A study on the localisation of the PHEX gene, 
and mutation analysis of patients with 
X-linked hypophosphataemic rickets

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Jonathan N Goulding

University College London
Gower Street
London WC1

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Abstract

X-linked hypophosphataemic rickets (HYP) is an X-linked dominant disorder that affects 1 in 20000 live births. The disease is characterised by a failure of the kidney to reabsorb phosphate from the glomerular filtrate, resulting in a phosphate leak and hypophosphataemia. The disease had previously been localised to the Xp22.1-22.2 region of the X chromosome, flanked by polymorphic markers DXS43 and DXS41.

Twenty affected kindreds were screened with an additional eleven polymorphic markers, which localised the gene to a 200-300 kb region in Xp22.1, which lay on a single YAC, A0472. To further order the markers a YAC and cosmid contig from the region were also screened with these markers, with the following marker order deemed the most likely:


Screening Southern blots of patient DNA with cosmids that overlapped YAC A0472, identified a cosmid that showed an altered banding pattern, and therefore a mutation, in two unrelated individuals. This cosmid was sequenced and a candidate gene (PHEX) identified. The gene showed homology at the amino acid level to a family of metalloendopeptidases. To confirm that PHEX was the gene involved in X-linked hypophosphataemic rickets, patient DNA from eighty unrelated families was screened by Single Stranded Conformational Polymorphism (SSCP). A total of twenty three different mutations were identified, which included both deletions and point mutations. This confirmed that PHEX was the gene involved in the HYP disorder.
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Chapter 1

Introduction
1.1 Rickets and Osteomalacia

Rickets is a bone disease found in children, caused by the defective mineralisation of bone during bone growth, which leads to the bones being soft and flexible. When this same defective mineralisation occurs in non-growing bones it is termed osteomalacia, which can affect both children and adults.

Rickets as a disease has been described for centuries, but it was not until the 19th century that the histopathology was first described, with the radiographic delineation being described early in the 20th century, and the biochemical profiles described in the 1920s. In this country, it was a major health problem at the turn of the century, mainly affecting poor children who lived in the heavily industrialised cities. Poor nutrition, and therefore a low dietary intake of vitamin D, coupled with poor living conditions restricting exposure to sunlight, made the disease prevalent. In this country, and in other developed countries, dietary rickets is no longer a major public health problem, although this is not necessarily true in the poorer developing countries.

The main clinical features of rickets and osteomalacia are bone pain and tenderness, skeletal deformity and muscle weakness. Rickets is a disease of children. It is caused by interference with endochondral bone formation; the sequence of events that takes place in the epiphyseal growth plates and results in the lengthening of the long bones. Osteomalacia is the excessive accumulation of osteoid tissue throughout the skeleton, due to altered mineralisation, and is found in both adults and children (Glorieux, 1991).
In children the areas of the most rapid bone growth differ with age. This means therefore that rickets will be manifested in different areas of the body at different ages. At birth it is the skull that grows the most rapidly, so neonatal rickets may show craniotabes, which is where the cranial vault is extremely soft. In the first year of life rickets is manifested in the swollen epiphyses at the wrist and in swelling of the costochondral junction. Also at this age, the inward pull of the diaphragm can produce a groove in the ribcage, known as Harrison’s sulcus. In toddlers, rickets can lead to bow leg deformities, whilst knock knees are more characteristic in older children.

In children that are affected by rickets, the growth plate is widened because cartilage cells in the growth plate proliferate, but fail to mineralise the surrounding matrix. Muscle myopathy is frequently found in children suffering from rickets (although this is not seen in children with X-linked hypophosphataemic rickets, section 1.5). If severe enough, this can prevent walking and therefore limit deformities of the lower limbs. Another characteristic of rickets is a short stature, which occurs to a variable degree. In extreme cases pathological fractures in the shafts of the long bones can occur, but these are only found in the severest cases.

Normal mineralisation of bones needs several components. There must be an adequate supply of both calcium and phosphate, bone cells must be able to synthesise alkaline phosphatase (required for bone mineralisation), and there should be no toxic factors present. Rickets is characterised by the elongation and distortion of the normal columnar arrangement of chondrocytes in the zone of hypertrophy of cartilaginous
growth plate. In the underlying zone of maturation, calcification is delayed or absent, and vascularisation via defective or obliterated channels is impaired or irregular, and the primary spongiosa is abnormal. The radiological assessment shows that the ricketic growth plate is always widened, splayed and cupped, frayed and ragged.

In osteomalacia the skeleton is demineralised and the cortices are thin, but these changes are not radiologically distinguishable from osteoporosis. The main features of osteomalacia are the presence of pseudo-fractures, known as Looser’s zones, or Milkman’s syndrome. These are discrete zones of bone rarefaction that have the appearance of non-displaced fractures and most often occur at sites where arteries cross the bones. They are usually bilaterally symmetrical, and are most frequently found at the femoral neck and shaft, the pubic and ischial rami, the clavicles, ribs and scapulae, and the radius and ulna. Whilst the cause of these fractures is unknown, they heal promptly with treatment. Vitamin D deficient osteomalacia may also show evidence of hyperparathyroidism, such as subperiosteal bone resorption.
1.2 Vitamin D

1.2.1 Overview of the metabolism of vitamin D

Vitamin D, in the form of vitamin D₃, is made from 7-dehydrocholesterol in the skin by exposure to ultraviolet light in the 270-300 nm range, or can be derived from dietary sources as either vitamin D₂ or vitamin D₃. Both vitamin D₃ and vitamin D₂ undergo the same processes, which involve 25-hydroxylation in the liver, followed by 1α-hydroxylation in the kidney, to make the biologically active compounds 1,25-(OH)₂D₃ and 1,25-(OH)₂D₂ respectively. There is little evidence that these two active forms differ in their mode of action. The metabolic activations of vitamin D₃ are carried out by cytochrome P-450 containing enzymes; the vitamin D₃-25-hydroxylase (CYP27) (and possibly another P-450 in the hepatocyte), and the 25-hydroxyvitamin D-1α-hydroxylase (CYP1α) in the renal proximal tubular cell (renal 1α-hydroxylase). Both of these enzymes are located in the inner mitochondrial membrane of these cells. The synthesis of 25-hydroxyvitamin D₃ appears to be loosely regulated in the liver, whereas the synthesis of 1,25-(OH)₂D₃ by renal 1α-hydroxylase is tightly regulated by the levels of plasma 1,25-(OH)₂D₃ and calcium. The renal enzyme is strongly upregulated by parathyroid hormone (PTH) and in combination these increase serum calcium levels. The current view is that both synthesis and degradation of 1,25-(OH)₂D₃ are tightly regulated events (Jones et al., 1998).
Chapter 1: Introduction

1.2.2 Hepatic 25-Hydroxylation

Vitamin D does not circulate in the bloodstream for long, but is taken up by either adipose tissue for storage, where it can be stored for months or even years in humans, or by the liver for further metabolism. Vitamin D₃ undergoes its first step of activation in the liver; 25-hydroxylation (Blunt et al., 1968). There has been some controversy as to whether one or two enzymes carry out the 25-hydroxylation, but currently only the mitochondrial 25-hydroxylase has been purified and cloned (Andersson et al., 1989). The product of the 25-hydroxylation step is the major circulating form of vitamin D₃ and is present in human plasma at concentrations in the range of 10-40 ng/ml (Hollis, 1997). The fate of 25-OH-D₃ is dependent upon the calcium requirements. An urgent need for calcium results in 1α-hydroxylation, whilst an abundance of calcium results in 24-hydroxylation (Jones et al., 1998).

1.2.3 Renal 1α-hydroxylation

The 1α-hydroxylase enzyme performs the tightly regulated step which introduces a 1α-hydroxyl group into the A-ring of 25-OH-D₃, creating the hormone 1,25-(OH)₂-D₃ (Jones et al., 1998). The cloning of the gene for this enzyme led to the mapping of the human gene to 12q13.1-q13.3 (St-Arnaud et al., 1997). This is the same locus where the gene defect which causes hereditary vitamin D dependent rickets type I (VDDR I) was found (section 1.4.3).
1.2.4 24 Hydroxylation

The discovery of 24,25-(OH)₂D₃ occurred before the identification of 1,25-(OH)₂D₃ (Holick et al., 1972). The ease in which 24,25-(OH)₂D₃ was generated in large amounts suggested that the metabolic step was upregulated rather than downregulated by vitamin D administration. It appears that 24-hydroxylation is the first step in the destruction of the vitamin D hormone; its main function is to convert 1,25-(OH)₂D₃ to calcitroic acid (Makin et al., 1989; Reddy and Tserng, 1989). In the early 1990s the gene was cloned and was found to classify as a cytochrome P-450, CYP24. It belongs to the same subfamily as the other two known vitamin D related cytochromes (Chen et al., 1993; Itoh et al., 1995; Ohyama et al., 1991). The structure of the gene has been described for rats, mice and humans, and has two vitamin D response elements (VDREs- section 1.2.12) in its proximal promoter. These allow the upregulation of CYP24 by 1,25-(OH)₂D₃ from undetectable to high levels of mRNA within 4 hours (Shinki et al., 1992). It seems that the C-24 oxidation pathway is a complex, self-induced mechanism for limiting the action of 1,25-(OH)₂D₃ in target cells once the initial wave of gene expression has been initiated. It is expressed in the kidney, and other classical vitamin D target tissues, including the intestine and bone (Jones et al., 1998).

1.2.5 Role of vitamin D in calcium homeostasis

Calcium is an extremely tightly regulated substance in the plasma of higher animals. It is important for a number of essential functions, such as neural transmission, muscle contraction and relaxation, exocrine secretion, blood clotting, the adhesion of cells to one another and the construction of structural elements, such as the skeleton. The
calcium homeostatic system is complex, and the vitamin D endocrine system is the basic system in managing the calcium of plasma, with important roles for the parathyroid hormone and calcitonin (Jones et al., 1998).

1.2.6 Role of the parathyroid gland and parathyroid hormone

The parathyroid gland is the body's calcium sensing organ. In response to hypocalcemia, even slight hypocalcemia, the parathyroid gland releases, within seconds, parathyroid hormone (PTH). This hormone acts to initiate the mobilisation of calcium to replace that which is lacking in the plasma, and also to increase the renal uptake of calcium. PTH is short-lived in plasma, lasting only seconds or minutes. PTH receptors are found in the kidney nephron and osteoblasts, but not in the small intestine or in osteoclasts.

PTH plays an important role in the kidney. It blocks reabsorption of phosphate, which causes a phosphate diuresis. In the proximal convoluted tubule cells it activates the enzyme 1α-hydroxylase, which converts 25-OH D₃ to the active hormone, 1,25-(OH)₂D₃. PTH activates the 1α-hydroxylase by increasing the mRNA encoding this enzyme. It also suppresses the 24-hydroxylase enzyme, which is the hormone involved in the destruction of the vitamin D hormone (section 1.2.4). How it does this has not yet been elucidated, although there is a decrease in the amount of mRNA (Jones et al., 1998).
The parathyroid gland is also a target of vitamin D action. When the parathyroid gene was cloned, a vitamin D response element (VDRE, section 1.2.12), was found in the promoter region of the gene. This acts to suppress transcription of the parathyroid gene.

1.2.7 Physiological actions of 1,25-Dihydroxyvitamin D₃

The hormone 1,25-(OH)₂D₃ acts by itself to initiate active intestinal calcium transport into the small intestine. It also activates osteoblasts, which then release osteoclast activating factors resulting in the stimulation of osteoclasts to resorb bone, and/or the activation of reverse transport of calcium to the bone fluid compartment (Suda et al., 1995). The final result of this is that calcium is mobilised from the skeleton into the plasma compartment by the action of vitamin D hormone and PTH. Vitamin D deficient animals and parathyroidectomised animals will not mobilise calcium unless vitamin D (in the case of vitamin D deficient animals) or PTH (for the parathyroidectomised animals) is provided.

In the distal renal tubule another mechanism of the two hormones acting in concert causes the reabsorption of the last 1% of the filtered load calcium into the plasma compartment. These sources of calcium cause a rise in serum calcium that clears the sensing point of the calcium receptor, which shuts down the secretion of PTH.
1.2.8 Calcitonin

Hypercalcemia is a potential problem, as it could lead to the calcification of soft tissue such as the kidney, heart, aorta and intestine, which could cause organ failure and death. To prevent this, not only is it important that PTH secretion is shut off, but also that calcitonin is secreted by the thyroid. Calcitonin is responsible for lowering serum calcium through its action on the skeleton; it works directly on osteoclasts and osteocytes to reduce the calcium mobilising activity and shut down the calcium coming out of the skeleton.

1.2.9 Intestinal calcium and phosphate absorption

Calcium absorption is one of the basic functions of 1,25-(OH)_2D_3. Independent of this is its role in stimulating intestinal absorption of phosphate. The mechanism by which it stimulates the absorption is unknown in both cases.

1.2.10 Vitamin D receptors

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily. It has the highest similarity with the subfamily containing the retinoic acid (RAR) and thyroid hormone receptors (TR). The VDR can be split functionally into different domains. The NH_2 terminus has little function ascribed to it as yet. There is a DNA binding domain (C domain), then a hinge domain (D domain), and a ligand binding domain (E domain). The ligand binding domain is a complex part of the protein which is
responsible for the high affinity binding of the ligand, the dimerisation with the retinoid X receptor (RXR), and binding to transcription factors. The wild type VDR binds to its ligand 1,25-(OH)₂D₃ with extremely high affinity, and both the 1α- and the 25-hydroxyl groups are critical for high affinity binding. The absence of either group reduces binding approximately 500 fold. The VDR is expressed generally at relatively low levels in vivo, although target tissues such as the kidney, bone and intestine, may have higher levels of receptor.

1.2.11 Mechanism of transcriptional regulation by vitamin D

Vitamin D shares many features with the other ligand-activated nuclear receptors, such as the retinoic acid receptor and the thyroid receptor. Target genes are upregulated through binding of the VDR protein to specific DNA sequences, termed response elements, in the promoter regions of these genes. Binding of the 1,25-(OH)₂D₃ to the receptor increases the heterodimerisation of VDR with a cofactor, the retinoid X receptor. This binding of the heterodimer to the response element induces a bend in the DNA of the promoter. It also appears to change the conformation of the COOH terminus of the VDR, allowing a region of this to interact with other transcription factors. It seems that these other transcription factors may help in the remodelling of the DNA structure through acetylation of histones, which leads to the promoter being opened to the transcriptional machinery (Jones et al., 1998).
Several genes, including PTH (Demay et al., 1992), have been found to be
downregulated by $1,25-(OH)_2D_3$. It is not certain how this occurs, but at least two
possible mechanisms exist. It may be that binding of VDR to a downregulatory vitamin
D response element (VDRE) may disrupt the binding of upregulating transcription
factors, or it could be that the VDR binds to an inhibitory response element, which
may lead to interactions with repressor proteins which decrease transcription of the
gene.

1.2.12 Vitamin D response elements

Vitamin D response elements are sequences of DNA bound by VDR, which are
isolated from the promoters of vitamin D responsive genes. Many response elements
consist of two repeats of the sequence AGGTCA separated by non specific bases
(Darwish and DeLuca, 1996). The number of nucleotides separating these half sites
depends upon the receptor that binds. RXR binds preferentially to a single base
separating the two half sites, VDR binds to half sites separated by 3 bases, and TR to
half sites separated by 4 bases. Almost all the VDR upregulated genes have VDREs
with 3 bases separating the half sites, although this may not be true for the
downregulated genes.
**1.3 Phosphate regulation**

Homeostasis of phosphate is inseparable from that of calcium for two reasons: the concentration of each in the body fluids is limited by the concentration of the other; and both are required in fixed proportions for the mineralisation of bone. As with other essential inorganic substances, the body content of PO₄ in general, and plasma phosphate (pPO₄) in particular, are determined by a balance between intestinal absorption and renal excretion (Wesson, 1997).

