) (normal = 1.0 to 5.3 pmol l\(^{-1}\)). Ten unrelated normocalcaemic Asian individuals were studied as controls.

DNA analysis, PCR amplification and direct DNA sequencing, Leukocyte DNA extractions and hybridization analysis were performed using standard procedures\(^8\). PCR amplifications of the 3 PTH gene segments were performed separately as described previously\(^8\) with 3 pairs of oligonucleotide primers (Fig.2): 1L consisted of 5'TGAGATCCAGAGAATTCGGAGTGAC3' and 1R of 5'AACCCATTAGTTAGTTAGTT3'; 2L consisted of 5'AATCTCATGGAATTACAGAA3' and 2R of 5'TGGCTCTCAAGCAAGACATTGTCTTG3'; 3L consisted of 5'TGGAGTCCAGAGAATTCGGAGTGAC3' and 3R of 5'GTGAGTCCAGAGAATTCGGAGTGAC3'.

**Fig. 3** The autoradiographs show the nucleotide sequences of the PTH exon 2–intron 2 boundary obtained from a normal individual and patient IV.1. The exon sequence is indicated by upper case letters, the intron sequence is indicated by lower case letters and the exon-intron boundary is shown (broken line). The patient has a single base substitution (g to c), and this has altered the normal consensus 5' donor splice site sequence (gtaagt). This mutation has resulted in the occurrence of a DdeI restriction enzyme site (ctaag) and this has facilitated the detection of this donor splice mutation in other members of the family (Fig.1).

**Fig. 4** The illegitimate transcription of the PTH gene in EBV transformed lymphocytes was detected by PCR amplification of PTH cDNA which had been synthesized by addition (+) of the AMV reverse transcriptase to extracts of RNA obtained from EBV-transformed lymphocytes of normal and affected individuals. In 10 normal individuals (N, to N\(_4\), shown), correctly spliced PTH cDNA was observed at the expected size of 258 bp. This product was not present when reverse transcriptase was omitted (-) from the reaction or when only genomic DNA (G) or a water blank (B) were used, thereby demonstrating that this product is not due to amplification of a genomic sequence but is RNA specific. In family A/89, the affected individuals (IV.1, IV.3 and IV.4) who were homozygous for the donor splice site mutation (Fig.1) were found to differ from the normals in having an abnormal PTH cDNA of 168 bp in size. The mutant (m) PTH cDNA differed from the normal or wild type (W) by 90 bp, which corresponds to the size of exon 2 (Fig. 2). The parents (III.1 and III.2) and the unaffected sibling (IV.2) who are heterozygous for the mutation (Fig.1) have both the mutant and wild type PTH cDNA.
The RNA was treated with DNase I (Boehringer Mannheim), to the initiation codon (ATG) and the 25 amino acid signal sequence to that of a normal (data not shown) and the published sequence reveals that, in the mutant PTH cDNA, exon 1 has been spliced to exon 3. Thus, exon skipping with a loss of the 90 bp of exon 2, which contains the initiation codon (ATG) and the 25 amino acid signal peptide, has occurred.

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Parathyroid hormone gene analysis in autosomal hypoparathyroidism using an intragenic tetranucleotide (AAAT)$_n$ polymorphism

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Molecular Medicine, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, UK

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Abstract. We have identified a polymorphic tetranucleotide consisting of (AAAT)$_n$ within the first intron of the parathyroid hormone (PTH) gene, and have used this to investigate the segregation of the PTH gene and idiopathic hypoparathyroidism in 7 affected and 21 unaffected members from three families. An association between the PTH locus and autosomal dominant idiopathic hypoparathyroidism in one family was excluded by observing recombination between the two loci. In the remaining two families with autosomal recessive idiopathic hypoparathyroidism, the PTH locus was not similarly excluded. We had previously demonstrated a donor splice site mutation of the PTH gene in one of these families, and PTH gene abnormalities were therefore sought in the second of these families. DNA sequence analysis of the three exons, together with 4 exon-intron boundaries and the promoter region of the PTH gene revealed no abnormalities, thereby indicating molecular pathology at another locus. Thus, our analysis of idiopathic hypoparathyroidism reveals genetic heterogeneity for this disorder. In addition, our indentification of a microsatellite polymorphism of the PTH gene should help further segregation studies of this locus in families with parathyroid disorders.

Introduction

Mutations of the parathyroid hormone (PTH) gene, which is located on 11p15 (Mayer et al. 1983; Naylor et al. 1983), have previously been reported to be associated with autosomal dominant (Arnold et al. 1990) and autosomal recessive (Parkinson and Thakker 1992) idiopathic hypoparathyroidism, a disorder that is characterised by hypocalcaemia and hyperphosphataemia because of PTH deficiency. We have therefore investigated additional families with idiopathic hypoparathyroidism for PTH gene abnormalities. In order to facilitate segregation studies at the PTH locus, a polymorphic microsatellite repetitive sequence was identified in intron 1 of the PTH gene. Polymorphisms caused by length variations in such microsatellite tandem repeats, which can be detected by the use of the polymerase chain reaction (PCR), occur frequently and are inherited in a Mendelian manner (Litt and Luty 1989; Weber and May 1989). Our identification of such a polymorphic microsatellite sequence associated with the PTH locus should facilitate investigation of PTH gene abnormalities in disorders of parathyroid activity.

Materials and methods

Patients and families

Twenty-eight members (7 affected, 21 unaffected) from three families, designated 1/89, 2/89 and 7/90 (Fig. 1) with idiopathic hypoparathyroidism were investigated. The clinical and biochemical details of families 1/89 and 2/89, which suffer from autosomal recessive hypoparathyroidism, have been previously described (Parkinson and Thakker 1992; Shaw et al. 1991). In family 7/90, autosomal dominant inheritance of hypoparathyroidism was observed, and the patients (1.1, II.1 and II.4) suffered in late childhood or adulthood from hypocalcaemic symptoms resulting from hypoparathyroidism. Clinical immunodeficiency or autoimmune-related deficiencies were not observed in any of the patients. In addition, these patients had none of the dysmorphic features associated with pseudohypoparathyroidism, and the renal cyclic adenosine monophosphate (cAMP) response to synthetic 1-38 human PTH (Roelen et al. 1989) was normal. Normocalcaemia was restored with oral 1α-hydroxycalciferol therapy. Unaffected relatives had no history of hypocalcaemic symptoms and were demonstrated to be normocalcaemic with normal circulating immunoreactive PTH concentrations (Nussbaum et al. 1987). In addition, 39 unrelated normal individuals (25 northern Europeans and 14 Bangladeshis) and six 3-generation families (48 members) were investigated as controls in studies of the PTH locus.