**1.3.1 Intestinal absorption of phosphate**

Approximately 70% of ingested PO₄ is absorbed, mainly in the duodenum and jejunum, in both humans and rats. The fraction absorbed varies inversely with the amount that is ingested. Active absorption is by sodium dependent cotransport, but the presence of calcium in the lumen is also necessary. In addition, significant amounts of sodium free absorption, possibly passive, have also been observed (Armbrecht, 1990). The principal control of intestinal absorption is by the active form of vitamin D, 1,25-(OH)₂D₃, although the mechanism is currently unknown. PTH has an indirect effect on intestinal absorption, as it stimulates renal synthesis of 1,25-(OH)₂D₃, thus constituting a feedback system. There is evidence of intestinal secretion of PO₄, although the only apparent physiological function of this is an emergency response to hyperphosphataemia (Wesson, 1997).
1.3.2 Renal excretion of phosphate

In contrast to renal uptake of phosphate, which is an active process, renal excretion of phosphate is passive. Renal absorptive transport in humans conforms to the pattern of most substances that are filtered and actively reabsorbed. At low filtered loads reabsorption is nearly complete; with increasing load the excretion accelerates as the limiting rate of absorption, or transport maximum \( (T_m) \) is approached. The \( T_m \) is measured under conditions of filtered load by either the subtraction of the excretion rate from the filtered load, or by graphically comparing the excretion rate against plasma concentration. Once the load/\( T_m \) ratio reaches between 1.2 and 1.5, there is no further reabsorption. \( T_m \)-PO\(_4\) in humans has been adjusted to surface area, and in normal subjects has ranged from 100 to 160 \( \mu \)mol/1.73 m\(^2\) (or to 100 ml/min glomerular filtration rate (GFR)). The actual \( T_m \) within an individual, as well as between individuals, is highly variable, and is subject to many stimulants and depressants (Wesson, 1997).

1.3.3 Calcitonin

As mentioned in section 1.2.8, calcitonin lowers serum calcium by its action on the skeleton. Calcitonin also has the effect of lowering pPO\(_4\) concentrations, and reduces osteoclast resorption of bone.
1.3.4 Vitamin D

It is uncertain as to whether vitamin D has a direct effect on the transport of phosphate (tPO$_4$), but both 25-(OH)D$_3$ and 24,25-(OH)$_2$D$_3$ as well as 1,25-(OH)$_2$D$_3$ enhance the phosphaturic effect of PTH (Friedlander, 1996; Puschett and Beck, 1975).

1.3.5 Intake of phosphate

Dietary intake of PO$_4$ presents a continuing challenge to homeostatic control, due to the variation in diet. The response to and elimination of acute phosphate overload can be described as the overflow of the $T_m$. Chronic overload of PO$_4$ in 4 normal subjects over 2 weeks resulted in a large rise in pPO$_4$ and no change to the $T_m$ in the first two days. Thereafter, the $T_m$ decreased greatly and the pPO$_4$ levels returned to control levels. In the same study three individuals with hypoparathyroidism showed a less marked decrease in $T_m$, although the response was still statistically within normal limits (Thompson and Hiatt, 1957).

1.3.6 Molecular diversity of the sodium-inorganic phosphate systems

There are three dissimilar families of sodium-inorganic phosphate (NaPi) transporters identified to date. Type I, NPT1, are found mainly in the kidney and do not appear to be regulated by dietary phosphate levels. Type II, NPT2, exist in different isoforms. The type II transporters have been shown to be the major functional Na$^+$-dependent phosphate cotransporter in the proximal tubules (Biber et al., 1996). They are
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expressed almost exclusively in the kidney cortex, although NaPi-3 has been detected in osteoclasts (Gupta et al., 1997). A new isoform of NaPi-3, NaPi-3b, was described by Feild et al. (Feild et al., 1999), where they isolated full length clones from human lung and small intestine. It seems likely that this protein may be the small intestine Na/Pi cotransporter, although it is expressed in other tissues, and may play a role in phosphate metabolism in many cell types. Furthermore, NaPi-2 has been found to have three related cDNA clones, NaPi-2α, β and γ. These clones appear to be splice variants of NaPi-2, and may modulate the function of NaPi-2 in the renal proximal tubules (Tatsumi et al., 1998). Both type I and type II contain potential N-glycosylation sites in the extracellular loops, and potential phosphorylation sites for protein kinase C, although there is currently no direct evidence that phosphorylation of NaPi isoforms are involved in the regulation of Pi transport.

The third type of cotransporter, type III, are present in many organs, including the brain, heart, lung, liver and kidney. They share with type II cotransporters the ability to adapt to extracellular Pi concentration, something the type I transporters cannot. It is not currently clear whether the type III transporters are a unique family of housekeeping NaPi cotransporters (Friedlander, 1996).
1.4 Causes of rickets and osteomalacia

There are numerous forms of rickets. In addition to dietary rickets, vitamin D abnormalities and phosphate deficiencies can also cause rickets and osteomalacia.

- Nutritional deficiency
- Malabsorption
- Impaired activation (Hereditary vitamin D-dependent rickets type I (VDDR I))
- End organ resistance (Hereditary vitamin D-dependent rickets type II (VDDR II))
- X-linked hypophosphataemic rickets
- Autosomal recessive hypophosphataemic rickets
- Autosomal dominant hypophosphataemic rickets
- Adult onset vitamin-D resistant hypophosphataemic osteomalacia
- Hypophosphataemic rickets with hypercalciuria
- Renal tubular acidosis (types I and II)
- Fanconi’s syndrome
- Hypophosphataemic bone disease
- Oncogenous osteomalacia

In addition to these, hereditary hypophosphatasia, a deficiency in alkaline phosphatase, can also cause rickets and osteomalacia.
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1.4.1 Nutritional deficiency in Vitamin D

Rickets arising from a nutritional deficiency in vitamin D is now rare, particularly in the developed world. It only occurs when both diet and UV light exposure are inadequate, and is only really seen in some vegans lacking vitamin D supplementation (Strewler, 1993).

1.4.2 Malabsorption of vitamin D

Vitamin D is a fat soluble substance that depends upon the action of pancreatic lipases and bile salts for its absorption. Malabsorption of vitamin D may be due to complications of pancreatic insufficiency, loss of bile salts, or diffuse disorders of the intestinal mucosa (e.g. celiac disease). Endogenous synthesis of vitamin D due to exposure to sunlight should compensate for impaired absorption. There is however evidence of enterohepatic recirculation of 25-hydroxyvitamin D, which suggests that endogenously synthesised vitamin D metabolites may be lost in malabsorption syndromes (Strewler, 1993).

1.4.3 Hereditary vitamin D-dependent rickets type I (VDDR I) (OMIM:264700)

VDDR I is a disorder characterised by muscle myopathy, rickets and osteomalacia, bowing of the legs, bone fractures, enamel hypoplasia, small stature and growth delay. Neurological problems, hypotonia, motor retardation, seizures and tetany, are also found. Individuals are hypercalcaemic, have hypophosphataemia, high urinary cyclic
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AMP, high serum parathyroid hormone (PTH), high alkaline phosphatase, defective intestinal absorption of calcium, and a deficiency in 1α-hydroxylase (Scriver, 1970; Strewler, 1993; Thakker and O'Riordan, 1988). A child born affected with VDDR I is clinically well at birth, but develops the symptoms over the first two years of its life.

It was found that massive doses of vitamin D and high doses of 25-OH-D₃ were needed to correct the defects, but only small doses of 1,25-(OH)₂D₃ were required (Fraser et al., 1973). The evidence therefore pointed to a defect in the renal 1α-hydroxylase enzyme, which converts 25-OH-D₃ to 1,25-(OH)₂D₃, (section 1.2.3). An analogous disease has been shown to affect pigs (Winkler et al., 1982). In VDDR I a defective intestinal absorption of calcium has been demonstrated (Hamilton et al., 1970). The defect is caused by defective 1-alpha-hydroxylation of 25(OH)D, and so it is thus accompanied by a low serum level of 1,25-(OH)₂D₃.

In 1961 it was suggested by Prader et al that VDDR I was inherited in an autosomal dominant pattern, but they later expressed doubts regarding this (cited by Dent et al. (Dent et al., 1968) ). It has since been shown that the disorder is inherited in an autosomal recessive mode. It is a rare disease, but VDDR I was found in an unusually high frequency in French Canadians that live in the Saguenay region of Quebec, with an estimated gene frequency of 0.02 (Bouchard et al., 1985). The estimated prevalence of VDDR I at birth in this area is 1 in 2358, which means an estimated carrier rate of 1 in 26 (De Braekeleer, 1991). This suggested that there is a founder effect in this region.
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Linkage analysis located the gene defect in VDDR I to the long arm of chromosome 12, and by further analysis narrowed the region down to 12q14. Linkage disequilibrium between VDDR I and a 3 marker haplotype, which flanked the locus, supported the notion of a founder effect in the French Canadian population studied.

The gene which encodes for the vitamin D 1α-hydroxylase gene (P450c1α) was found to be located on chromosome 12q and was cloned in 1997 by Fu et al., using a combination of degenerate PCR, cDNA library screening and 5' RACE (Fu et al., 1997a; Monkawa et al., 1997; St-Arnaud et al., 1997). The gene was mapped to 12q13.1-ql3.3 by FISH, which is the same region of the chromosome to which VDDR I was mapped. The genomic sequence for the gene consisted of 9 exons, spanning 4.8 kb. Mutations in the gene which would inactivate it were described in a patient by Fu et al. (Fu et al., 1997b). The patient described in the study had two frameshift mutations in the gene, meaning a different frameshift mutation was inherited from each parent. This gave evidence that VDDR I was caused through a mutation in the gene encoding 1α-hydroxylase. Wang et al. (Wang et al., 1998) confirmed this, describing twelve new mutations in seventeen families from eight ethnic groups, including the common French-Canadian mutation from the Charlevoix-Sanguenay region. The Charlevoix haplotype was associated with the deletion of guanine 958 (codon 88) which disrupted the recognition sequence for the restriction endonucleases TaqI and MaeII. This discovery meant that a rapid restriction fragment length polymorphism (RFLP) diagnosis is possible for this mutation. In the other ethnic groups, six of the families had an additional copy of a 7 bp duplicated sequence which altered the
downstream reading frame, creating a premature stop signal. Another patient had, in addition to the 7 bp duplication, a 2 bp duplication in the same region, suggesting that this region may be a mutational hotspot. The remaining mutations described by Wang et al. were unique mutations.

As VDDR I is caused by mutations in the 1α-hydroxylase gene, recommended treatment is physiological doses of 1,25-(OH)₂D₃ administered on a daily basis, which is efficient replacement therapy.

1.4.4 Hereditary vitamin D-dependent rickets type II; VDDR II (OMIM:277440)

VDDR II is a disease which resembles VDDR I. The clinical symptoms are rickets, progressive loss of teeth, seizures and tetany, hypercalcemia unresponsive to 1,25-dihydroxycholecalciferol, normal serum 25-hydroxyvitamin D, and high serum 1,25-(OH)₂-cholecalciferol and often alopecia. Two forms of this disease have been described, those with alopecia, VDDR IIA; and those without, VDDR IIB.

End organ hyposensitivity to 1,25-(OH)₂D₃ was first reported in 1978 (Brooks et al., 1978; Marx et al., 1984). A 13 year old female was described who suffered from total alopecia, rickets that was unresponsive to vitamin D₂, and hypocalcemia unresponsive to several agents including 1,25 cholecalciferol. The majority of patients reported were the offspring of consanguineous marriages, suggesting that an autosomal recessive mutation was likely. The elevated serum concentrations of 1,25-(OH)₂D₃ in patients
suffering from VDDR II indicated that there was an abnormality in the mode of action of 1,25-(OH)2D3 in the target tissues. The functions of 1,25-(OH)2D3 are mediated by an intracellular receptor protein that binds DNA and concentrates the hormone in the nucleus, in a manner analogous to the classic steroid hormones. A receptor mutation was postulated (Haussler et al., 1980; Thakker and O'Riordan, 1988).

Studies of skin fibroblasts from VDDR II patients demonstrated that the disorder results from either a defect in the vitamin D receptor interactions with 1,25-(OH)2D3, or its nuclear site of action.

The vitamin D receptor (VDR) is an intracellular polypeptide of 50-60 kD that specifically binds 1,25-(OH)2D3, and interacts with target cell nuclei, section 1.2.10. The gene was cloned by Baker et al. (Baker et al., 1988) and by analysis of somatic cell hybrids the gene was assigned to chromosome 12q. This is the same region of the chromosome where the gene causing VDDR I was located. When linkage studies between these loci were done, no recombination was found between VDR and VDDR I.

Mutations in the VDR were first described by Hughes et al. (Hughes et al., 1988), in two families, both with mutations in the DNA binding domain. Since then several different mutations have been described in the VDR gene, all of which cause VDDR II. Most of the mutations described were found in the highly conserved N-terminal DNA binding domain, but some have been described that affect the ligand binding
domain (Malloy et al., 1997). Splice site mutations have also been described (Hawa et al., 1996) and there has been one case of a major structural mutation of the VDR gene, a deletion spanning exons 7, 8 and 9 (Malloy et al., 1999; Thompson et al., 1991).

1.4.5 Autosomal recessive hypophosphataemic rickets (OMIM:241520)

Stamp and Baker (Stamp and Baker, 1976), described the case of a brother and sister who presented with severe rickets in infancy. Their disease showed continued activity, resistance to vitamin D, early fusion of cranial sutures, increased bone density, nerve deafness and lifelong hypophosphataemia, which was unaffected by treatment. They were children of a first cousin marriage, and neither parent, or a third sibling, showed any evidence of the disease. Blood grouping, HLA typing and genetic fingerprinting supported paternity and consanguinity (Baker and Stamp, 1989).

1.4.6 Autosomal dominant hypophosphataemic rickets (OMIM:193100)

Autosomal dominant hypophosphataemic rickets is a disease characterised by vitamin D resistant rickets and osteomalacia, hypophosphataemia and inappropriately normal calcitriol concentrations. The disorder has incomplete penetrance, and occasionally has a delayed onset. Some affected children have lost the phosphate wasting defect after puberty. Econs et al. (Econs and McEnery, 1997; Econs et al., 1997), looked at linkage analysis in a large kindred, and found no linkage with two known sodium-
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dependent inorganic phosphate transports; SLC17A2, located on 5q35, or the sodium phosphate transport 1 gene, located on 6p. They performed a genome wide search using microsatellite markers and demonstrated linkage to markers on 12p, with a maximum LOD score ($z_{max}$, chapter 3, section 3.1.2) =5.65 at a $\theta=0.0$ for marker VWF, and a $z_{max}=3.73$ at a $\theta=0.0$ for marker CD4. Multipoint analysis indicated that the most likely location for the gene is a 18 cM interval between markers D12S100 (telomeric) and D12S397 (centromeric) with a $z_{max}=8.13$.

1.4.7 Adult onset vitamin D-resistant hypophosphataemic osteomalacia

Adult onset vitamin D-resistant hypophosphataemic osteomalacia was first described by Frymoyer and Hodgkin (Frymoyer and Hodgkin, 1977). They described a large kindred with an X-linked dominant disorder of isolated phosphate wasting. They said this disorder was distinct from Hypophosphataemic rickets since the affected children lacked radiographic evidence of rickets, and affected individuals typically presented with the clinical manifestations of the disease in the fourth or fifth decade of life. It appeared therefore, that there might be two forms of X-linked dominant hypophosphataemic rickets/osteomalacia arising from mutations in two different genes on the X-chromosome. With the identification of the gene involved in X-linked hypophosphataemic rickets (HYP) (chapter 3), Econs et al. (Econs et al., 1998), showed that the mutation causing the adult onset syndrome described by Frymoyer and Hodgkin was in the same gene as that causing hypophosphataemic rickets, and
that this syndrome was indeed Hypophosphataemic rickets and not a second X-linked dominant form (chapter 4).