DNA hybridisation analysis

Leukocyte DNA extraction, restriction endonuclease digestion, gel electrophoresis and hybridisation analysis were performed as previously described (Thakker et al. 1990). The PTH genomic probe

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Microsatellite polymorphisms

An examination of the published PTH genomic sequence (Reis et al. 1990) revealed a tandem repetitive sequence consisting of the tetranucleotide (AAAT)$_n$ at position 2859–2874 of intron 1. Oligonucleotide primers (L and R) flanking this microsatellite were synthesised to enable PCR amplification of the tandem repeat. Primer L extended from position 2729 to 2751 and consisted of 5'-GGTTAAGTTG-CCTGGAATATC-3', whereas primer R extended from position 3005 to 2982 and consisted of 5'-CACGAGAAGTTTGGAAATCCA-3'. Primer L (10 pmol) was 5'-labelled by the use of 5 units of the enzyme T4 polynucleotide kinase, T4 PNK (BRL), in a 10-μl reaction containing 50 mM TRIS·HCl pH 7.5, 10 mM MgCl$_2$, 5 mM dithiothreitol, 50 μCi of γ$^32$P ATP. PCR amplification was performed by adding 250 ng genomic DNA to a 25-μl volume containing 12.5 pmol each unlabelled primer, 0.1 pmol γ$^32$P end-labelled primer L, 50 mM KCl, 10 mM TRIS·HCl pH 8.4, 2.5 mM MgCl$_2$, 1 unit heat-stable DNA polymerase from *Thermus aquaticus* (Taq polymerase, BRL) and 0.2 mM each of dATP, dCTP, dTTP and dGTP. After an initial denaturation for 5 min at 94°C, 30 cycles of PCR amplification were performed. Each cycle consisted of 30 s at 92°C to denature double-stranded DNA, 30 s at 66°C for primers L and R to anneal to their respective complementary sequences, and 1 min at 72°C for the extension of the DNA strands. At the end of the 30 cycles, a period of 9 min at 72°C was allowed for final extension of the DNA strands. The PCR products were electrophoresed on 6% denaturing polyacrylamide gels at 1.4 kV and 35 mA for 4 h, and vacuum-dried. The PTH polymorphisms were detected by autoradiography at room temperature. In addition, polymorphisms in the repetitive sequence of a heterozygous individual (II.2 from family 1/89) were confirmed by DNA sequencing using previously described methods (Parkinson and Thakker 1992; Bilous et al. 1992). Primers L as the sequencing primer.

**PCR amplification and direct DNA sequencing of the PTH gene**

Leukocyte DNA from patients and normal individuals was used for PCR amplification and direct DNA sequencing of the three exons, the 4 exon-intron boundaries and the promoter region of the PTH gene, utilising previously described oligonucleotides and conditions (Parkinson and Thakker 1992; Bilous et al. 1992).

**Linkage disequilibrium analysis**

The ASSOC (version 2.2) computer program (Ott 1985), available from the Medical Research Council at the UK Human Genome Mapping Project Resource Centre (HGMP), was utilised to detect linkage disequilibrium between the (AAAT)$_n$, TaqI and PstI polymorphisms of the PTH gene. Chi squared values ($\chi^2$) at 4 degrees of freedom, together with $P$-values and standardised disequilibrium coefficients ($\Delta$) were ascertained. Linkage disequilibrium was established by a significant $P$-value; a $\Delta$ value greater than zero was indicative of the strength of the association.

**Results and discussion**

**Tetranucleotide polymorphism**

A tetranucleotide (AAAT)$_n$ polymorphism with two alleles was observed in intron 1 of the PTH gene; the occurrence of (AAAT)$_2$, which was designated allele 1 and that of (AAAT)$_3$ was designated allele 2. Mendelian inheritance of these alleles, which were 281 bp and 277 bp in size, was observed in 48 individuals from six 3-generation families. An analysis of 39 normal unrelated individuals (25 Europeans and 14 Asians) demonstrated the frequency for allele 1 to be 0.49 and that for allele 2 to be 0.51, thereby yielding a polymorphism information content (PIC) value (Botstein et al. 1980) of 0.375. The genotypes obtained with the (AAAT)$_n$ polymorphism in 36 unrelated individuals were compared with those obtained with PstI and TaqI RFLPs. The genotypes obtained with each of the three polymorphisms were the same in 21 individuals but, in the remaining 15 individuals, the (AAAT)$_n$, and TaqI genotypes differed in 13 individuals, the (AAAT)$_n$, and PstI genotypes differed in one individual, and the (AAAT)$_n$ genotype differed from both the PstI and TaqI genotypes in another individual. The combined use of these three polymorphisms of the PTH gene enabled the detection of heterozygosity for segregation studies in 66% of individuals. Linkage disequilibrium analysis of the (AAAT)$_n$, polymorphism and the TaqI and PstI RFLPs yielded similar results from the European and Asian populations (Table 1), and significant linkage disequilibrium between these three polymorphisms was observed.

Linkage disequilibrium is usually observed between markers that have a low mutation rate and that are closely
linked (Snell et al. 1989). The (AAAT)$_n$ polymorphism in intron 1 of the PTH gene is 383 bp upstream from the polymorphic TaqI site in intron 2, and 2.4 kb upstream from the polymorphic PstI site. Thus, our finding of linkage disequilibrium is in keeping with the close proximity of these three polymorphic sites and our results provide indirect evidence for a low mutation rate at these sites. However, linkage disequilibrium has not been previously observed (Mirc and Levine 1992) between two internal polymorphic mutations of the PTH gene designated Mir1 and Mir2. The Mir1 mutation occurs in intron 1 and the Mir2 mutation occurs 364 bp downstream in exon 3; the order and distances between the five PTH polymorphic sites is: (AAAT)$_n$, 231 bp – Mir1 – 152 bp – TaqI – 212 bp – Mir2 – 1821 bp – PstI. The absence of linkage disequilibrium between the two physically close Mir sites suggests that these sites may be associated with high mutation rates. This complex pattern of linkage disequilibrium at the PTH locus, in which some polymorphisms display a random association and are interspersed among those that display significant linkage disequilibrium, is analogous to that observed at the Huntington disease locus (MacDonald et al. 1991). The absence of linkage disequilibrium of the Mir1 and Mir2 polymorphisms may limit the use of these for haplotype analysis of the PTH locus. However, the absence of linkage disequilibrium between these two polymorphisms does not necessarily imply that it does not exist, but rather that it has not been detected. Further combined studies utilising the Mir1, Mir2, (AAAT)$_n$, TaqI and PstI polymorphisms associated with the PTH gene should help to elucidate the degree of linkage disequilibrium and mutation rates amongst these PTH gene polymorphisms.

### References


### Table 1. Linkage disequilibrium for PTH polymorphisms

<table>
<thead>
<tr>
<th>PTH polymorphisms</th>
<th>Group</th>
<th>E (n = 25)</th>
<th>A (n = 11)</th>
<th>E + A (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$P$</td>
<td>$\Delta$</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>(AAAT)$_n$ vs TaqI</td>
<td>18.7</td>
<td>&lt;0.001</td>
<td>0.12</td>
<td>11.6</td>
</tr>
<tr>
<td>(AAAT)$_n$ vs PstI</td>
<td>43.7</td>
<td>&lt;10^-6</td>
<td>0.24</td>
<td>16.1</td>
</tr>
<tr>
<td>PstI vs TaqI</td>
<td>19.6</td>
<td>&lt;0.001</td>
<td>0.13</td>
<td>8.2</td>
</tr>
</tbody>
</table>


Ott J (1985) A chi square test to distinguish allelic association from other causes of phenotypic association between 2 loci. Genet Epidemiol 2:79–84


Familial hypoparathyroidism is an unusual condition that can present at any time from early infancy until well into adulthood. It can be inherited in an autosomal dominant, autosomal recessive, or X-linked recessive pattern. Its presence has been associated with other congenital abnormalities, such as absence of the thymus (DiGeorge's syndrome).

We recently encountered a patient with longstanding sensorineural deafness who presented with symptoms of thirst and polyuria and was found to have diabetes mellitus. He also had hypocalcemia, subsequently proved to be due to hypoparathyroidism, and a high serum creatinine concentration; further studies revealed small kidneys with a large right-sided cyst of the renal pelvis. There was a family history of sensorineural deafness in a brother and two nieces, and further investigation revealed that they also had hypoparathyroidism and renal abnormalities. In this report we describe the family in detail and demonstrate that they have a combination of autosomal dominant hypoparathyroidism, sensorineural deafness, and renal dysplasia.

Methods

Initially, routine serum biochemical tests were performed in the three members of the index patient's family (Subjects III-2, IV-5, and IV-6) (Table 1) known to have sensorineural deafness; all had hypocalcemia. The brother (Subject III-2) of the index patient (Subject III-3) had a poorly functioning left kidney with a large cyst of the renal pelvis, discovered during an investigation of flank pain. At this point we decided to investigate parathyroid, auditory, and renal function formally in as many family members as possible or their parents gave informed consent to the studies.

Parathyroid function was assessed by measuring serum ionized calcium (with an ion-specific electrode [ICA 1, Radiometer, Copenhagen, Denmark]) and concentrations of intact parathyroid hormone (Allegro Intact PTH Assay, Nichols Institute Diagnostics, San Juan Capistrano, Calif.). Responses of serum cyclic AMP to 1-38 human parathyroid hormone (Shire Pharmaceuticals, Andover, United Kingdom) given in a dose of 0.5 μg per kilogram of body weight intravenously were measured by radioimmunoassay. The tubular maximal reabsorptive capacity for phosphate was calculated from measurements of phosphate in simultaneously obtained urine and serum samples.

The parathyroid hormone genes of eight family members were analyzed with the use of restriction-fragment–length polymorphisms (RFLPs) (Fig. 1), and products obtained by polymerase-chain-reaction (PCR) amplification of the three exons and four exon-intron boundaries from the affected Subject III-2 and an unrelated normal subject were subjected to direct DNA-sequence analysis, as previously described. The sequence of the parathyroid hormone–gene promoter was similarly analyzed, but the temperature during the annealing step was 62°C, with use of the primers L-5'AGTGAAGGCGATGCAGTACG-3' and R-5'AATCTCATGAATTTCAGAAGTGA-3'. This PCR product was cloned into a modified Blue-script vector, and the DNA sequences from seven independent clones were determined.

Hearing was assessed by pure-tone audiometry in the range of 125 to 8000 Hz (Fig. 2).

The glomerular filtration rate was measured directly in Subjects III-2 and III-3 by determining the 24-hour creatinine clearance and 51Cr-labeled EDTA clearance, respectively, but it was estimated in Subjects IV-3, IV-5, and IV-6 on the basis of their heights and serum creatinine concentrations. Subject III-3 underwent renal ultrasonography, and Subject III-2 had intravenous urography. Subjects III-1, III-4, IV-3, IV-5, and IV-6 underwent scanning with 99mTc-labeled dimercaptosuccinic acid and intravenous urography. Subject IV-6, who had the lowest glomerular filtration rate and the most abnormal results on intravenous urography, underwent percutaneous renal biopsy.