1.4.8 Hypophosphataemic rickets with hypercalciuria (HHRH) (OMIM:241530)

Tieder et al. (Tieder et al., 1985) described six individuals from an inbred Bedouin kindred with a hereditary syndrome of hypophosphataemic rickets and hypercalciuria. There were five males and one female affected, and the disorder began in early childhood. Characteristic features were rickets, short stature, increased renal clearance of phosphate, hypercalciuria, normocalcemia, increased intestinal absorption of calcium and phosphorus, increased serum concentration of 1,25-(OH)_{2}D_{3} and suppressed parathyroid hormone production. Reversal of all the abnormalities, except the decreased glomerular filtration rate, $T_{mP}/GFR$, was achieved by long term phosphorus supplementation. The primary defect was thought to be a phosphate leak in the kidney, which resulted in hypophosphataemia, with the appropriate increase in 1,25-(OH)_{2}D_{3}, which in turn increased calcium absorption, parathyroid suppression and hypercalciuria. Inheritance of the disease was thought to be autosomal recessive. When more asymptomatic members of the tribe were studied, 40% had a pattern of biochemical abnormalities similar to their relatives with HHRH, these were thought to be heterozygotes for the gene defect.
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1.4.9 Renal tubule acidosis type I (RTA I) (OMIM:179800)

Different types of renal tubule acidosis have been recognised (Morris et al., 1965). In RTA I the bicarbonate threshold is normal, the defect is in the distal tubule. As well as the renal tubule acidosis, affected individuals also have nephrocalcinosis, osteomalacia and growth failure, hypokalemic muscle weakness, periodic paralysis, high serum chloride, low serum bicarbonate and hypocalcemia.

Although the disease has been recognised for many years (Randall and Targgart, 1961), Chaabani et al. (Chaabani et al., 1994) reported a large family (which, they suggested, had been the only large family reported) that suffered from primary RTA I. Bruce et al. (Bruce et al., 1997) and Karet et al. (Karet et al., 1998) found that the disease is associated with mutations in the chloride-bicarbonate exchanger gene SLC4A1. There were four amino acid substitutions in the gene identified, 3 of them involved arginine-589.

It has been proposed by Lewis (Lewis, 1992) that Tiny Tim, a character in Charles Dickens’ ‘A Christmas Carol’ (1843) was affected by RTA I, as his symptoms were growth failure, osteomalacia with pathological fractures, hypokalemic muscle weakness and periodic paralysis, all of which are characteristic of RTA I.
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1.4.10 Renal tubular acidosis type II (RTA II) (OMIM:312400)

Unlike RTA I (OMIM:79800), in RTA II, excretion of acid in the distal tubule is normal, and the urine is normally acidic. The disease is characterised by the partial failure to reabsorb bicarbonate in the proximal tubule. It can occur as an isolated defect, with growth retardation as the main clinical feature, but is more typically a feature of the Fanconi syndrome (section 1.4.11). Production of 1,25-(OH)\textsubscript{2}D\textsubscript{3} in RTA II is variable (Grossman, 1998).

1.4.11 Fanconi syndrome

Fanconi syndrome is a term used to describe the coexistence of multiple proximal tubular reabsorptive defects, mainly involving phosphate, glucose, amino acids and bicarbonate, but also extending to calcium, potassium, urate and water. Fanconi syndrome exists in many forms, some of which are hereditary, but some forms are acquired, and can be due to toxicity from heavy metals and outdated tetracycline among others.

1.4.11.1 Fanconi renotubular syndrome I (OMIM:227700)

Fanconi renotubular syndrome type I is a childhood and infantile form of Fanconi syndrome, without cystinosis. Tieder et al. (Tieder et al., 1988) described a brother and sister, the offspring of consanguineous parents, who had Fanconi syndrome, severe bone disease and marked hypocalciuria without renal tubular acidosis.
However, in contrast to other reports of this syndrome, \(1,25-(OH)_2D_3\) serum levels were elevated in these patients.

**1.4.11.2 Fanconi renotubular syndrome II (OMIM:227800)**

This form of the Fanconi syndrome affects adults, and, as with Fanconi renotubular syndrome I, there is no cystinosis. The disease presents at around 40 years of age, the bones become looser, and there is a loss of height, and muscle weakness. The urine contains high amounts of amino acids and glucose, similar to the childhood form. There was evidence that inheritance of this disorder is recessive, but a study by Brenton et al. (Brenton et al., 1981), showed that in the family they studied the disorder was autosomal dominant and the authors concluded that there was no evidence of recessive inheritance in the literature.

**1.4.11.3 Fanconi-Bickel syndrome (FBS) (OMIM:227810)**

Fanconi-Bickel syndrome is extremely rare, characterised by hepatorenal glycogen accumulation, proximal renal tubular dysfunction and impaired utilisation of glucose and galactose. Initial symptoms are fever, vomiting, growth failure and rickets at the age of 3 to 10 months. The patients later present with dwarfism, protuberant abdomens, hepatomegaly, moon shaped faces, and fat deposition on the shoulders and abdomen. The cutting of teeth, and puberty are both retarded, and fractures and pancreatitis are complications, with rickets and osteoporosis constant features later in life. There is no specific therapy available (Manz et al., 1987).
Evidence was found that FBS is inherited in an autosomal recessive fashion, was supported by consanguinity in families and the occurrence in sibs (Manz et al., 1987). Santer et al. (Santer et al., 1997) noted that the functional loss of the facilitative glucose transporter 2 (GLUT2) gene was comparable to the clinical symptoms observed in FBS patients. The GLUT2 gene had been localised to chromosome 3q26.1-q26.3 by Fukumoto et al. (Fukumoto et al., 1988), by somatic cell hybridisation. Matsutani et al. (Matsutani et al., 1992) positioned the gene on 3q using a (CA)$_n$ microsatellite repeat polymorphism adjacent to the end of exon 4a. Santer et al. (Santer et al., 1997), reported 3 homozygous mutations in the GLUT2 gene, confirming that this was the gene involved in FBS.

1.4.12 Hypophosphataemic bone disease (HBD) (OMIM:146350)

Scriver et al. (Scriver et al., 1977) identified a disorder that was characterised by modest shortening of stature, bowing of the lower limbs, non-rachitic bone changes and hypophosphataemia. The defect appeared to be different to X-linked hypophosphataemic rickets, and, for the same level serum phosphate, the bone disease was milder in HBD than in HYP. In two out of the five cases described, the disease was found to be autosomal dominant. These cases showed phosphataemic response to 1-α-OH analogues of vitamin D$_3$, whilst one of the other patients, that had no family history, failed to respond; HBD may therefore be heterogeneous. It is unclear whether
this disorder is separate from the autosomal dominant form of vitamin D resistant rickets (section 1.4.6).

1.4.13 Oncogenic Hypophosphataemic Osteomalacia

Oncogenic hypophosphataemic osteomalacia is a rare tumour-derived syndrome, which results in severe rachitic bone disease (70 cases reported between 1959 and 1990) (Drezner, 1990; Hewison et al., 1992). The main features of the disease are reduced $T_m$P/GFR (renal phosphate leak), abnormally low levels of 1,25-(OH)$_2$D$_3$, elevated alkaline phosphatase, and osteomalacia. Tumours that cause osteomalacia are mainly mesenchymal. The primary tumour-derived factor that causes the bone defects are unknown, although there is convincing evidence for the presence of a circulating humoral component or components. The remission of the symptoms after tumour resection support the present of a humoral factor. A study by Rowe et al. (Rowe et al., 1996b) presented supporting evidence of a humoral factor, and described 56-58 kDa proteins on a western blot of tumour conditioned media. They concluded that it seemed likely that these proteins were involved in transducing the tumour-derived phosphopenic effects. With the identification of the gene involved in X-linked hypophosphataemic rickets, PHEX (chapters 3 and 4), and the fact that this gene has high homology to a family of zinc metallopeptidases suggests that the tumour derived factors may represent unprocessed hormone unmodified by the PHEX gene (Rowe, 1998).
1.5 X-linked hypophosphataemic Rickets

X-linked hypophosphataemic rickets (HYP) is the commonest form of inherited rickets, affecting approximately 1 in 20000 live births. The symptoms include vitamin D resistant rickets, spinal stenosis, bowed legs, short stature, defective intestinal absorption of phosphate, hypophosphataemia, high alkaline phosphatase, reduced renal phosphate \( T_m \). Muscle myopathy and tetany are not associated with HYP.

Rickets that was associated with resistance to vitamin D therapy was first described in 1937 by Albright et al. (Albright, 1937). HYP was originally thought to be inherited in an autosomal fashion, with the affectation status being determined by bone abnormalities (Christensen, 1941). However, Winters et al. (Scriver et al., 1991; Winters et al., 1958), in 1958 proposed that renal loss of phosphate was the origin of the hypophosphataemia. They commented that all the patients that they used in their survey had hypophosphataemia, and that their \( T_mP \) was set at a new lower level, and that the percent of the filtered phosphate which was reabsorbed (%TRP) was lower than expected in all patients. They observed that vitamin D responsive rickets had been replaced by rickets that was resistant to vitamin D. This transition reflected an increase in the heritability of rickets. Winters et al. used the term “vitamin D resistant rickets” to imply a hereditary disease characterised by hypophosphataemia and diminished renal tubular reabsorption of phosphate. They also noted that there were no instances of male to male transmission, and that all daughters of affected males exhibited hypophosphataemia. They performed a literature review, where they noted that there
was a report of male to male transmission of vitamin D resistant rickets, which was incompatible with the disease being X-linked; it was, however, compatible to there being an autosomal form of the disease.

Berndt et al. (Berndt et al., 1996), in 1996 performed a retrospective study on twenty three adult patients with X-linked hypophosphataemic rickets. They said that HYP was clearly a chronic disease with an important psychosocial impact that extended into adulthood. The patients judged themselves less able to cope with physical and psychological stress, which was mainly contributed to bone pain, disproportionate growth failure, restricted mobility, waddling gait and multiple hospitalisations, leading to a lack of schooling and vocational training. The study group was small, and so may not have been valid for demographic analyses, but they did find a indication that unemployment and early retirement were seen more often than was found in a healthy population. Almost all of the patients were married, or wanted to be married, however a proportion of the women refused to have children, due to the risk of an affected baby.

1.5.1 Treatment of X-linked hypophosphataemic rickets

Treatment of HYP has been reported to have limited success. Treatment with ergocalciferol (vitamin D₂) alone and at the necessarily high doses to have an effect, was only partially successful at neutralising the effects of the disease, and it was frequently complicated by vitamin D toxicity. The infusion of phosphate can stimulate
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bone mineralisation, but is impractical and readily complicated by hypocalcemia and secondary hyperparathyroidism (Costa et al., 1981).

The efficacy of vitamin D$_2$ and oral phosphate therapy was examined by Lyles et al. (Lyles et al., 1982). They noted that long term observations indicated that treatment with vitamin D$_2$ alone failed to cure HYP, and posed a serious problem due to the problem of vitamin D intoxication and renal damage. They looked at combination treatment with oral phosphate supplement and vitamin D$_2$. The oral phosphate was employed to raise the serum P$_i$ concentration and pharmacological amounts of vitamin D$_2$ were used to prevent secondary hyperparathyroidism, which is attendant upon administration of oral P$_i$. This combination treatment resulted in the radiographic healing of the rachitic lesions and accelerated growth in affected subjects. Although previous reports had documented that oral P$_i$ and vitamin D$_2$ therapy resulted in the amelioration of the hypophosphataemia and acceleration of growth in affected children, the authors commented that the evidence that bone becomes normal in response to the P$_i$ and vitamin D$_2$ therapy was inferential and based on the changes in the radiographic appearance of the rachitic lesions. They claimed that the results of their study indicated that long term treatment with oral P$_i$ and vitamin D$_2$ therapy did not eliminate the osteomalacic bone defect, and that analysis of bone biopsies obtained after 18-36 months showed unequivocal evidence of persistent osteomalacia.

However, despite the persistence of bone disease in the treated subjects, the dynamics of bone mineralisation were significantly improved.
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Improved healing of bone lesions and more effective control of hypophosphataemia was reported when phosphate therapy was combined with calcitriol. A child suffering from HYP was treated for three years with vitamin D-inorganic phosphate supplement, and then by 1,25(OH)₂D₃ and half of the previous phosphate supplement. Whilst the initial treatment did not correct either the hypophosphataemia or the alkaline phosphatase elevation, the 1,25(OH)₂D₃-inorganic phosphate regimen was well tolerated and effective in normalising these variables. There was also improved growth and healing of the rickets, which was further evidence of the efficacy of this treatment (Hirschman et al., 1978).

Treatment with calcitriol was found to improve the net retention of phosphate, primarily in preadolescents: the levels of serum calcium were found to rise and alkaline phosphatase fell in the patients thus treated, but renal handling of phosphate was not restored to normal (Costa et al., 1981). In patients that were treated with phosphate and ergocalciferol, osteomalacia was found. When the ergocalciferol was replaced with calcitriol the same patients had improved bone mineralisation, an improvement which had been reported in earlier studies (Drezner et al., 1980; Glorieux et al., 1980).

A report by Peacock et al. (Peacock et al., 1977) reported a positive correlation between a change in serum phosphorus and an improvement in bone mineralisation. However, some of the patients studied by Peacock et al. had muscle myopathy, which is not found in HYP, and so it seems likely that these patients had an alternative form of rickets. In patients treated with calcitriol rather than ergocalciferol a lower dose of phosphate was needed, decreasing the risk of secondary hyperparathyroidism during
therapy (section 1.5.6). The undesirable side effects of treatment with calcitriol include hypercalcemia and changes in serum creatinine. However, in the study by Costa et al., hypercalcemia was mild, infrequent, found only in those patients given the highest doses of calcitriol, and was eased by lowering the dose. They suggested that for the initial treatment dosage should be small, and that the increments should also be small. They concluded that although calcitriol did not correct the basic defect in HYP, it was the vitamin D metabolite of choice for the treatment of HYP.

Patients with HYP have inappropriately low concentrations of calcitriol, despite manifesting hypophosphataemia. A comparison of the response of both control and HYP patients to calcitonin was performed (Econs et al., 1992). It was found that the response did not differ between the control or the HYP patients. Calcitonin stimulation in both groups resulted in a comparable increment of calcitriol concentration. They concluded that the defect in calcitriol production in HYP was incomplete, limited to the proximal convoluted tubule and probably a secondary effect, rather than a primary defect.

Treatment of HYP in children with 1,25(OH)2D3 and phosphate has been shown to be of clinical value. Growth rates of patients have been shown to improve with treatment (Chesney et al., 1983; Glorieux et al., 1980; Scriver et al., 1991), with the bone deformities being less severe (Glorieux, 1990; Glorieux et al., 1980). The effect of treatment on adults was investigated by Sullivan et al. (Sullivan et al., 1992) on patients who had active bone disease. They found that treatment of symptomatic adults
led to improvements (although the study was not placebo controlled), and the biochemical markers were only slightly affected. There were no serious complications of treatment reported, except in one patient. They therefore concluded that treatment of symptomatic HYP adults with 1,25-(OH)$_2$D$_3$ and phosphate led to clinical and histomorphometric improvements, but that patients being treated must be carefully monitored. They found no cases with nephrocalcinosis, but acknowledged that as this was a problem in children there was a need to monitor for it.

1.5.2 Problems with treatment

There are three main complications of the treatment of HYP. Hypervitaminosis D, nephrocalcinosis (section 1.5.3) and hyperparathyroidism (section 1.5.6) (Carpenter, 1997).

Hypervitaminosis D manifests itself by hypercalcemia and hypercalciuria. The occurrence of these was more frequent with the use of high doses of fat soluble vitamin D and infrequent monitoring of the serum and urine biochemistries. Although there have been deaths due to this, they are rare today due to the availability and use of 1α-hydroxylated vitamin D metabolites (Carpenter, 1997).