Results

The family tree, with the eight affected and five unaffected members, is shown in Figure 1, and the salient clinical and biochemical characteristics of each member are shown in Table 1. Four subjects (III-2, III-3, IV-5, and IV-6) were considered fully affected because they had hypoparathyroidism, sensorineural deafness, and renal dysplasia. Two subjects (IV-3 and IV-4) were considered partially affected because they had isolated renal dysplasia, normal hearing, and (in Subject IV-3) normal serum calcium concentrations. Two other family members (IV-1 and IV-2) may have had hypoparathyroidism, on the basis of low serum calcium concentrations and sudden death during infancy, respectively. All the remaining family members had normal serum calcium concentrations and normal results of audiometry (Subjects II-1, III-1, III-4, and III-5), and there were no abnormalities on renal imaging (Subjects III-1 and III-4), except in Subject II-1, who had undergone a nephrectomy for a staghorn calculus in 1977. Subject II-2, who died of a brain-stem infarction, had normal kidneys and parathyroid glands on postmortem examination.

None of the fully affected family members had symptomatic hypocalcemia or any clinical features of hypoparathyroidism. In particular, none had a history of epilepsy, basal-ganglia calcification on x-ray films of the skull, or cataracts. None had clinical features of pseudohypoparathyroidism or any suggestion of branchial-arch dysgenesis, and all had normal x-ray films of the hands and normal T-lymphocyte subgroups.

These same four subjects had unequivocally low
Table 1. Clinical and Biochemical Characteristics of a Family with Hypoparathyroidism, Sensorineural Hearing Loss, and Renal Dysplasia.*

<table>
<thead>
<tr>
<th>FAMILY MEMBER</th>
<th>AGE</th>
<th>HYPOPARATHYROIDISM</th>
<th>SENSORINEURAL DEAFNESS</th>
<th>RENAL DYSPLASIA</th>
<th>TOTAL CALCIUM</th>
<th>IONIZED CALCIUM</th>
<th>INORGANIC PHOSPHATE</th>
<th>CREATININE</th>
<th>PTH</th>
<th>PEAK cAMP</th>
<th>CLINICAL DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mg/dl</td>
<td>ng/liter</td>
<td>mg/liter</td>
<td>mg/liter</td>
<td>pg/ml</td>
<td>mg/liter</td>
<td></td>
</tr>
<tr>
<td>III-2</td>
<td>38 yr</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>7.7</td>
<td>4.1</td>
<td>5.2</td>
<td>1.2</td>
<td>18</td>
<td>102</td>
<td>Deafness noted at 1 yr of age</td>
</tr>
<tr>
<td>III-3</td>
<td>27 yr</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>6.6</td>
<td>3.7</td>
<td>4.7</td>
<td>1.2</td>
<td>&lt;10</td>
<td>324</td>
<td>Index patient; presented with diabetes, which is now controlled by diet; deafness noted in adulthood</td>
</tr>
<tr>
<td>IV-5</td>
<td>10 yr</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>8.1</td>
<td>4.3</td>
<td>7.1</td>
<td>0.6</td>
<td>24</td>
<td>216</td>
<td>Deaf since infancy</td>
</tr>
<tr>
<td>IV-6</td>
<td>9 yr</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>9.2</td>
<td>4.6</td>
<td>5.8</td>
<td>0.8</td>
<td>30</td>
<td>172</td>
<td>Deafness noted at 5 yr of age; micturating cystogram normal; renal biopsy confirmed dysplastic kidneys (Fig. 3)</td>
</tr>
<tr>
<td>Partially affected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-3</td>
<td>12 yr</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>9.7</td>
<td>5.2</td>
<td>4.5</td>
<td>0.8</td>
<td>25</td>
<td>—</td>
<td>Normal audiogram; renal dysplasia</td>
</tr>
<tr>
<td>IV-4</td>
<td>Neonatal death</td>
<td>?</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>76</td>
<td>Postmortem examination reported solid lungs and severe renal dysplasia with no visible normal renal tissue; parathyroid glands not mentioned</td>
</tr>
<tr>
<td>Possibly affected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-1</td>
<td>3 mo</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>7.5</td>
<td>—</td>
<td>5.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Admitted to hospital at 3 mo of age in collapsed state; possible encephalitis; severely handicapped after this event; serum calcium remained &lt;8.8 mg/dl for 1 mo; no subsequent tests; died at age of 8 yr; no postmortem examination</td>
</tr>
<tr>
<td>IV-2</td>
<td>3 mo</td>
<td>?</td>
<td>No</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Sudden death in infancy; kidneys normal macroscopically on postmortem examination; parathyroid glands not mentioned</td>
</tr>
<tr>
<td>Unaffected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1</td>
<td>61 yr</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>9.6</td>
<td>5.1</td>
<td>4.0</td>
<td>1.1</td>
<td>34</td>
<td>—</td>
<td>Left nephrectomy in 1977 for staghorn calculi with perinephric abscess</td>
</tr>
<tr>
<td>II-2</td>
<td>63 yr</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Died of brain-stem infarct</td>
</tr>
<tr>
<td>III-1</td>
<td>40 yr</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>9.5</td>
<td>5.0</td>
<td>3.4</td>
<td>1.0</td>
<td>31</td>
<td>—</td>
<td>Low renal threshold for glucose; normal audiographic and renal imaging results; father of 2 children with no hearing problems</td>
</tr>
<tr>
<td>III-4</td>
<td>32 yr</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>8.8</td>
<td>4.8</td>
<td>3.4</td>
<td>0.8</td>
<td>31</td>
<td>—</td>
<td>Normal audiographic and renal imaging results; mother of 3 children with no hearing problems</td>
</tr>
<tr>
<td>III-5</td>
<td>35 yr</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>9.0</td>
<td>4.8</td>
<td>2.7</td>
<td>0.8</td>
<td>—</td>
<td>—</td>
<td>Mother of Subjects IV-3, IV-4, IV-5, and IV-6; normal audiogram</td>
</tr>
<tr>
<td>Normal range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.6-9.8</td>
<td>4.8-5.5</td>
<td>2.5-4.5</td>
<td>0.8-1.2</td>
<td>10-65</td>
<td>2.5-4.0</td>
<td></td>
</tr>
</tbody>
</table>