The value of treatment has been questioned for patients with HYP, with a certain amount of debate on the merits of treatment. In 1989 Stickler and Morgenstern (Stickler and Morgenstern, 1989), queried the value of treatment of HYP patients.
They performed a retrospective analysis on fifty two adult patients, and they concluded that there was no evidence that any form of treatment (high doses of vitamin D; vitamin D plus phosphate supplements; or calcitriol plus phosphate), had any effect on adult height, symptoms or alkaline phosphatase levels. They admit that their study had shortcomings, in that it was a retrospective study, and that there was no biochemical data to assess the extent of correction of the mineral imbalances or radiological data to assess the resolution of rickets. They found that three patients in the study had had renal failure in their twenties. Twenty three patients had nephrocalcinosis, whereas patients who had not undergone treatment and were first seen in their clinic as adults, showed no evidence of nephrocalcinosis. However they did not look any further at nephrocalcinosis in any of the patients. They concluded that neither form or duration of treatment had any effect on the outcome of hypophosphataemic rickets, and that because of the possibility of renal failure and nephrocalcinosis, treatment might do more harm than good. This was refuted by Stamp and Goldstein (Stamp and Goldstein, 1990), who stated that only 6 out of 16 patients that they had treated with phosphate supplements showed no detectable improvement in the rate of growth. They suggested that a careful tailoring of pharmacological vitamin D or calcitriol at the earliest possible age until the end of growth was needed, and that the differences between the two groups studied could be due to an increased severity of the disease in the USA, or differences in management and patient selection. They said that Stickler and Morgenstern had shown only that delayed diagnosis and treatment of HYP was no better than not treating at all. They suggested that a controlled trial of vitamin D therapy, which Stickler and Morgenstern had proposed, would pose serious ethical
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questions. Reusz et al. (Reusz et al., 1990a) agreed that the discontinuation of treatment was not justified; they stated that in the group studied by Stickler and Morgenstern the hypophosphataemia was detectable only by laboratory tests in some of the families, and that there were no bone deformities. However, the patient group they examined all had rickets and suffered growth retardation. They showed that there was a height gain in the treated patients, and thus concluded that treatment was justified. Stickler and Morgenstern (Stickler and Morgenstern, 1991), countered this, stating that as there was no treatment which made short children grow significantly, together with the frequent observation of nephrocalcinosis in treated children, therapy should not be automatic. They also called for a controlled treatment trial, saying that this was the only ethical solution.

Berndt et al. (Berndt et al., 1996), also performed a retrospective study on twenty three adult patients. They said that HYP was clearly a disease that affected children and extended into adult life. The consequences of early bone deformities, decreased final height, multiple operations and treatment side effects had a severe effect on patients, and that secondary degenerative alterations of the skeletal systems could worsen with age. Some of the patients they examined had episodes of pain and increasingly limited joint mobility, which had greatly impaired their quality of life. Although treatment with vitamin D and phosphate had a positive effect on growth (see above), many of these patients still had growth deficiency and showed bone deformities in spite of therapy. They proposed that patients with a low $T_{mP}/GFR$ might be at a higher risk of developing the more severe symptoms. They found a trend that
patients with a moderate urinary loss of phosphate who received early vitamin D and phosphate treatment did better than children with a severely reduced $T_mP/GFR$ receiving late or no therapy. Their conclusion was that patients affected by HYP should be treated with vitamin D and phosphate during childhood, and that they should receive interdisciplinary care by nephrologists, orthopaedic surgeons, physiotherapists and dentists throughout child- and adulthood.

1.5.3 Nephrocalcinosis

Although treatment with 1,25-(OH)$_2$D$_3$ and phosphate is successful in treating HYP, treatment has been reported to cause renal damage. Renal calcification leads to nephrocalcinosis, which has been observed in children treated for HYP (Goodyer et al., 1987; Reusz et al., 1990a; Weber et al., 1988). Reusz et al. (Reusz et al., 1990b), looked at the possible causes of nephrocalcinosis, to see if other factors other than calcium excretion could be the cause. They looked at 12 patients, 6 of whom had nephrocalcinosis detectable by ultrasound. Urinary calcium excretion in all but one of these patients was normal, and that individual did not have detectable nephrocalcinosis. They then looked at oxylate excretion, and suggested that the enteric hyperabsorption and renal hyperexcretion of oxalate (due to the excessive phosphate supplementation), led to crystal formation even at the normal rates of calcium excretion. Since the relative phosphate intake falls with age, this could also explain why progressive renal calcification was not found.
Verge et al. (Verge et al., 1991), demonstrated that nephrocalcinosis was a complication of combination therapy with calcitriol and phosphate. They found an incidence of 79% of nephrocalcinosis in patients with HYP who were receiving combination therapy. This was higher than had been previously reported (11 out of 23 patients (Goodyer et al., 1987) and 6 out of 12 patients (Reusz et al., 1990b)). They concluded that nephrocalcinosis in children was a complication of therapy associated with large doses of phosphate. This was supported by Taylor et al. (Taylor et al., 1995) who compared ten adults and four children who had HYP but who did not receive treatment, with ten adults and eight children with HYP who underwent medical therapy. None of the untreated patients had nephrocalcinosis, whereas five of the treated adults, and five of the treated children did have nephrocalcinosis. Of these treated patients, the phosphate dose was significantly higher in those with nephrocalcinosis. The height increase in treated children must be balanced against the risk of nephrocalcinosis. In one patient with nephrocalcinosis whose therapy was stopped, the glomerular filtration rate was stabilised. This suggested that it may be best to cease therapy in patients who have achieved full growth, or who have decreased renal function.

Kidney function remains normal in most children with nephrocalcinosis, but irreversible histologic damage and a reduction of glomerular filtration rate have been observed (Alon et al., 1983). The nephrocalcinosis was demonstrated to be due to precipitation of calcium phosphate crystals in the kidney. They also found the higher phosphate intake was associated with decreased calcium excretion, leading to a
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reduction in urine calcium oxylate saturation and thus the tendency towards crystallisation (Alon et al., 1992). They also showed that by providing high concentration of phosphate in the drinking water of Hyp mice (a murine model for the disease (section 1.6)) a reduction in the urinary excretion of calcium was found, and the excretion of oxalate nearly doubled. As with humans, the renal calcifications were composed of calcium phosphate. These renal calcifications were located intratubular, indicating that the nephrocalcinosis was likely to be due to hyperphosphaturia. This suggested that the increase in urinary phosphate excretion after oral phosphate administration greatly surpasses oxalate excretion, resulting in a condition that favours inhibition of calcium oxalate precipitation and promotes the precipitation of calcium phosphate crystals.

Seikaly et al. (Seikaly et al., 1996), performed a retrospective evaluation of the medical records of thirty four HYP patients looking for evidence of nephrocalcinosis and renal tubular acidosis. Their data demonstrated an association between the development of nephrocalcinosis and hyperchloremic metabolic acidosis but without an effect on glomerular filtration rate. They also found an association between the duration of therapy and the degree of nephrocalcinosis, as had been previously reported by Goodyer et al. (Goodyer et al., 1987). In total, eighty four percent of the patients they studied that had nephrocalcinosis also had a hyperchloremic metabolic acidosis. There was a positive correlation between the degree of nephrocalcinosis and hypercalcuria. They concluded that therapy of X-linked hypophosphataemia is often associated with nephrocalcinosis, and that the development of nephrocalcinosis is
associated with renal tubular acidosis in HYP patients. Further investigation would be required to determine whether alkali therapy in patients who develop the renal tubular acidosis would affect growth, metabolic bone disease or the progression of nephrocalcinosis.

1.5.4 Growth Hormone Therapy

Wilson et al. (Wilson et al., 1991) looked at the effect of growth hormone treatment. They noted that early observations of growth hormone treatment increased renal phosphate reabsorption and calcitriol levels, which led to the speculation that it may have beneficial effects on HYP patients by positively affecting phosphate balance by increasing the levels of 1α-hydroxylation of vitamin D. Although it is unlikely that abnormalities in growth hormone secretion plays a part in HYP, they found that there were elevations of serum phosphate levels in all the subjects examined, to levels that were not significantly different from those seen with conventional therapy. Although they did not investigate growth rates when growth hormone only was administered, or alter the doses of phosphate or calcitriol during the study, they suggested that growth hormone may stimulate skeletal growth in HYP. Also, growth hormone therapy may have the additional effect of stimulating growth, although the data were too preliminary to suggest that this may affect adult height. A longer term study was undertaken by Saggese et al. (Saggese et al., 1995), that looked at the effect of growth hormone treatment on HYP patients, in combination with the conventional treatment. They found that adding growth hormone therapy to conventional treatment
improved the growth rate, phosphate retention and also increased bone mineral density, BMD. Their conclusion to the study was that in HYP children with poor growth, long term growth hormone treatment, combined with conventional treatment, had a positive effect on growth, phosphate metabolism and BMD. Another study also found that growth hormone may have partially corrected the defects in renal tubular transport, and may have acted as a phosphate sparing agent, meaning that treatment with lower doses of phosphate may be possible. This would have the advantage of reducing the adverse affects of phosphate treatment such as nephrocalcinosis (Patel et al., 1996). Seikaly et al. (Seikaly et al., 1997) performed a randomised study, which compared the effect of growth hormone to a placebo. They found that treatment with growth hormone enhanced linear growth, was well tolerated in HYP patients and had no effect on the grade of nephrocalcinosis. Twelve months of treatment with growth hormone increased bone mass and width, but not density. Administration of the placebo for this time had no effect on bone mass, width or density.

A later study by Cameron et al. (Cameron et al., 1999) questioned these findings. They looked at the effect of growth hormone therapy on HYP patients who were well-controlled on conventional therapy and found that adjunctive GH therapy appeared to offer no benefits with respect to linear growth, doses of conventional therapeutic agents or rachitic disease markers. They suggested that the compliance with conventional therapy may not have been optimal in the Saggese study. They concluded that despite the theoretical advantages of growth hormone therapy, adjunctive growth
hormone therapy would offer no benefits to HYP patients who are well-controlled on conventional therapy.

1.5.5 Parathyroid Hormone effects

HYP patients do not have elevated serum levels of the active vitamin D metabolite, 1,25-(OH)₂D₃, which would be expected with hypophosphatemia and phosphate depletion. Lyles and Drezner (Lyles and Drezner, 1982), looked to see if PTH, the principal stimulant of 1,25-(OH)₂D₃ activity, would increase serum 1,25-(OH)₂D₃ levels in HYP patients. They found that when parathyroid extract was administered, the 1,25-(OH)₂D₃ levels rose far less in the HYP group than in the control group. The excretion of cAMP, an index of PTH action in the kidneys, did not differ in either group, which suggested that the ability of the kidneys in each group to respond were the same; so they concluded that the disparate effects that they had observed were due to a defect in the enzyme activity.

1.5.6 Hyperparathyroidism and HYP

Hyperparathyroidism is a recognised complication of HYP (Davies, 1995) due to the chronic phosphate therapy, with CaPO₄ sequestration and the apparent lowering of pCa⁺⁺. Severe hyperparathyroidism may occur after treatment with high dose phosphate and vitamin D therapy, showing a need to monitor serum phosphate levels during treatment of the disorder (Firth et al., 1985). Oral phosphate therapy may mask the hyperparathyroidism by lowering serum calcium levels, so routine assessment of
parathyroid activity may be important during treatment to detect parathyroid hyperactivity. This may help detect hyperparathyroidism in the early stages so that it may be treated with calcitriol and a reduction in the phosphate dose before the hyperparathyroid state leads to a significant amount of skeletal demineralisation and becomes irreversible (Rivkees et al., 1992). Exaggerated nocturnal increases in serum PTH have been demonstrated by Carpenter et al. (Carpenter et al., 1994), and they speculated that this may have resulted from impaired calcium mobilisation from the osteomalacic bone, and that there may thus be a tendency to secondary hyperparathyroidism inherent to HYP. That hyperparathyroidism should be included as part on the clinical synopsis of HYP and that it should be looked for in HYP patients was supported by Knudtzon et al. (Knudtzon et al., 1995). Hyperparathyroidism in HYP patients is possibly due to chronic stimulation of PTH secretion, as is seen in renal osteodystrophy or after long standing vitamin D deficient rickets (Delmez and Slatopolsky, 1991; Sultan et al., 1989). This increased PTH secretion can be explained by three interrelated mechanisms: 1- relatively low calcium levels may stimulate PTH secretion. 2- the defect in vitamin D metabolism and/or action may result in reduced vitamin D inhibition of PTH secretion. 3- phosphate treatment may result in reduced levels of vitamin D and calcium, and stimulate PTH secretion (Knudtzon et al., 1995). It has been proposed that untreated HYP patients have normal levels of PTH and that the hyperparathyroidism may be the result of unbalanced treatment with vitamin D and phosphate (Rivkees et al., 1992).
1.5.7 Hypoparathyroidism in a HYP patient

Lyles et al. (Lyles et al., 1985), described an individual who had hypoparathyroidism and hypophosphataemic rickets; due to the hypoparathyroidism the renal leak was masked, and identification of the individual as suffering from HYP was determined by examination of the teeth. When hypophosphataemia is present during the development of the teeth the calcospheres fail to coalesce, and the pattern of interglobular dentine persists for the entire life of the tooth (Nikiforuk and Fraser, 1979). The patient described by Lyles et al. had hyperphosphataemia and an elevated $T_m\text{P/GFR}$ in the range found in patients with hypoparathyroidism alone. With treatment there was a fall in the serum phosphorus and in the $T_m\text{P/GFR}$ to that found in HYP patients. Their data suggested that the normal concentrations of calcium or vitamin D metabolite are necessary for the complete expression of the renal phosphate leak when endogenous PTH was absent. They also looked at the osteomalacic characteristic of the disorder, which had been ascribed to the presence of the hypophosphataemia. In this patient the osteomalacia persisted despite many years of the hyperphosphataemia. Whilst it was possible that this was due to the hypocalcemia, this was very mild due to the therapy given. They concluded that their observations in this unique individual highlighted the important role of calcium, PTH and possibly $1,25-(\text{OH})_2\text{D}_3$ in HYP.
1.5.8 Cardiovascular abnormalities

Cardiovascular abnormalities such as valvular disease, myocardial dysfunction, and hypertension occur in association with complications such as hypercalcemia, hypercalciuria, nephrocalcinosis and hyperparathyroidism, but were not reported in HYP patients (Nehgme et al., 1997). However, the group of patients that they examined showed diastolic hypertension and attenuated increases in systolic blood pressure with exercise. All the patients had nephrocalcinosis, so it is possible that that renal calcinosis influenced the regulators of vascular tone. The abnormal blood pressure may be explained by microdeposition of calcium on the arterial walls, or in peripheral blood vessels. They suggested that HYP patients should undergo periodic cardiology evaluations, as the abnormal findings were noted by cardiac ultrasonography and exercise stress testing, things not performed routinely in the clinical setting.

1.5.9 Dentition in HYP

In HYP the characteristic dental abnormalities, both in the primary and permanent teeth are draining abscesses. The defective dentin formation and extension of the pulpal horns into the cusp tips facilitate the entry of bacteria and cause spontaneous abscesses in caries free teeth. Most patients need to undergo prophylactic dental procedures throughout their lives to reduce the incidence of dental disease (Stratakis et al., 1995).
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The tooth abscesses in the absence of dental caries or periodontal disease that are characteristic of HYP develop as a result of pulpal infections through abnormal globular dentin, and the clinical treatment given did not have any effect on the dental calcification problems (Seow, 1991). Stratakis et al. (Stratakis et al., 1995) described a patient prone to recurring oral giant cell lesions, who also had a detectable elevation in PTH levels, which became normal after adjustment to his therapy. They hypothesised that the presence of these giant cell lesions may be an indication of occult or developing hyperparathyroidism.

Shields et al. (Shields et al., 1990) suggested that the defective development and mineralisation of teeth in HYP patients was a consequence of two separate processes which act in parallel, one affecting renal transport and homeostasis of phosphate, and the other directly affecting the developmental processes, which include the formation and mineralisation of hard tissues. Evidence to support this is that there is evidence of a gene dosage effect on serum Pi in affected males and females, which does not correlate with the severity of the bone disease. They looked at the PRATIO ratio, a two dimensional representation of pulp size relative to the tooth size which measures the outcome of tooth development. These values showed that there was a gene dosage effect in the male and female HYP patients examined, which was contrary to the serum Pi levels. They said that the large pulp size they observed in HYP teeth was not due to dentine resorption or pulp hypertrophy, but that the pulp space abnormality was due to either deficient mineralisation or a global dysplasia of the secondary dentin forming in
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the odontoblasts. This effect was more severe in the males, the females were presumably mineralising small segments of the secondary dentin.
1.6 Hypophosphataemic rickets in the mouse

In 1976 Eicher et al. (Eicher et al., 1976), reported a mouse model for X-linked Hypophosphataemia, Hyp. They had identified a dominant mutation which was located on the distal end of the X chromosome. The mutant mice were hypophosphataemic, had changes in bone resembling rickets, and had a high fractional excretion of phosphate anion (low net tubular reabsorption). They reported that phosphate supplementation in the diet from weaning helped prevent the severe skeletal abnormalities. They concluded that as the mouse resembled the male hypophosphateamic human the genes were probably homologous.