*All affected family members had normal skull and hand x-ray films and dental examinations. Thyroid-function tests, serum calcitonin and vitamin D concentrations, and blood counts with lymphocyte-subgroup analysis were all normal. Parathyroid and other autoantibody tests and syphilis tests were negative. All subjects with hypocalcemia are now taking 1 to 4 µg of cholecalciferol daily and are normocalcemic. PTH denotes parathyroid hormone, TmP tubular maximal reabsorptive capacity for phosphate (in milligrams per liter of glomerular filtrate), and GFR glomerular filtration rate. To convert values for calcium to millimoles per liter, multiply by 0.25; to convert values for phosphate to millimoles per liter, multiply by 0.32; and to convert values for creatinine to micromoles per liter, multiply by 88.4. The GFR was estimated in Subjects IV-3, IV-5, and IV-6 on the basis of height and serum creatinine levels. Peak cAMP denotes the peak cyclic AMP response to an injection of 0.5 µg of 1-38 human parathyroid hormone per kilogram.

The clinical details of affected family members include:
- **Affected III-2**: Deafness noted at 1 yr of age. Index patient; presented with diabetes, which is now controlled by diet; deafness noted in adulthood.
- **Affected III-3**: Deafness noted in adulthood.
- **Affected IV-5**: Deafness noted at 5 yr of age; micturating cystogram normal; renal biopsy confirmed dysplastic kidneys (Fig. 3).
- **Partially affected IV-3**: Normal audiogram; renal dysplasia.
- **Partially affected IV-4**: Postmortem examination reported solid lungs and severe renal dysplasia with no visible normal renal tissue; parathyroid glands not mentioned.

Possible causes of hypocalcemia include:
- **Affected IV-1**: Admitted to hospital at 3 mo of age in collapsed state; possible encephalitis; severely handicapped after this event; serum calcium remained <8.8 mg/dl for 1 mo; no subsequent tests; died at age of 8 yr; no postmortem examination.
- **Affected IV-2**: Sudden death in infancy; kidneys normal macroscopically on postmortem examination; parathyroid glands not mentioned.

Serum concentrations of ionized calcium, with undetectable (Subject III-3), low (Subject III-2), or inappropriately normal (Subjects IV-5 and IV-6) concentrations of intact parathyroid hormone and increased phosphate concentrations (Table 1). In addition, all four had brisk increases in serum cyclic AMP concentrations in response to the infusion of parathyroid hormone and high renal tubular maximal reabsorp-
tion of phosphate. Serum concentrations of magnesium and alkaline phosphatase were normal in all subjects.

An analysis of TaqI- and PstI-derived RFLPs in eight family members (Fig. 1) revealed similar results, and an association between the disease and the parathyroid hormone locus therefore could not be excluded. However, a comparison of 1130 nucleotides of the DNA sequence of parathyroid hormone from Subject III-2 with that from an unrelated normal subject revealed no abnormalities of the parathyroid hormone gene. In addition, Subject III-2 had a normal karyotype.

All four fully affected subjects had remarkably similar patterns of sensorineural deafness. The pattern (Fig. 2) was that of a bilateral, symmetric, sensorineural deficit affecting all frequencies but slightly more marked at the higher end of the frequency range. The similarity of this deficit in the adults and children suggests that the deafness was not progressive, and the patients did not believe that their hearing loss had changed with age.

Serum creatinine concentrations and glomerular filtration rates were within the normal range in Subjects III-2 and III-3, despite the presence of large cysts in the left and right kidneys. Both of the fully affected children (Subjects IV-5 and IV-6) and the surviving partially affected subject (IV-3) had abnormal serum creatinine concentrations and reduced estimated glomerular filtration rates (Table 1). The results of intravenous urography in these three subjects were consistent with the presence of bilateral renal dysplasia; they had small, irregular kidneys and abnormally compressed collecting systems. (Confirmation of this diagnosis depends on the histologic demonstration of generalized disorganization with primitive ducts and even islands of hyaline cartilage, and cortical and medullary cysts may be present.) Scanning with 99mTc-labeled dimercapto-succinic acid in Subject IV-6 suggested that the left kidney was responsible for only 16 percent of total renal function, and a percutaneous biopsy was thus performed at this site.

The biopsy specimen was clearly demarcated into areas that appeared relatively normal and areas that were dysplastic (Fig. 3). In the normal areas, there were approximately 10 normal-sized glomeruli with preserved tubules and interstitium. In the dysplastic areas, the glomeruli were compressed and partially

Figure 1. Pedigree of a Family with Hypoparathyroidism, Sensorineural Deafness, and Renal Dysplasia. Squares denote male family members; circles female family members; slashes deceased family members (with the cause of death if known listed below the symbol); solid symbols family members with hypoparathyroidism, deafness, and renal dysplasia; and striped symbols family members who were possibly or partially affected. The arrow indicates the index patient. MI denotes myocardial infarction, and SIDS sudden infant death syndrome.

*Tested; normocalcemia with normal audiometric results
†Normal kidneys and parathyroid glands on postmortem examination
♦Analysis of parathyroid hormone gene performed with RFLPs
sclerosed, the tubules were atrophic, and the interstitium was fibrotic, with a scanty focal lymphocytic infiltrate.

**DISCUSSION**

The unique combination of hypoparathyroidism, sensorineural deafness, and renal dysplasia transmitted as an autosomal dominant trait appears to constitute a new syndrome. Four members of this family had hypoparathyroidism, although none had symptomatic hypocalcemia. Other recognized causes of hypocalcemia, such as hypomagnesemia, malabsorption, and renal failure, were excluded on the basis of the clinical history and appropriate studies. Pseudohypoparathyroidism was excluded by the absence of any of its dysmorphic features, such as metacarpal shortening, and by a normal serum cyclic AMP response to parathyroid hormone. An abnormal parathyroid hormone molecule has been described as a cause of familial hypoparathyroidism, but serum parathyroid hormone concentrations were high in the affected subjects.