Another X-linked dominant mouse mutation was found that suffered from hypophosphataemia and rickets/osteomalacia, and in addition had a circling behaviour, inner ear abnormalities and sterility in males, with a milder phenotype in the females. This was named the Gyro (Gy) mutation. Gy was found to be linked very closely to the Hyp locus, and they both had a similar quantitative expression of serum phosphorus levels, renal excretion of phosphate and impairment to the Na dependent phosphate cotransport of the renal brush border membranes (Lyon et al., 1986).

1.6.1 Vitamin D metabolism in the Hyp mouse

In the Hyp mouse 1,25-(OH)₂D₃ does not stimulate intestinal transport of phosphate. O’Doherty et al. (O. Doherty et al., 1977), proposed that it was a defect in the
metabolism of the vitamin to its hormonal form, and so investigated the effect of 1,25-(OH)$_2$D$_3$ on intestinal transport of both calcium and phosphate. They found that 1,25-(OH)$_2$D$_3$ stimulated calcium transport in the intestine in both Hyp and control mice. In the control mice administration of 1,25-(OH)$_2$D$_3$ stimulated intestinal phosphate transport in a dose dependent manner, but failed to increase intestinal transport of phosphate in the Hyp mouse. Also, dietary supplementation of phosphate did not effect phosphate transport in the Hyp mouse, but did in the control group. These experiments supported the theory that the defect in the Hyp mouse is a phosphate transport defect.

Hyp mice have abnormal vitamin D metabolism, which was demonstrated by Meyer et al. (Meyer et al., 1980). The Hyp mouse has normal circulating levels of 1,25-(OH)$_2$D$_3$, which were not expected as either low levels of 1,25-(OH)$_2$D$_3$ (had the disease been caused by deficiency in 1,25-(OH)$_2$D$_3$), or high levels (due to the low plasma phosphate), would be expected. They fed both control and Hyp mice phosphate depleted diets, and the control group showed the expected increase in 1,25-(OH)$_2$D$_3$ levels, which was lacking in the Hyp mouse. Lobaugh and Drezner (Lobaugh and Drezner, 1983) showed that phosphate deficiency increased 1α-hydroxylase activity in normal mouse kidney, but not in Hyp mice.

Treatment of the Hyp mouse was found to be more successful with 1α-hydroxyvitamin D$_3$ than with 1,25-(OH)$_2$D$_3$. Both compounds have been found to increase the conservation of urinary phosphate and to improve the rachitic bone morphology.
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However, 1α-hydroxyvitamin D₃ was reported to repair the hypophosphataemia and increase the intestinal transport of phosphate, which 1,25-(OH)₂D₃ did not do (Beamer et al., 1980). The group hypothesised that this was not due to a genetic deletion in the intestinal phosphate transport system but an inability to respond to 1,25-(OH)₂D₃. Long term treatment with 1α-hydroxyvitamin D₃ resulted in hypercalcemia, and it does have the disadvantage that it can be toxic (although more so in the control males than the Hyp males). Both 1α-hydroxyvitamin D₃ and 1,25-(OH)₂D₃ had the same effect in the bone and kidney.

Work by Tenenhouse and Scriver (Tenenhouse and Scriver, 1981), showed that the renal transport defect in Hyp mice was not corrected by treatment with 1,25-(OH)₂D₃, which was consistent with findings on the human form of the disease, where 1,25-(OH)₂D₃ therapy improves the mineralisation of bone and the linear growth rate without any repair to the renal handling of Pi (section 1.5.1). There was clear improvement in Pi homeostasis during the infusion of 1,25-(OH)₂D₃ which they explained as being due to the enhancement of absorption of Pi in the intestine. This contrasted with the report of O’Doherty et al. (O. Doherty et al., 1977).

Tenenhouse (Tenenhouse, 1983) looked at the activity of 1α-hydroxylase by rearing the mice on a vitamin D deficient, low calcium diet. Both the control and Hyp mice fed this diet showed an increase in 1,25-(OH)₂D₃ and a corresponding decrease in the synthesis of 24,25-(OH)₂D₃, although the Hyp mouse did have a depressed 1α-hydroxylase response for a corresponding fall in serum calcium. Tenenhouse suggested
that this was due to abnormal regulation of vitamin D hormone biosynthesis in the Hyp mouse.

The experiments described above by Tenenhouse contrasted with a study by Meyer et al. (Meyer et al., 1982). They fed control and Hyp mice a low calcium diet and found that the concentrations of plasma calcium and phosphate were not changed significantly. They reported that the 25-OH D levels were lower in Hyp mice than in the controls, but that the decrease, whilst significant, was not pathogenic. Also, plasma 1,25-(OH)2D3 levels were not significantly different in either the control and Hyp mice fed a control diet, whilst the low calcium diet resulted in an increase of plasma 1,25-(OH)2D3 levels in both genotypes, which was greater in the Hyp mice. They concluded that the failure of Hyp mice to respond to low phosphate diet was a specific failure of the system over plasma 1,25-(OH)2D3 rather than a generalised failure of the 1,25-(OH)2D3 system. The difference in the findings between these experiments and those by Tenenhouse (see above) were explained by Tenenhouse as possibly being due to either differences in experimental protocols, or that the concentration of 1,25-(OH)2D3 in plasma is not a reliable estimate of 1α-hydroxylase activity, as there are other factors which contribute to the plasma steady state. Work by Seino et al. (Seino et al., 1982) showed that the defect was not in the 1,25-(OH)2D3 receptor, and so must be due to other cellular events other than the binding of 1,25-(OH)2D3 to its receptor.

Evidence that the control of 1α-hydroxylase activity is abnormal in the Hyp mouse was demonstrated by Fukase et al. (Fukase et al., 1984), who found that whilst the
enzyme activity increased in renal cortical cells from control mice in low calcium medium, and decreased in a high calcium medium, the enzymes activity in cells from the Hyp mouse did not alter with similar changes in calcium. When control, phosphate depleted and Hyp mice were stimulated with cAMP it was found that this did not alter the plasma calcium concentrations after 24 hours. Plasma phosphorus levels dropped in the control and increased in the phosphate depleted mice, whilst the Hyp mice remained unchanged. Control mice responded to the cAMP stimulus with a 10 fold increase in renal 1α- hydroxylase activity and the Hyp mice responded with a significantly lesser response. The lack of response in the Hyp mice was not due to the hypophosphataemia, as the phosphate depleted mice also responded to a greater amount than the Hyp mice. These data suggested that abnormal regulation of 1,25-(OH)2D3 results from aberrant intracellular regulation of 1α- hydroxylase (Nesbitt et al., 1989). Tenenhouse et al. (Tenenhouse et al., 1992) found that when Gy mice were reared on a low phosphate diet they exhibited a fall in plasma 1,25-(OH)2D3 and a rise in the renal degradative pathway, which contrasted to the results found in control mice. These experiments were evidence that Hyp and Gy are phenotypically similar.

Tenenhouse and Henry (Tenenhouse and Henry, 1985) found evidence that there was a significant increase in protein kinase C activity in the 20000 x g supernatant fraction from Hyp kidneys in comparison to the control littermates, and that this increase was specific to the kidney; other organs where the enzyme is active did not show this increase. They suggested that this showed that inappropriately high protein kinase C activity is specific to the Hyp kidney, and so may be related to the renal defects in the
brush border membrane phosphate transport, and vitamin D metabolism.

The effect of 1,25-(OH)\(_2\)D\(_3\) on the rachitic lesions in Hyp mice was looked at by Marie et al. (Marie et al., 1982). This treatment had been found to be beneficial on bone mineralisation in human HYP patients (section 1.5.1). They found that the rachitic and osteomalacic lesions were healed in young Hyp mice when treated with 1,25-(OH)\(_2\)D\(_3\), and that endosteal bone mineralisation was restored at doses that did not induce hypercalcemia or diminish renal function.

There is an increase in renal 24-hydroxylase mRNA in Hyp mice. This increase was found to be due to transcriptional activation of the 24-hydroxylase gene. The 24-hydroxylase transcripts were found to be localised to the proximal renal tubule in both Hyp and normal mice fed control and low phosphate diets, and 1,25-(OH)\(_2\)D\(_3\) elicits a dose-dependent increase in 24-hydroxylase mRNA in the same nephron segment (Roy and Tenenhouse, 1996)

1.6.2 Hyp Osteoblasts

Evidence of an intrinsic osteoblast defect was demonstrated by Ecarot-Charrier et al. (Ecarot-Charrier et al., 1988). They transplanted periosteal and osteoblast cells from control and Hyp mice into both genotypes and analysed the bone formed. They found that the extracellular environment was critical for the formation of bone in transplants, but that the Hyp cells transplanted into control mice did not form normal bone which
suggested that there are intrinsic defects in osteoblasts in Hyp mice. Transplanted osteoblasts from phosphate depleted normal mice into normal mice produced normal bone, which showed that the inability of Hyp bone cells to produce normal bone was not due to the environment the cells were in before transplant, but more likely due to a defect in the bone (Ecarot et al., 1992a). In later experiments periostea from control and Hyp mice were pair transplanted into control or phosphate supplemented Hyp mice, or into control or phosphate deprived normal mice. They looked at the bone nodules that were formed in the transplants after two weeks by measuring the thickness of the surrounding osteoid seams and relative osteoid volumes. In the phosphate deprived normal mice bone formation by normal cells was impaired, and the defective bone formation by the Hyp cells was worsened. Phosphate supplementation of the Hyp mice normalised the bone formation of the normal cells, but not the Hyp cells. This showed that the abnormal bone formation was not due to the hypophosphataemia, but a defect in the osteoblast (Ecarot et al., 1992b).

Ecarot et al. (Ecarot et al., 1995) also looked at whether the bone formation by the mutant cells could be corrected by 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Control and Hyp mice were infused continuously with 1,25-(OH)\textsubscript{2}D\textsubscript{3} in supraphysiological concentrations. It was found that the defective bone formation by normal cells was corrected in Hyp mice, but not the defective bone formation by the Hyp cells, although it was improved.

Further evidence for an osteoblast defect was shown by Yamamoto et al. (Yamamoto et al., 1992), who looked at the response of isolated Hyp osteoblasts to 1,25-(OH)\textsubscript{2}D\textsubscript{3}.
They found that the extracellular phosphate modulates the action of 1,25-(OH)₂D₃ on osteoblasts, and that this modulation was altered in Hyp osteoblasts. This work supported earlier work that had suggested that there was a diminished response of the osteoblast to 1,25-(OH)₂D₃ in Hyp (Glorieux et al., 1980).

The phospholipid content of the bones of male Hyp mice were found to be reduced relative to those of the control animals. These complexes are thought to have a role in mineral deposition and so this change may relate to the mineralisation defect, and were thought to be due to the phosphate deficiencies at the sites of bone formation (Boskey et al., 1991).

1.6.3 Vitamin D and Calcium Binding proteins

Children with HYP suffer from an impaired intestinal mineral absorption, and it has been proposed that this is also found in Hyp mouse during the growth phases, and that it is associated with low calcium binding protein (CaBP) levels (Bruns et al., 1984). Hyp mice also have also been reported to have a significant decrease in the nuclear uptake of 1,25-(OH)₂D₃ by the duodenal mucosal cells (Yamamoto et al., 1985), which they proposed was the reason for the decreased synthesis of CaBP. Later work by Bruns et al. (Bruns et al., 1987) showed that the levels of the 9 kd and the 28 kb CaBP in both the kidneys and intestine of the juvenile Hyp mouse were reduced; more so in the intestine than in the kidney. The levels in the adult were in the normal range. Administration of 1,25-(OH)₂D₃ induced normal or supranormal levels of the 9 kd
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CaBP, whilst the 28 kd CaBP was unaffected by 1,25-(OH)₂D₃ administration. The reduced levels of CaBP were found to correlate with the observed intestinal malabsorption of calcium.

Young (5 week old), Hyp mice have been shown to have a lower balance of Ca, with higher faecal Ca and low urinary Ca, whilst older (12 week old), mice had balances that were not significantly different to the control mice. Meyer et al. (Meyer et al., 1984) concluded that it was the low levels of duodenal CaBP that caused this low intestinal absorption, but that it was likely that this defective absorption was due to a lack of plasma 1,25-(OH)₂D₃. This intestinal malabsorption was found in Gy mice as well as Hyp mice (Woodward et al., 1993).

1.6.4 Phosphate handling

Harvey and Tenenhouse (Harvey and Tenenhouse, 1992) looked at the Na⁺-phosphate cotransport system in the renal brush border membranes in the Hyp mouse. They found that the Hyp mouse responded normally to the Na⁺ gradient driving force, membrane potential and pH; which ruled out several possible mechanisms for the phosphate transport defect in the Hyp mouse.

1.6.5 PTH levels in the Hyp mouse

Kiebzak et al. (Kiebzak et al., 1982), reported that Hyp mice have significantly higher plasma PTH levels than normal mice, and suggested that this was due to secondary
hyperparathyroidism and concluded that this secondary hyperparathyroidism may be
due to skeletal resistance to endogenous PTH, and that this may have been a
contributing factor to their characteristically elevated renal excretion of phosphate. In
another report Kiebzak and Meyer (Kiebzak and Meyer, 1982), looked at
thyroparathyroidectomised (TPTX) mice to test for renal hypersensitivity and skeletal
resistance to PTH, but showed that the Hyp mice were not hypersensitive.

1.6.6 Parabiosis in the Hyp mouse

Meyer et al. (Meyer et al., 1989a), surgically joined normal and Hyp mice by
parabiosis at 4 weeks of age. The normal mice joined to Hyp mice had a progressive
diminution of plasma phosphate over the following 3 week period, which approached
that of Hyp mice. These normal mice had a greater renal phosphate excretion index
(urine P/plasma P/urine creatinine) than normal parabiosed mice, which suggested a
reduced renal tubular reabsorption of phosphate. There was no rise in plasma calcium
or plasma 1,25-(OH)2D3, and there was a significant reduction in femoral mineral
content, although not in femoral length or body growth, in comparison to the normal-
normal linked pairs. The change in renal handling was specific since urinary loss of
potassium and magnesium were not affected. Separation of the normal-Hyp pairs after
3 and 6 weeks after parabiosis caused the normal mice to achieve normal plasma
phosphate levels within 24 hours, and they had plasma phosphate levels higher than
normal mice separated from normal-normal parabiosed pairs. They concluded that
these data suggested the presence of a phosphaturic factor in the Hyp mice that
crossed the parabiotic union into normal mice, and induced many of the symptoms of X-linked hypophosphataemia. In other words, the defect causing X-linked hypophosphataemia is due to a humoral factor, as oppose to an intrinsic kidney defect.

To determine whether PTH was the hormone involved Meyer et al. (Meyer et al., 1989b), once again linked mice by parabiosis, but this time with mice that had either undergone surgical parathyroidectomy (PTX) or sham surgery. The surgery was carried out three weeks after the mice had been linked by parabiosis. It was found that PTX mice did not eliminate the changes in the normal mice that had been parabiosed to Hyp mice, the normal mice remained hypocalcemic relative to the normal-normal controls in both the sham operated and the PTX mice. It was also found that in the PTX mice, the plasma phosphate was still lower in the normal mice parabiosed to Hyp mice when compared to the normal-normal mice. They concluded that as the renal handling of phosphate caused by parabiosis to a Hyp mouse persisted after PTX, it was unlikely that the humoral factor that causes the renal phosphate loss was PTH.

1.6.7 Kidney crosstransplantation

In an attempt to determine whether the defect in X linked hypophosphataemia is due to an intrinsic kidney defect, or due to a humoral factor, Nesbitt et al. (Nesbitt et al., 1992), crosstransplanted kidneys from Hyp mice and normal mice and examined phosphate homeostasis. They first looked at the effects of uninephrectomy on the
indices of phosphate metabolism that identified the mutant phenotype. They found no differences in the serum phosphate concentration, fractional excretion of phosphate (FEP), or tubular reabsorption of phosphate per millilitre of glomerular filtrate (TRP) in uninephrectomised normal and Hyp mice, when compared to the sham operated controls. They then transplanted kidneys from normal and Hyp mice into normal and Hyp recipients. Normal mice transplanted with normal kidneys, and Hyp mice transplanted with Hyp kidneys showed no differences in serum phosphorus, FEP and TRP when compared to normal and Hyp mice respectively. Engraftment of normal kidneys into Hyp mice, and mutant kidneys into normal mice did not correct or transfer the defect, serum phosphorus, FEP and TRP were not affected in the recipient. They concluded that the Hyp mouse defect is not due to a kidney defect but instead to a humoral factor.
1.7 Aims of this project

In this chapter I have described the change in the clinical appearance of clinical rickets from a nutritional disease to an inherited one. I have described the different forms of rickets that are found, particularly X-linked hypophosphataemic rickets, the most frequent cause of rickets in humans, and I have given a description of vitamin D and phosphate metabolism in humans, as both of these are important in HYP. The murine models Hyp and Gy have also been described, as many experiments on them have helped to elucidate many facts about X-linked hypophosphataemic rickets.

The actual genetic defect that causes X-linked hypophosphataemic rickets was unknown, and the aim of this project was to help isolate the gene that is involved in this disease. The work that I carried out on this is described in Chapter 3, and the work I performed on identifying the mutations in HYP patients is described in Chapter 4. I have also suggested a possible mechanism for the action of the gene involved in HYP.
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Unless otherwise stated, all procedures were carried out at room temperature. All water was double distilled in glass apparatus.

2.1 Preparation of DNA from whole blood.

10 mls of blood, containing 100 µl 0.5M EDTA (as anticoagulant) was mixed with 90 mls cell lysis buffer, (0.32M sucrose, 10 mM tris-HCl pH 7.5, 5 mM MgCl₂, 1% triton X 100) at 4°C. The sample was centrifuged at 1500 x g for 15 minutes at 4°C. The resultant pellet of nuclei was resuspended in a solution containing 4.5 ml of 0.075M NaCl; 0.024 M EDTA pH 8.0, 0.5 ml of a solution containing 5% SDS with 2 mg/ml proteinase K added, and the resultant solution incubated for 12-16 hours at 50°C. Following this digestion, the solution was mixed gently with 5 ml of phenol (Tris saturated, pH 8.0), centrifuged briefly, and the aqueous phase collected. The extraction procedure was repeated once on the aqueous phase and again the aqueous phase was collected. To this aqueous phase, 5 mls of chloroform and iso-amyl-alcohol (24:1 v/v) was added and mixed gently. Following a brief centrifugation the aqueous phase was once again collected and 0.5 ml 3M sodium acetate added together with 11 mls absolute ethanol. The tube was inverted gently until the DNA precipitated. The DNA was harvested using an inoculating loop and placed into 0.8 ml of TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and left at 4°C for 2 days to dissolve.
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2.2 Small scale preparation of plasmid and cosmid DNA.

The bacterial clones were grown in 10 mls LB broth (1% Bacto-tryptone, 1% NaCl, 0.5% Bacto-yeast extract) containing 100 μg/ml ampicillin antibiotic overnight at 37°C. The cells were harvested by centrifugation at 1500 x g for 10 minutes and resuspended in 400 μl TEG buffer (50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). The sample was transferred to a 1.5 ml Eppendorf tube and 400 μl of a solution containing 0.2 M NaOH, 1% SDS was added and mixed. 200 μl of 3M Potassium acetate was then added and the tubes vortexed to mix. The tubes were centrifuged at 12000 x g and the supernatant collected. The solution was then extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 v/v/v) (PIC), the aqueous layer collected and the extraction procedure repeated. An equal volume of aqueous ether was added, mixed and the upper ether level discarded. 450 μl (0.6 volumes) of isoproponol was added, vortexed to mix and this was then incubated at room temperature for 30 min. The incubations were centrifuged at 12000 x g for 15 minutes and the resultant pellet washed with 70% ethanol, dried, and finally resuspended in 50 μl TE buffer.

2.2.1 Preparation of P1 DNA

The bacterial clones were grown in 10 mls LB broth containing 30 μg/ml kanamycin antibiotic overnight at 37°C. The cells were harvested by centrifugation at 1500 x g for
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10 minutes and resuspended in 300 μl TEG buffer. The sample was transferred to a 1.5 ml Eppendorf tube and 600 μl of a solution containing 0.2 M NaOH, 1% SDS was added and mixed. 450 μl of 3M potassium acetate was then added and the tubes vortexed to mix. The tubes were centrifuged at 12000 x g and the supernatant collected. The solution was then extracted with an equal volume of PIC, the aqueous layer collected, and the extraction procedure repeated. 0.6 volumes of isopropanol was added to the aqueous phase, vortexed to mix and then incubated at room temperature for 20 min. The incubations were centrifuged at 12000 x g for 15 minutes and the resultant pellet washed with 70% ethanol, dried and resuspended in 50 μl TE buffer.

2.3 Purification of DNA from agarose gels using GeneClean II

DNA was electrophoresed on agarose gels in 0.5 x E buffer (0.4 M Tris, 0.2 M sodium acetate 3H₂O, 0.02 M EDTA disodium salt) containing 0.5 μg/ml ethidium bromide. The DNA was visualised under UV light and the bands excised from the gel. The gel slices were weighed, three volumes of 6 M sodium iodide solution added and incubated for 5 minutes or until the gel had melted. 5 μl of Glassmilk (suspension of silica matrix in water) was added to the solution and this was incubated for 5 minutes at room temperature until the DNA was adsorbed on the silica matrix. The solution was centrifuged at 12000 x g for 5 seconds to pellet the silica, the supernatant discarded, and the pellet washed three times with New Wash solution, (50% alcohol,
with NaCl, Tris, EDTA). 10 μl of TE buffer was added to the pellet and incubated at 50°C for 5 minutes to elute the DNA. The supernatant containing the DNA was collected in a fresh tube and the elution step repeated.

2.4 Polymerase Chain Reaction (PCR)

Standard PCRs were performed in a total volume of 25 μl, containing 50 mM KCl, 10 mM Tris-HCl, 200 μM dNTPs, 1 μM of each primer (table 2.1), and 25 U/ml of Taq polymerase. The PCR cycle consisted of a 2 minute predenaturation step at 94°C, followed by 35 cycles, each one consisting of 1 minute at 94°C, 1 minute at the applicable annealing temperature (table 2.1), and 1 minute at 72°C. The reaction was finished with a final extension step consisting of 7 minutes at 72°C. The samples were then cooled to 4°C. The PCR conditions for two of the primer pairs used differed from the above: DXS1052 had an extension temperature of 67°C, and M13 had an extension at 72°C for 2 minutes, and only had 25 cycles (section 2.13).

2.4.1 Hotstart PCR

For hotstart PCR, the PCRs were set up as outlined above (section 2.4) but without the Taq polymerase added. The PCR tubes were heated to 95°C before the enzyme was added, and then cycled as detailed above.
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<table>
<thead>
<tr>
<th>Marker Name</th>
<th>Primer Sequence</th>
<th>Annealing temperature</th>
<th>MgCl₂</th>
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<td>1.6 mM</td>
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<tr>
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<td>DXS999</td>
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<td>1.25 mM</td>
</tr>
</tbody>
</table>

Table 2.1. Primers used for PCR.

2.5 End labelling of oligonucleotides for linkage analysis using microsatellite markers.

110 pmoles (1-3 μl) of one of the oligonucleotides for the PCR was added to 3.5 μl

10 x kinase buffer (0.5 M Tris-HCl pH 7.6, 0.1 M MgCl₂, 50 mM dithiothreitol

(DTT), 1 mM spermidine, 1 mM EDTA pH 8.0), 10 U T4-polynucleotide kinase,
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140 μCi γ ^{32}P ATP (3000 Ci/mmol) (Amersham), the volume made up to 35 μl with H₂O and was incubated at 37°C for 1 hour, followed by heat denaturation at 95°C for 5 minutes. This produced enough labelled oligonucleotide for a 50 x 25 μl PCRs with no further purification needed. The final volume of radiolabelled oligonucleotide per 25 μl reaction was 2.2 pmoles, and this volume was increased to a final concentration of 1 μM by the addition of unlabelled oligonucleotide. The PCR was then performed as outlined in section 2.4, using DNA from the patients families. Once complete 4.5 μl of the PCR was added to 5 μl of Stop solution, (0.3% Bromophenol Blue and Xylene Cyanol FF, 10 mM EDTA (pH 7.5) and 97.5% deionised formamide). The samples were then heated to 80°C for 2 minutes to denature them, placed on ice and loaded onto a 6% polyacrylamide gel (all markers except DXS7474 which was loaded onto a 4% gel). The gel was electrophoresed at 60 W for 1½ to 3 hours (depending upon the size of the product expected) in 1 x TBE (0.09 M Tris borate, 0.002 EDTA). The gel was then dried onto Whatman 3 MM paper, and a photographic film placed over it for 1 to 3 days until the image had developed.

2.6 Southern Blotting

DNA was electrophoresed on an agarose gel, either 2.5% Nusieve agarose (FMC Biotechnologies) for fragment sizes of less than 1 kb, or 0.8% Seakem agarose (FMC Biotechnologies) for fragments larger than 1 kb. The buffer used for electrophoresis was 0.5 x E containing 0.5 μg / ml ethidium bromide added to stain the DNA. The conditions for electrophoresis differed depending upon the size and composition of the
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gel. Once the DNA had electrophoresed into the gel, the gel was destained for 1 hour in H$_2$O. If the fragments were of a large size the gel was depurinated in HCl for 10 minutes, for smaller fragments this step was omitted. The gel was then soaked in 0.4M NaOH for 20 minutes. A nylon filter, Hybond N+ (Amersham), was pre-wetted in H$_2$O and soaked for 5 minutes in 0.4 M NaOH. A wick of blotting paper (3MM paper, Whatman) was soaked in 0.4 M NaOH. The gel was placed upon this wick, the Hybond filter was placed on top of the gel and covered with 5-10 sheets of pre-soaked blotting paper, and absorbent tissue. A 1 kg weight was placed on top of the tissue and this was left overnight at room temperature whilst the DNA transferred to the Hybond filter. Once the DNA had transferred, it was washed twice in 2 x SSC (20 x SSC 3 M sodium chloride, 0.3 M sodium citrate) and stored, wrapped in Saran wrap, at either 4°C for short term storage, or at -70°C for long term storage. To label the DNA, the filter was rinsed in 2 x SSC and placed in a hybridisation tube with hybridisation buffer, either standard hybridisation buffer [4 x SSC, 10 x Denhardt's {2% (w/v) Polyvinyl pyrolidine, 2% (w/v) Ficoll, 2% (w/v) bovine serum albumin (BSA)}, 0.1% (w/v) sodium pyrophosphate, 5% (w/v) dextran sulphate, 50 µg / ml sonicated salmon sperm DNA], or when labelling with a d(C.A)$_n$-d(G.T)$_n$ heteropolymer (Pharmacia Biotech), using Church buffer (0.5 M disodium hydrogen phosphate, 7% (w/v) SDS, 1 % (w/v) BSA, 1 mM EDTA). The filters were incubated in this buffer for 6 hours at 65°C.

The probe for hybridisation was prepared using one of two methods, random primer labelling (section 2.6.1) or nick translation (section 2.6.2).
The labelled probe was heat denatured at 95°C for 3 minutes, then placed on ice for a further 2 minutes. The probe was then added to prewarmed hybridisation buffer, (Church buffer was used for the d(C.A)n-d(G.T)n heteropolymer). The filters to be probed were conditioned in prehybridisation buffer for 6 hours at 65°C. When ready to perform the hybridisation reaction the prehybridisation buffer was removed from the filters and 10 ml of fresh hybridisation buffer containing the denatured probe were added. The DNA bound to the filters was hybridised for 12 hours at 65°C. When using labelled cosmids as probes, or other DNA that contained (or was likely to contain) repeat sequences, the protocol was modified to remove the repeat sequences by incubating the labelled probe with total placental human DNA (Sigma) prior to adding it to the filter (section 2.6.3).

After hybridisation the filters were washed twice for 15 minutes in 2 x SSC 0.1% SDS (w/v), twice in 1 x SSC 0.1% SDS, and twice in 0.1 x SSC 0.1% SDS at room temperature, then twice in 0.1 x SSC 0.1% SDS at 65°C. The filters were wrapped in Saran wrap and exposed to photographic film.

2.6.1 Random primer labelling.

Labelling was carried out using the Amersham Megaprime kit. 25 ng of DNA was added to 5 μl of nonomer primer and sufficient H₂O added to make the final volume
50 μl. The sample was incubated at 95°C for 5 minutes to separate the DNA strands and allowed to cool to room temperature to allow the nonomer primers to anneal to the DNA. 5μl of labelling buffer (10 x concentrated buffer containing Tris/HCl pH 7.5, β- mercaptoethanol and MgCl₂) was added, together with 4 μl of each dNTP, (omitting the dNTP(s) used to label the DNA), 5μl of the labelled dNTP, (³²P dNTP, 50 μCi, 3000 Ci/mmol (Amersham)) and 2μl of Klenow fragment DNA polymerase. The sample was mixed gently and incubated at 37°C for 10 min. The labelled DNA fragment was purified by centrifugation at 1000 x g for 6 minutes at room temperature through a pre-prepared Sephadex spin column, which retains free nucleotides in the column matrix.

2.6.2 Labelling by nick translation.

150 ng of the probe DNA to be labelled was added to 20 μl of nucleotide labelling buffer (100 μM each of dATP, dGTP, and dTTP in Tris/HCl pH 7.8, 2-mercaptoethanol, and MgCl₂, 10 μl ³²P dCTP [50 μCi, 3000 Ci / mmol (Amersham)], and H₂O was added to give a final volume of 90 μl. 10 μl of enzyme (DNA polymerase I and DNase I) was added and the sample incubated for two hours at 15°C and the labelled DNA fragment purified by centrifugation using a Sephadex spin column (see above).
2.6.3 Preparing DNA with repeat sequences for Southern blots

For DNA with repeat sequences, the DNA was labelled as described above, and the Southern filters prepared in the same way. When the filters were prehybridised, sonicated human placental DNA (Sigma) at 200 μg/ml was added. The labelled DNA probe was prepared as described above, and 6 mg of sonicated human placental DNA added. The sample was heated to 100°C for 5 minutes, cooled to 65°C and maintained at this temperature for 5 minutes. The DNA probe was then added to a solution containing 400 μl 20 x SSC and 400 μl of 25% dextran sulphate (to give a final concentration of 5 x SSC, 5% (w/v) dextran sulphate), with sufficient H₂O to make a total volume of 2 ml. This was incubated at 65°C for 6 hours before being added to the hybridisation buffer, which was then added to the filter on which the DNA to be probed was bound.

2.7 isolation of microsatellite (CA) repeats (Rowe et al., 1994)

DNA was isolated from P1 clones (section 2.2.1), separated using pulse field gel electrophoresis (PFGE section 2.15) in 1% low melt agarose using 0.5 x TBE as the running buffer. The gel was electrophoresed for 30 hours at 165 volts (18 watts, 111 amps, step time of 90 seconds). The gel was stained for 30 minutes in 0.5 x TBE containing 0.5 μg/ml ethidium bromide, the DNA visualised using UV light, and the P1 DNA excised from the gel. The gel slices were then melted at 65°C for 5 minutes and 17 μl of the preparation aliquoted into separate tubes containing 2 μl of low salt.
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restriction enzyme buffer. The DNA was digested at 37°C for 4 hours with restriction enzymes (10 U), which give blunt ends after digestion, and work optimally in low salt buffers (SnaB1, Dra1, Sma 1, Stu1, Rsa1) and then cooled to room temperature.