All four family members with hypoparathyroidism had almost identical patterns of sensorineural deafness, whereas their normocalcemic relatives had normal hearing. The third feature was renal dysplasia, confirmed by renal biopsy in Subject IV-6 and diagnosed radiologically in her two sisters, one of whom had normal hearing and normal serum calcium concentrations. Their father and uncle also had cystic renal abnormalities, and their brother, Subject IV-4, who died within hours of birth, had severe renal dysplasia; there was no mention of his parathyroid glands in the postmortem report.

Three types of familial hypoparathyroidism are now recognized: an X-linked recessive type described in two large North American families; an autosomal recessive type, in which hypoparathyroidism forms part of the autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy syndrome; and an autosomal dominant type. The third type is the one affecting the family that we studied and is the most common, with a reported annual incidence of 2 to 4 per 100,000 hospital referrals. Molecular genetic analysis in several families with autosomal dominant
hypoparathyroidism failed to show any linkage with the parathyroid hormone gene on chromosome 11, although a mutation of the signal peptide-encoding region of the preproparathyroid hormone gene has been described in one of these families. Our analysis of the parathyroid hormone gene in this family with hypoparathyroidism, sensorineural deafness, and renal dysplasia revealed no abnormalities. The combined results of our study and previous reports confirm that there must be considerable genetic and molecular heterogeneity in familial hypoparathyroidism.

There have been several case reports of an association between familial hypoparathyroidism and sensorineural deafness, although the genetic basis for this association is unclear. Weidauer et al. described a father and son with symptomatic hypocalcemia and nonprogressive sensorineural hearing loss; the father also had vitiligo. Yumita et al. described two families in which the hearing loss in affected subjects was progressive, but they did not include any details of the audiometric results. In addition, in one family, at least one subject with hearing loss had normal serum calcium concentrations, and two affected subjects had other types of endocrine disease— one had thyrotoxicosis and the other azoospermia. A more extensive study of subjects with different types of hypoparathyroidism revealed several with different degrees of sensorineural hearing loss, suggesting that hypocalcemia may aggravate deafness. However, deafness can worsen despite the correction of hypocalcemia, and none of the subjects with hormone loss that we studied had any subjective change during treatment of hypocalcemia. Lehnhardt has reported sensorineural deafness and pseudohypoparathyroidism in two families with an affected parent and child, and the hearing loss in these subjects was similar to that in the family we studied. No mention was made of their response to treatment.

One subject studied by Yumita et al. had a hypoplastic right kidney. There have been six other reports of an association between parathyroid disease, deafness, and renal abnormalities. Barakat et al. described a family in which four brothers, each of whom had symptomatic hypocalcemia and severe sensorineural deafness, died of renal failure in early childhood. Serum parathyroid hormone was not measured; however, one of the patients had a normal phosphaturic response to an infusion of parathyroid hormone. Shaw et al. reported hypoparathyroidism and renal insufficiency due to tubular dysfunction in four cousins, two of whom had sensorineural deafness, and Edwards et al. described a family with hyperparathyroidism, nephropathy, and deafness. The pattern of inheritance was thought to be autosomal recessive in both these families. Dahlberg et al. described two brothers with a number of abnormalities, including hypoparathyroidism and renal failure, and Hunter et al. reported a case of nephrogenic diabetes insipidus and hypoparathyroidism in a family with autosomal dominant hypoparathyroidism. Finally, Kunstadter et al. described a neonate with hypoparathyroidism and a nonfunctioning kidney that may have been due to ureteral obstruction.

It is not clear why there should be a genetic link between parathyroid disease, deafness, and nephropathy. The parathyroid hormone and nephroblastoma genes are both present on chromosome 11, and the latter gene is thought to have a major role in renal growth and development. However, the two genes are widely separated at sites p11 and p13, respectively. We are not aware of the identification of any gene related to auditory development on chromosome 11.

Several families with hypoparathyroidism described in the literature had a history of unexplained sudden death in infancy, and one subject (IV-2) in the family we studied died suddenly in infancy. Although an early report of absent parathyroid glands in infants who died suddenly has not been confirmed, it is possible that undiagnosed hypocalcemia may be a factor in some unexpected deaths in infancy.

In conclusion, we have described a family with a distinct autosomal dominant syndrome of hypoparathyroidism, sensorineural deafness, and renal dysplasia. This syndrome represents another example of the curious and yet unexplained association of inherited abnormalities in the parathyroid glands, auditory system, and renal tract.

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References

Ilegitimate transcription of the parathyroid hormone gene in lymphocytes from normal and hypoparathyroid individuals

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Introduction

The detection by the polymerase chain reaction (PCR) of a low level of transcription of a tissue-specific gene in cells that do not exhibit a physiological expression of the gene has been referred to as either "nontissue specific" or "ectopic" or "illegitimate" transcription [1-3]. For example, illegitimate transcription of the Duchenne muscular dystrophy (DMD) gene encoding dystrophin, which is physiologically expressed only in muscle, has been observed to occur in fibroblasts, lymphoblastoid cells, HepG2 hepatoma cell lines and peripheral blood lymphocytes [4,5]. Additional studies have demonstrated that illegitimate transcription with correct splicing of the mRNA also occurs for other highly tissue-specific genes which encode clotting factor VIIIc, β globin, anti-Mullerian hormone and aldolase A [2]. The demonstration of such illegitimate transcription is of medical importance as it enables the use of easily accessible peripheral blood lymphocytes for the detection of abnormalities in mRNA processing, and thereby avoids the requirement for expressing tissue that may only be obtainable by biopsy. We have therefore investigated lymphocyte cell cultures for illegitimate transcription of the parathyroid hormone (PTH) gene in order to facilitate studies of PTH messenger RNA (mRNA) in patients with parathyroid disorders [6] and from whom parathyroid cells may not be available.

Materials and Methods

Epstein-Barr virus (EBV) transformed lymphocytes from 10 unrelated normal individuals and three patients with autosomal recessive idiopathic hypoparathyroidism [6] were cultured, and total RNA was extracted [7] and treated with DNase I to remove any contaminating DNA. A specific first strand complementary DNA (cDNA) copy of the PTH mRNA sequence was made [6] by using the oligonucleotide 5'TCAACCAAGACATTGCTTTC3', which is from a region of exon 3 that is complementary to the 3' domain of PTH mRNA, as a primer for the avian myeloblastosis virus (AMV) reverse transcriptase (Fig. 1). The yield of the
reverse transcribed PTH cDNA was increased by two rounds of PCR amplification in which two pairs of primers were used. Contamination of the solutions was checked for by using controls which either consisted solely of genomic DNA, or contained only the buffers and enzymes, or in which the AMV reverse transcriptase had been previously omitted. On completion the PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide to reveal the cDNA fragments under ultraviolet light (Fig. 2). In addition, these DNA fragments were isolated and utilized for direct double-stranded DNA sequencing [8]. The details of the PCR primers, reaction conditions and sequencing reactions are described by Parkinson and Thakker 1992 [6].

**Results**

Illegitimate transcription of the PTH gene was demonstrated by obtaining a PTH cDNA of 258 base pairs (bp) from lymphocyte cultures established from normal individuals (Fig. 2). This PTH cDNA was not observed either when RNA was absent, as in the genomic (G) DNA control, or when the reverse transcription step was omitted (—). Thus, this PTH cDNA arose from amplification of illegitimately transcribed PTH mRNA and not from amplification of the genomic sequence, whose PCR product would exceed 3000 bp. A similar analysis in a patient with autosomal recessive isolated hypoparathyroidism due to a donor splice site mutation at the exon 2-intron 2 boundary revealed a smaller mutant PTH cDNA of 168 bp (Fig. 2) [6].

**Fig. 2.** Illegitimate transcription of the PTH gene revealed by detection of PTH cDNA in cultured lymphocytes.
DNA sequence analysis of the 258 bp cDNA obtained from a normal individual revealed a correctly spliced PTH cDNA with the order exon 1-exon 2-exon 3 (Fig. 3). DNA sequence analysis of the mutant PTH cDNA from the patient with autosomal recessive hypoparathyroidism demonstrated exon skipping in which exon 2 was lost and exon 1 was spliced to exon 3 [6]. The loss of exon 2 which encodes the initiation codon (ATG) and the 25 amino acid signal peptide would respectively prevent the commencement of PTH mRNA translation and the translocation of any PTH peptide through the endoplasmic reticulum, thereby resulting in PTH deficiency.

In Fig. 2 illegitimate transcription of the PTH gene revealed by detection of PTH cDNA in cultured lymphocytes. The illegitimate transcription of the PTH gene in normal individuals is associated with correct splicing of PTH mRNA. 

Fig. 3. The autoradiograph shows a 123 bp segment from the total 258 bp DNA sequence of the normal PTH cDNA synthesized from illegitimately transcribed PTH mRNA. A comparison of the published sequence [9] to the sequence shown: GTTCATAAGACATTCTTGATGGAAGATCTACTGTGA reveals correct splicing in the order exon 1-exon 2-exon 3. Thus, illegitimate transcription of the PTH gene in cultured lymphocytes from normal individuals is associated with correct splicing of PTH mRNA.
EBV-transformed lymphocytes was detected by PCR amplification of PTH cDNA which has been synthesized by addition (+) of the enzyme reverse transcriptase to extracts of RNA obtained from EBV-transformed lymphocytes of normal (N) individuals and a patient (P) suffering from autosomal recessive hypoparathyroidism [6]. The samples were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide to enable visualization of the PTH cDNA fragments which are shown on the lower panel with the respective individual shown above. In 10 normal individuals (N1 and N2 shown), the PCR-amplified PTH cDNA was observed at the expected size of 258 bp. This product was not present when reverse transcriptase was omitted (−) from the reaction or when only genomic DNA (G) or a water blank (B) were used, thereby demonstrating that this product is not due to amplification of a genomic sequence but is RNA-specific. Thus, illegitimate transcription of the PTH gene has been demonstrated to occur in lymphocytes. In the patient (P), who is homozygous for a donor splice site mutation at the exon 2-intron 2 boundary, an abnormal PTH cDNA of 168 bp was found. Thus the mutant (m) PTH cDNA differed from the normal or wild type (W) by 90 bp, which corresponds to the size of exon 2. The father (F) and mother (M) of the patient, who were heterozygous for the mutation, have both the mutant and wild type PTH cDNA. Thus, both normal and abnormal copies of PTH cDNA resulting from the respective illegitimate transcription of wild type and mutant PTH genes can be detected to elucidate defects in the processing of PTH mRNA.

Discussion

Parathyroid hormone gene expression is usually confined to the parathyroid glands and our results, which reveal transcription of the PTH gene in cultured lymphocytes, therefore represent the first demonstration of illegitimate transcription of the PTH gene. In addition, our study has established that this illegitimate transcription is associated with correct splicing of the PTH mRNA in normal individuals. The extent of illegitimate transcription of other genes has been previously estimated [2–4] to be one molecule of correctly spliced mRNA per 1000 cells, and the physiological relevance and mechanisms involved in this low level of illegitimate transcription are not known. It has been postulated that the promoter regions of a tissue-specific gene may be activated by some of the ubiquitous transcriptional factors, for example TATA box factors and CAAT box binding proteins in the absence of the respective tissue-specific transcriptional factors [2]. The binding of these ubiquitous transcriptional factors to their respective DNA elements would be facilitated by the chromatin disruption that occurs during DNA replication, and nontissue-specific, i.e., illegitimate, transcription has been observed to be greater in actively proliferating lymphoblasts than in confluent fibroblasts [2]. Our demonstration of the illegitimate transcription of the PTH gene has facilitated our investigation of abnormalities in the processing of PTH mRNA in a patient from whom parathyroid glands were not available and who suffered from autosomal
recessive idiopathic hypoparathyroidism due to a donor splice site mutation of the PTH gene [6]. Exon skipping with a loss of exon 2 was revealed to be the cause of hypoparathyroidism in this patient. Thus, our demonstration of the illegitimate transcription of the PTH gene in cultured lymphocytes has avoided the requirement for parathyroid tissue biopsies, and this opens the way to further elucidate defects in the processing of PTH mRNA that may cause disorders of parathyroid activity.