Figure 2.1: Protocol for isolation of CA microsatellite repeats. The Cosmid / P1 DNA is digested with blunt ended restriction enzymes and the preannealed linker ligated on. The ends of the linker are then filled in and the DNA fragments amplified by PCR.

2.5μl of 10 x ligase buffer (0.5 M Tris-HCl, 100 mM MgCl₂, 100 mM dithiothreitol, 500 μg/ml BSA), 2 pmol of linker, (which was prepared by annealing two oligonucleotides, [the linker long strand (LLS) 5'-gcg gtg acc egg gag ate tga att tga atc-3' and linker short strand (LSS) 5'-gaa ttc aga tc-3'] and the two primers were mixed in equimolar amounts, heated to 65°C for 5 minutes and left to cool to room temperature. The resultant double stranded linker was adjusted to 10μM) and 2 U T4 ligase were added to the digested DNA. The samples were incubated for 12 hours at
room temperature and the reaction mixes of each restriction digest were melted at 65°C for 5 minutes. 2 µl were added to PCR reaction mixtures containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM tris-HCl, 5 mM NH₄Cl, 125 µM dNTPs, 40 pmol of LLS primer and 25U/ml Amplitaq DNA polymerase. The linker was filled in by incubating at 65°C for 5 minutes (figure 2.1) and the fragments amplified by PCR (figure 2.1). The PCR was as follows: 2 minutes 95°C predenaturation, 35 cycles of PCR (1' 95°C, 2' 65°C, 2' 72°C) with a final extension round of 72°C for 7'. The PCR product was then electrophoresed on a 2.5% Nusieve agarose gel, blotted onto Hybond N+ and screened for the presence of a CA microsatellite repeat with nick translated radiolabelled d(C.A)ₙ-d(G.T)ₙ heteropolymer (Pharmacia Biotech). Positive bands to which the probe annealed were excised from duplicate gels and subcloned into a PCR cloning vector (Invitrogen PCRᵀᴹ). 15 recombinants were selected using blue/white colour selection for the presence of an insert in the plasmid, amplified by PCR using M13 Rev and M13 Fwd primers (table 2.1) with the M13 Fwd primer being biotinylated. One half of the product was electrophoresed on a gel, blotted onto Hybond N+ (section 2.6) and screened with the CA heteropolymer. The stored aliquots of the samples to which the probe annealed and that were therefore positive for CA microsatellites, were sequenced (figure 2.2) using solid phase sequencing (section 2.8).
Figure 2.2 Section of sequencing gel showing the CA repeat of DXS7473
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2.8 Sequencing DNA using magnetic beads: Solid phase Sequencing.

The DNA samples for sequencing were amplified by PCR, using primers where one was 5' biotinylated. The products were electrophoresed on an agarose gel, the bands excised, and purified by GeneClean (section 2.3). 20 μl of streptavadin-bound magnetic beads (Dynabeads M-280 (Dynal)) were washed for each template to be sequenced in 20 μl of 1 x Binding and Washing (B&W) buffer, (2 x Binding and Washing buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2M NaCl) and then resuspended in 40 μl 2 x B&W. The washed beads were added to 40 μl of the purified PCR product, mixed, and incubated at room temperature for 15 min. For products >1 kb the incubation time and temperature was increased to 37°C for at least 30 minutes. During the incubation the tubes were mixed gently to keep the beads resuspended. The beads were then harvested using a magnetic particle collector (MPC), washed with 40 μl 1 x B&W and then stored at 4°C until ready to sequence.

The beads with the DNA bound onto them, were collected, the supernatant discarded and 8 μl of 0.1 M NaOH added and incubated at room temp for 10 min. The supernatant was again discarded and the beads washed once with 50 μl NaOH, once with 40 μl 1 x B&W and once with 50 μl TE. The beads were finally resuspended in 10 μl of water. For the sequencing, 2 μl of sequencing primer (0.8 μM) and 2 μl of annealing buffer (1 M Tris-HCl (pH 7.6), 100 mM MgCl₂ and 160 mM DTT) were
added to the DNA which was bound to the beads, and mixed gently. The samples were incubated at 60°C for 10 minutes, allowed to cool to room temperature for 10 minutes. 3 μl of labelling mix (1.375 μM each of dCTP, dGTP, and dTTP, and 333.5 mM NaCl), 1 μl of 35S ATP (Amersham) and 2 μl of diluted T7 polymerase (1:5) were added and the samples incubated at room temperature for five minutes. Four tubes containing 2.5 μl of each ddNTP (A, C, G, or T) were prepared and warmed to 37°C. 4.5 μl of the labelling reaction was added to each of the four tubes containing the ddNTP's and incubated at 37°C for 5 minutes. The reactions were terminated by the addition of 5 μl Stop Solution. The products were heated to 80°C, placed on ice, and loaded on an 8% polyacrylamide gel, which had been prerun at 60 watts for 30 minutes prior to loading. The polyacrylamide gel was run in 1 x TBE at 60 watts for between 3 and 6 hours.

2.9 Automated sequencing on the PE Biosystems 373.

The product to be sequenced (either plasmid or PCR product) was electrophoresed on an agarose gel and purified using GeneClean II (section 2.3). The DNA (for a PCR product approximately 15 ng/100 bp was added; for plasmids 0.5-1 μg was used), was added to 9.5 μl reaction premix (1.58 μM A-DyeDeoxy, 94.74 μM T-DyeDeoxy, 0.42 μM G-DyeDeoxy, 47.37 μM C-DyeDeoxy, 78.95 μM dITP, 15.79 μM dATP, 15.79 μM dCTP, 15.79 μM dTTP, 168.42 mM Tris-HCl (pH 9.0), 4.21 mM (NH4)2SO4, 42.10 mM MgCl2, 0.42 U/μl AmpliTaq DNA polymerase ), 3.2 pmol sequencing primer and water to a total volume of 20 μl. This was overlaid with 40 μl
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mineral oil and the tubes were placed in a Perkin Elmer Cetus 480 thermal cycler preheated to 96°C and cycled for 25 cycles at 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The sequencing products were purified by phenol/chloroform extraction. For this procedure 80 μl water was added to the reaction mix along with 100 μl chloroform, to dissolve the mineral oil. The chloroform layer was removed, the sample extracted with 100 μl phenol/H₂O/chloroform (68:18:14 v/v/v), vortexed and centrifuged at 12000 x g for 1 min. The lower organic phase was discarded and the extraction procedure repeated on the aqueous phase with a second aliquot of phenol / H₂O / chloroform mix. After the second extraction procedure, the sample was again vortexed and centrifuged at 12000 x g for 1 minute, and the upper aqueous phase collected. The products were precipitated by the addition of 15 μl 2M sodium acetate, pH 4.5, and 300 μl absolute ethanol. The samples were vortexed, centrifuged at 12000 x g for 15 min, and the resultant pellets washed with 70% ethanol and dried. 4 μl of loading dye was used to redissolve the pellets prior to loading onto the PE Biosystems 373 automated sequencer.

2.10 Single Stranded Conformational Polymorphism (SSCP)

The DNA from 81 patients was amplified by PCR using primers that flank each exon of the PHEX gene (table 2.2). The DNA was electrophoresed on a Multiphor II electrophoresis unit (Pharmacia Biotech) using the DNA Fragment Analysis Kit (Pharmacia Biotech). The gels used for the run were premade dehydrated gels (CleanGels) which were rehydrated in 25 ml of rehydration buffer. The CleanGel
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Table 2.2. Primer sets used for SSCP. Primers SSCP6 and SSCP7 were supplied by Dr Tim Strom. Primers SSCP1 gave poor amplification, and so alternative primers SSCP9 were designed.

was placed on the Multiphor II Electrophoresis Unit with two pre-soaked electrode strips placed 4 mm from the top and bottom of the gel. 2-3 µl of the PCR product was added to the loading buffer and heated to 95°C for 3 minutes, cooled on ice, and loaded onto the gels. The gel was prerun for 10 minutes at 200 V, and then the
voltage was increased to 600 V for 45 minutes. The bands were visualised by silver staining using the Stratagene silver staining kit.

2.11 Silver staining of SSCP gels using the Stratagene kit

The gel to be stained was fixed for 30 minutes in 50% methanol, washed in water for 1 x 30 minutes, and 2 x 15 minutes. The gel was then submerged in reducing solution (Stratagene) and incubated for 20 minutes with shaking. The reducing solution was drained off and 200 ml of silver staining solution (Stratagene) added, and this incubated with shaking for 20 minutes. The silver staining solution was then drained off, the gel washed for 10 seconds in water and 50 mls of developer solution (Stratagene) added. When the developing solution began to change colour it was drained off and a further 50 mls added until that started to change colour. This was also drained, 200 mls of the developer solution added, and the gel incubated in this with shaking until the desired staining intensity was achieved. 25 mls of stop solution (Stratagene) were added and the gel incubated in this with shaking for 5 to 10 minutes. The solution was drained and the gel rinsed in water. The gel was then wrapped in cellophane for storage at room temperature.
Chapter 2: Materials and Methods

2.12 Cloning of PCR products using Invitrogen's TA cloning system

DNA for cloning was amplified by PCR in a 25 µl reaction (section 2.4), and gel purified using GeneClean II (section 2.3). 2 µl of fresh, purified, PCR product was added to 1 µl ligation buffer (60 mM Tris-HCl, pH 8.3 (at 42°C); 60 mM MgCl₂; 50 mM NaCl; 1 mg/ml BSA; 70 mM β-mercaptoethanol; 1 mM ATP; 20 mM DTT; 10 mM spermidine), 2 µl pCR™ vector (25 ng/µl), 1 µl T4 ligase and 4 µl water and incubated at 14°C overnight. It was critical that the PCR amplicon to be cloned was fresh, as the T overhang is lost with time. For each transformation reaction, one vial of OneSHOT™ cells was thawed on ice and 2 µl β-mercaptoethanol was added and mixed by tapping the tube. 1 µl of the ligation mix was added and the cells once more mixed gently by tapping. The cells were incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, incubated on ice for 2 minutes and rescued by the addition of 450 µl of SOC medium (2% tryptone; 0.5% Yeast extract, 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄; 20 mM glucose (dextrose)). The cells were then incubated at 37°C for 1 hour with shaking, 200 µl of the cells were plated onto LB agar plates (LB broth with 15g/l agar added) containing 50 µg/ml ampicillin or kanamycin and 25 µl X-gal (40 mg/ml stock in dimethylformamide) and the plates incubated at 37°C overnight. The plates were placed in a fridge at 4°C for up to 3 hours to allow the blue colour to develop in background colonies.
Chapter 2: Materials and Methods

2.13 Amplification of plasmid DNA by Polymerase Chain Reaction

A white colony from the LB plates was picked on an inoculating loop, and transferred into 50 µl of PCR mix (50 mM KCl, 10 mM tris-HCl, 200 µM dNTPs, 1.25 mM MgCl₂, 1 µM M13 fwd primer, 1 µM M13 rev primer (table 2.1), and 25 U/ml Taq polymerase) and then into 5 mls LB + amp (50 µg/ml). The broth was incubated overnight at 37°C and the cells frozen at -70°C in 15% glycerol for long term storage. The PCR cycle consisted of a 10 minute predenaturation step at 94°C, followed by 25 cycles, each one consisting of 1 minute at 94°C, 1 minute at 51°C and 2 minutes at 72°C. The reaction was finished with 1 cycle at 72°C for 7 minutes. The samples were then cooled to 4°C. Broth cultures and frozen stocks were also amplified in this manner, using 1 µl of cells grown in LB broth, or using a scraping of frozen stocks.

2.14 Extraction of yeast DNA

The yeast was grown in AHC media [0.67% yeast nitrogen base without amino acids (Difco), 1% acid hydrolised casein (Difco), 20 mg/l adenine hemisulphate, pH adjusted to 5.8 with approximately 70 µl 12N HCl, 50 mls 40% (w/v) D-glucose (filter sterile)] at 30°C for 48 hours. The cells were harvested by centrifugation at 900 x g and the pellet rinsed twice in distilled water. The yeast cells were then resuspended in 1 ml distilled water, 1 ml of 1% Incert agarose (FMC) added, and poured into plug moulds at 4°C. Once the plugs had set, they were transferred to 10 volumes of SCEM (1M sorbitol, 10 mM EDTA pH 8.0, 0.1M sodium citrate pH 5.8, 30 mM...
β-mercaptoethanol) with 1 U/ml Zymolase 20-T and incubated at 37°C for 3 hours.

The SCEM was replaced with an equal volume of DLS [1% (w/v) dodecyl lithium
sulphate, 50 mM NaCl, 10 mM Tris-HCl pH 7.8], incubated 50°C for 2 hours,
replaced with a fresh volume of DLS and incubated overnight at 50°C. The plugs were
then rinsed several times with TE and stored in TE at 4°C until used.

2.15 Pulse Field Gel Electrophoresis (PFGE)

YAC and P1 DNA were separated on pulse field gels. The gels were 1% gels, using
either normal or low melt agarose, with 0.5 x TBE as the running buffer and a running
temperature of 9°C. The DNA was either loaded using agarose blocks (section 2.14) or
loaded directly onto the gel. For P1 clones and the smaller YACs (≈500 kb) the gel
was electrophoresed for 30 hours at 165 volts (18 watts, 111 milliamps, step time of
90 seconds). For YAC clones that were larger (YAC Y4), ≈1.2 mb, the gel was
electrophoresed at 130 volts (9 watts, 74 milliamps, step time of 125 seconds for
48 hours and 300 seconds for 24 hours.) The gels were stained in buffer containing
0.5 μg/ml ethidium bromide, visualised under UV light, the bands excised and the
DNA either purified by GeneClean (section 2.3) or the agarose melted at 65°C and
used with no further purification.
Chapter 3

Mapping of the HYP locus by linkage analysis
Chapter 3 : Mapping of the HYP locus by linkage analysis

3.1 Introduction

3.1.1 Identification of disease genes

Human disease genes can be identified using two approaches; functional cloning and positional cloning. In functional cloning the gene is isolated based on information regarding the protein product and/or its function. In positional cloning, the isolation of the disease gene starts from the knowledge of its genetic or physical location in the genome, usually with little or no knowledge of its function. The first step in positional cloning is gene mapping; the assignment of the gene to a specific chromosome and part of a chromosome. The most powerful tool for this is linkage analysis (Adolph 1996).

The human genome is estimated to be in the order of 3000 megabases (mb), comprising of an estimated 60000 to 100000 genes; which corresponds to a gene approximately every 40 to 50 kilobases (kb) (Adolph 1996). The disease X-linked hypophosphataemic rickets is, as the name implies, an X-linked dominant disorder. A male therefore will always pass the disease onto his daughters (as they will inherit his X chromosome), but never onto his sons, as they inherit his Y chromosome. A female has a 50-50 chance of passing the disease onto her children regardless of sex. This thesis describes some of the efforts made towards identifying the gene defect causing HYP by linkage analysis.
Chapter 3: Mapping of the HYP locus by linkage analysis

3.1.2 Linkage analysis

Genetic linkage analysis is a technique that allows mapping of disease genes which are detectable only as a phenotypic characteristic. Linkage was first demonstrated by Morgan in 1910, working on fruit flies. Human linkage was described in 1911, with the realisation that colour blindness was expressed only in males, but was inherited through the female line, and was therefore sex linked. Autosomal linkage was first described in 1968 by Donahue, who looked at cosegregation of blood group antigens and a distinctive abnormal chromosome 1 (Adolph 1996).