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References

Linkage analysis of three cloned DNA sequences, DXS294, CDR and DXS105, in X-linked recessive hypoparathyroid families

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Idiopathic hypoparathyroidism (HPT) has been reported to occur as an X-linked recessive disorder (MIM No:30770) in two multigeneration kindreds (1,2). Affected males suffer from infantile onset of epilepsy and hypocalcaemia, which appears to be due to an isolated congenital defect of parathyroid gland development. We have previously localised HPT by linkage analysis to Xq26-Xq27 and have established the locus order Xcen-DXS37-F9-HPT-DXS98-DXS52-DXS15-Xqter (3). However, additional linked markers are being sought as the genetic distance between HPT and F9 is 4cM and that between HPT and DXS98 is 3cM. The polymorphic DNA probes DXS294, CDR and DXS105 have been localised to Xq26-Xq27 (4) and we have used these for further linkage studies in our previously reported HPT families (3) to define the genetic map in relation to HPT. Linkage was established between HPT and DXS105 (peak LOD score = 4.2, at 0% recombination) and between HPT and CDR (peak LOD score = 3.0, at 0% recombination). A peak LOD score of 2.0, at 5% recombination was observed between HPT and DXS294. These results indicate the locus order Xcen-DXS294-F9-HPT-DXS98-Xqter (4). Deletion mapping studies in a patient with haemophilia B (factor IX deficiency) who did not suffer from HPT but had a deletion involving the F9 locus (4) revealed positive hybridisation signals with DXS98 but an absence of hybridisation signals with F9, CDR and DXS105. Thus, the combined results of the linkage and deletion mapping studies demonstrate the locus order Xcen-DXS294-F9-(CDR, DXS105)-HPT-DXS98-Xqter. The physical distance between the flanking markers DXS105 and DXS98 has been previously estimated to be 400 Kbp (5) by pulsed field gel electrophoresis (PFGE). Defining this 0.4 Mbp region containing the HPT locus, will open the way for elucidating the factors controlling the embryological development of the parathyroid glands.
HYPOPARATHYROIDISM DUE TO A DONOR SPlice SITE MUTATION IN THE PARATHYROID HORMONE GENE. D.B. Parkinson* and R.V. Thakker, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, UK.

We have investigated one kindred with autosomal recessive idiopathic hypoparathyroidism for abnormalities in the parathyroid hormone (PTH) gene. The affected children, which were from a consanguineous marriage, had suffered from neonatal onset hypocalcaemia due to PTH deficiency. Leucocyte DNA was extracted and an analysis of PTH gene restriction fragment length polymorphisms (RFLPs) indicated segregation of the PTH locus with hypoparathyroidism. The PTH gene was further investigated by using the polymerase chain reaction (PCR) to separately amplify the 3 exons and exon-intron boundaries of the gene from an affected member and an unrelated normal individual. The DNA sequences of the 3 resulting PCR products, which were 228 bp, 474 bp and 565 bp in size, were determined by direct double stranded DNA sequencing. The patient's DNA sequence differed from the normal by a single base substitution (g>c) at the first base of intron 2, thereby altering the 5' donor splice site. This mutation resulted in the occurrence of a DdeI restriction endonuclease site (ctagaac) in the patient's DNA and this was used to detect the mutation in the remainder of the patient's family and 10 unrelated normal individuals. The DdeI site was present in both alleles of affected individuals (mutant alleles) but absent in both alleles of normal unrelated control individuals (wild type alleles). The parents and unaffected relatives were found to be heterozygous in having wild type and mutant alleles. Analysis of the mRNA transcript from the mutant and wild type PTH genes has demonstrated that the mutation results in abnormal processing of the mRNA in which exon 1 is spliced to exon 3. Thus, autosomal recessive hypoparathyroidism in this kindred is due to a novel mutation involving the 5' donor splice site of intron 2 of the PTH gene.
ECTOPIC TRANSCRIPTION OF THE PARATHYROID HORMONE GENE IN LYMPHOCYTES FROM NORMAL AND HYPOPARATHYROID INDIVIDUALS.

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Ectopic transcription, which is also referred to as non-tissue specific expression, has been detected by the use of the polymerase chain reaction (PCR) for the genes encoding factor VIII and dystrophin. We have investigated lymphocyte cell cultures for ectopic transcription of the parathyroid hormone (PTH) gene in order to facilitate studies of PTH messenger RNA (mRNA) in patients with parathyroid disorders. Lymphoblastoids from 10 unrelated normal individuals and 3 patients with autosomal recessive idiopathic hypoparathyroidism were cultured and total RNA was extracted and treated with DNaseI to remove any contaminating DNA. The first strand of PTH complementary DNA (cDNA) was synthesised by using a PTH gene specific primer and the enzyme reverse transcriptase from the Avian Myeloblastosis virus. The yield of PTH cDNA was increased by 2 rounds of PCR amplification and the resulting product was visualised by electrophoresis through an ethidium-bromide stained 1.5% agarose gel. The DNA sequence of this PCR product was determined by direct double stranded DNA sequencing. Ectopic transcription of the PTH gene was demonstrated by obtaining a PTH cDNA of 258 bp from normal individuals; DNA sequence analysis of this revealed correct splicing of the 3 exons. A study of the hypoparathyroid patients revealed a PTH cDNA of 168 bp and DNA sequence analysis of this transcript demonstrated a loss of exon 2. This abnormal PTH cDNA correlated with a donor splice site mutation (g→c) at the exon 2/intron 2 boundary of the PTH gene. The loss of exon 2 which encodes the initiation codon and the signal peptide will respectively prevent translation of the PTH mRNA and translocation of the PTH peptide, thereby causing PTH deficiency. Thus, our results represent the first demonstration of non-tissue specific or ectopic transcription of the PTH gene. In addition our findings open the way for further elucidation of the molecular basis associated with parathyroid disorders.