Linkage analysis is based upon the observed segregation of homologous chromosomes and the alleles they carry, during meiosis in family studies. Genetic linkage can be defined as the tendency for alleles at loci closely located to one another on the same chromosome, to be transmitted together during meiosis. During meiosis, the two sister chromatids align themselves, and exchange sections, in a process known as crossing-over. Breakages occur along the chromatids at the same sites, and the opposite ends are joined, which creates recombinant chromatids. These sites are termed chiasma (figure 3.1). The probability of crossing-over occurring between any two genes increases the further apart they are located on the chromosome. Thus, genes that lie on the same chromosome can still assort independently, particularly if they are a large distance apart. The likelihood of crossovers occurring decreases the closer gene loci are together on a chromosome.
Chapter 3: Mapping of the HYP locus by linkage analysis

Figure 3.1 Crossing over during meiosis. A) The two sister chromatids align. Two alleles for two loci are marked A and B on one chromatid, and a and b on the other. B) Breakages occur, and the opposite ends become joined. C) This produces two recombinant chromatids, which have alleles Ab and aB.

The observed number of recombinants with respect to any two loci is termed the recombination fraction, denoted by the Greek letter theta (θ). If one crossover takes place between two loci then the average number of germ cells which contain a recombinant chromosome is two. If an additional crossover occurs, the probability that the same sister chromatids will be involved is 0.25, which would convert the recombinants to non-recombinants. However, there is also a 0.25 probability that the non-recombinant chromatids will crossover, becoming recombinant. Also, there is a 0.5 probability that one originally non-recombinant will crossover with one of the recombinants, which, whilst this
would alter the recombinant status of the chromatids, does not alter the number of recombinants. This means that the mean number of recombinant chromosomes will be 50% (when looking at two loci). This gives the upper limit of $\theta = 0.5$ when two loci are not linked, for example, when they are on separate chromosomes. Linked loci will have a lower value, reaching zero when the loci coincide; the theta value will be lower the closer two loci are located on a chromosome.

Evidence for linkage is measured by the log likelihood of linkage, or LOD score. This is the log10 of the ratio of the probability that the data would have arisen from linked loci, to the probability that the data would have arisen from unlinked loci. The minimum accepted LOD score taken to show evidence of linkage is 3. This does not however signify a 1 in a $10^3$ possibility of the results having arisen by chance, since any two loci have approximately a 50 times greater probability of not being linked than being linked due to the size of the genome. Therefore, a LOD score of 3 means that the likelihood is 1 in 20 that the data suggests linkage. Maximum LOD scores are shown as $z_{\text{max}}$.

### 3.1.3 Genetic markers used in linkage analysis

For a marker loci to be of use for linkage analysis it must be polymorphic; the higher the level of polymorphism in a population, the more useful the marker. For this study on X-linked hypophosphataemic rickets two types of markers were used, Restriction Fragment Length Polymorphisms (RFLPs) and microsatellites. RFLPs are due to alterations in the target sequence of a restriction enzyme. They are biallelic, each
polymorphism will either have or lack the restriction site. The other type of marker used were microsatellites which are areas of small repeats (usually two or three bases) repeated a large number of times. Microsatellites are usually multiallelic, and so often have a higher rate of polymorphism than RFLPs. They are therefore of great value in a linkage study.

3.1.4 Localisation of the HYP locus by linkage analysis

The gene which causes the inherited disease X-linked hypophosphataemic rickets was first shown to be flanked by the RFLP markers DXS41 and DXS43 (Machler et al. 1986; Read et al. 1986; Thakker et al. 1987). An additional two markers, both RFLPs, were found to be tightly linked to the DXS43 locus, these were DXS197 and DXS207. Markers DXS274 and DXS208, also RFLPs, were found to be closely linked to DXS41 (Rowe et al. 1992).

Initial work on the localisation of the HYP gene was performed on 15 kindreds.

Recombinations in these families localised the HYP locus to lying proximal to DXS43 and DXS197, and distal to DXS274 and DXS41. Recombinations were found in these families that separated markers DXS43 and DXS197, with DXS197 lying closer to the HYP locus, table 3.1. No recombinants were found that separated DXS41 and DXS274. Therefore the most likely gene order was:

Xpter - DXS43 - DXS197 - HYP - (DXS274, DXS41) - Xcen (Rowe et al. 1992)
Ten new markers were used to screen the same group of families, as well as five additional kindreds for whom DNA became available, to identify individuals who were recombinant for the markers. These recombinants were used to order the markers and identify the closest flanking markers.

The family trees of the twenty families used in the study are shown at the end of the chapter, 3.25 to 3.45. Subsets of the families showing key individuals and recombinations are shown in figures throughout this chapter. For all the family trees, males are represented as squares, and females as circles. For affected individuals the shapes are filled in, for unaffected individuals they are open. A diagonal line bisecting the shape shows that the person is deceased.
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Table 3.1: Key recombinants against HYP with the markers studied. The allelic segregation is coded as follows: R recombinant against HYP, N non recombinant against HYP, U uninformative, P phase unknown (refers only to family XHYP06 where the parents have died, and so the alleles for them can only be inferred) - indicates the experiment was not carried out. DXS451 was only used for family E to confirm that the recombination was centromeric to the disease.
3.2 Materials and Methods

Twenty families were used in this study, with a total of 260 individuals. These consisted of 139 males (46 affected, 93 unaffected) and 121 females (88 affected and 33 unaffected). Initially only DNA from 15 of the families were available (a total of 212 individuals, 117 males (39 affected, 78 unaffected) and 95 females (69 affected, 26 unaffected). The additional five families were not screened with markers that were already located as lying outside the closest flanking markers of the HYP region. DNA was prepared from whole blood, amplified by PCR using radiolabelled primers, and the products electrophoresed on a polyacrylamide gel as outlined in chapter 2, section 2.5. The computer software used to compute two-point data LOD scores was linkage package 5.2 of Lathrop et al (Lathrop et al. 1984). A Dell 486D/33 personal microcomputer networked to the Human Genome Mapping project CRC SUN UNIX system via the Joint Academic Network (JANET) was used to compute the analyses. HYP gene frequency was set to 0.0001, with penetrances of 1 in males and .99 in heterozygous females (Rowe et al. 1996).
3.3 Mapping and identification of the HYP locus

3.3.1 Linkage analysis of affected families using a microsatellite marker derived from DXS274

To increase the informativity of the DXS274 locus, a microsatellite, mpa274 (GDB:345239), was isolated. This microsatellite marker was derived from DXS274, and used to screen the available families (Rowe et al. 1993). This marker had five alleles, in the range of 134 to 144 bp, with a maximum heterozygosity of 0.5500. When the data for this marker was combined with the data derived from the RFLP marker DXS274, only three families, F, Z and XH04, were uninformative.

Figure 3.2: Subsection of family XH05 screened with marker mpa274. The number to the left of each individual (274,X) indicated the marker, DXS274, and the allele that that individual had (either 2, 3 or 2 and 3). The recombinant allele is shown in red.
Chapter 3: Mapping of the HYP locus by linkage analysis

There was a single recombinant with DXS274, table 3.1. In family XH05 individual III-1 was recombinant, as his mother (II-2) had alleles 2 and 3, and he had inherited allele 3, whilst his three sibs, all male, had inherited allele 2. As all four children were affected, III-1 was recombinant for this marker against HYP. This placed mpa274 as segregating with marker DXS41, which was also recombinant in this individual (figure 3.2).

There were no recombinants with family P, table 3.1. The mother (I-1) had alleles 1 and 2, and the father (I-2) had allele 2. In the first generation of children, the unaffected sibs (II-5, II-8, II-9) all inherited allele 1 from their mother (as the unaffected female (II-9) must have inherited allele 2 from her father), and all the affected sibs in this generation (II-1, II-3, II-6, II-10) had inherited allele 2 from their mother. When this family had been screened with DXS41, individual II-6 was recombinant for that marker (figure 3.3), which she was not for DXS274. This thus placed the crossover in this individual between markers DXS41 and DXS274, which separated them and placed DXS274 as the most proximal marker to HYP.

Figure 3.3: A section of family P showing key individuals, with the results of screening the family with markers DXS41 and DXS274. The numbers to the side of the individual family members refer to the marker (marker DXS41 represented as 41, and marker DXS274 represented as 274), and the allele that each member had (either 1, 2 or 1 and 2). The recombinant allele is shown in red.

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In family E the grandmother (I-2) was homozygous for allele 2, and so was therefore uninformative. Her daughters were all informative, as they had inherited alleles 1 and 2. Their affected children inherited allele 2, whilst none of the unaffected children did. Individual III-8 was recombinant for marker DXS43 and, as he was not for DXS274, this confirmed the position of DXS274 proximal to DXS43.

In family N the entire family was informative. DNA was not available for the first generation male (I-1), but as the daughters (II-2 and II-3) both had alleles 1 and 3, and his wife had alleles 3 and 4, the daughters must have inherited allele 3, and allele 1 from their father. Similarly, individual II-1 must have had allele 4, as his daughter had alleles 3 and 4, and must have inherited allele 3 from their mother (II-2). All the affected individuals had inherited allele 3, showing that this allele segregated with the HYP locus in this family.

In family T the first generation female (I-2) was uninformative as she was homozygous for allele 3. Her daughter, II-6, was affected and had inherited alleles 3 and 4 from her parents, and her two affected sons, III-4 and III-5 had also both inherited allele 3, which showed that the disease segregated with allele 3. Individual II-1 was recombinant with marker DXS43, but as his mother (I-2) was homozygous, it was not possible to determine if this individual was recombinant for DXS274.

In family U the affected father, I-2, passed allele 4 onto his daughters, II-1, II-2 and II-4, who in turn passed this allele on to their affected offspring, III-1 and III-3. The unaffected
daughter, III-2 inherited allele 3 from her mother. The disease therefore segregated with allele 4 in this family.

The affected father in family V, I-1, passed allele 1 onto both his daughters (II-2 and II-3). Two of their sons, III-2 and III-3 were affected, and had inherited allele 1 from their mothers, whilst the unaffected son, III-1, inherited allele 2 from his mother. The disease therefore segregated with allele 1 in this family.

The affected mother, I-1, in family W passed allele 2 onto her offspring, II-1 and II-2. The affected male, II-2, passed allele 2 onto all his daughters, who were all affected. The only affected member of the fourth generation, IV-1, had also inherited allele 2 from his mother, whereas none of the unaffected offspring had. The disease therefore segregated with allele 2.

In family XH02 the affected mother passed allele 3 onto her affected daughter, II-3, and allele 2 onto her unaffected children, II-1 and II-2. DNA was not available for the remainder of the family, II-4, III-1 and III-2. The disease therefore, segregated with allele 3 in this family.

In family XH06 the affected mother, I-1, passed allele 3 onto both her affected daughters, II-2 and II-4, and allele 3 onto her 3 unaffected children, II-3, II-5 and II-6. Individual II-2 passed allele 2 onto her affected son, III-2, and allele 3 onto her unaffected daughter, III-1. The disease segregated with allele 2 in this family. Individual II-4 had been previously
found to be recombinant with markers DXS43 and DXS197 (table 3.1). As she was not recombinant with marker DXS274, this confirmed the proximal location of DXS274 with respect to DXS43 and DXS197, figure 3.4.

Figure 3.4: A section of family XH06 showing key individuals, with the results of screening the family with markers DXS43, DXS197 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, marker DXS197 represented as 197 and marker DXS365 represented as 365), and the allele that each member had (either 1, 2 or 3; or a combination of two for the females). The recombinant alleles are shown in red.

DNA was only available for a small subset of family XH09 (III-1, III-2 and their children, IV-1, IV-2 and IV-3). In this family the mother was affected, and had alleles 1 and 3. Her affected offspring inherited allele 1 from her, and her unaffected son inherited allele 3. The HYP locus therefore segregated with allele 1. Individual IV-3 was recombinant with markers DXS43 and DXS197 (table 3.1), but was not recombinant with DXS274 (figure 3.5). This again confirmed the proximal location of DXS274 with respect to DXS43 and DXS197.

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Figure 3.5: A section of family XH09 showing key individuals, with the results of screening the family with markers DXS43, DXS197 and DXS274. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, marker DXS197 represented as 197 and marker DXS274 represented as 274), and the allele that each member had (either 1, 2 or 3; or a combination of two for the females). The recombinant alleles are shown in red.

In family XH13 the affected mother, II-2 had alleles 2 and 3, and her two affected sons had inherited allele 3 from her. The disease thus segregated with allele 3 in this family.

When the data derived from DXS274 and mpa274 was combined, it was found that the marker was tightly linked to the HYP locus and had a maximum LOD score \( (z_{\text{max}}) = 9.6 \), and a \( \theta_{\text{max}} \) of 0.02 (Table 3.2), and the following gene order was inferred (Rowe et al. 1993):

\[
\text{Xpter} - \text{DXS43} - \text{DXS197} - \text{HYP} - \text{DXS274} - \text{DXS41} - \text{Xcen}
\]
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<table>
<thead>
<tr>
<th>Locus</th>
<th>$Z_{\text{max}}$</th>
<th>$\theta_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXS43</td>
<td>4.5</td>
<td>0.140</td>
</tr>
<tr>
<td>DXS197</td>
<td>3.4</td>
<td>0.130</td>
</tr>
<tr>
<td>DXS274</td>
<td>9.6</td>
<td>0.020</td>
</tr>
<tr>
<td>DXS41</td>
<td>8.3</td>
<td>0.040</td>
</tr>
<tr>
<td>DXS1052</td>
<td>9.9</td>
<td>0.000</td>
</tr>
<tr>
<td>DXS365</td>
<td>18.1</td>
<td>0.000</td>
</tr>
<tr>
<td>DXS443</td>
<td>16.0</td>
<td>0.000</td>
</tr>
<tr>
<td>DXS999</td>
<td>9.6</td>
<td>0.040</td>
</tr>
<tr>
<td>DXS7473</td>
<td>12.02</td>
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<td>11.93</td>
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<td>0.000</td>
</tr>
<tr>
<td>DXS7101</td>
<td>11.09</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Table 3.2: Two point peak lod scores for markers against HYP. DXS274 refers to the combined data from DXS274 and mpa274. DXS365 is the combined data from both the RFLP and microsatellite marker.

3.3.2 Screening an additional five kindreds with marker mpa274

DNA from an additional five kindreds became available after the initial study with mpa274, and so these kindreds were analysed at a later date. The data generated were not used to calculate LOD scores, the study was used to look for recombination events, and thus order the markers.

Families XHYP01 and XHYP06 were uninformative for this marker. With family XFG8, DNA was not available for the mother, I-2. The father had allele 2, and his children, all of whom were affected, had allele 3, which they inherited from their mother. The disease therefore segregated with allele 3.
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With family XFG9 the affected mother, II-2, had alleles 2 and 4. Her affected son, III-2, had allele 4, whilst his two unaffected sibs, III-1 and III-3 had inherited allele 2 from their mother. The disease therefore segregated with allele 4.

In family XFG10 the affected mother, I-2, had alleles 2 and 3, and all her children, who were affected, had inherited allele 2 from her. The disease therefore segregated with allele 2. There were no recombinants found in these families.

3.3.3 Screening the HYP families with marker DXS365

Marker DXS365 was obtained from the American Type Tissue Culture Collection. The marker was a RFLP marker, and was used to screen Msp1 digests of DNA from the families, as outlined in materials and methods, chapter 2. However, when this marker was tested on Southern blots of the patient DNA, it was found that repeat sequences in the probe bound to the DNA on the Southern blots causing very high background, which made interpretation of these blots extremely difficult. To overcome this problem, the probe was pre-hybridised with total human DNA (materials and methods 2.6.3). However, this still gave poor results; the blots still had extremely high background which prevented successful analysis of the data. Therefore, DXS365 was partially sequenced and primers were designed to amplify by PCR a 96 bp region which contained no repeat sequences. This 96 bp site targeted sequence (sts) was radiolabelled, and used to screen Msp1 Southern blots of the families DNA. This greatly facilitated interpretation of the blots, and allowed this marker to be used to screen the families.
When the families were screened with this marker, families N, T, Z, XH04, XH05, XH09, and XH13 were all uninformative. The remaining families were all informative, and no recombinants found. Thus, marker DXS365 was tightly linked to HYP ($z_{\text{max}} = 5.4$, $\theta_{\text{max}} = 0.0$) and was therefore potentially the closest marker. These data suggested the following marker order:

\[
\text{Xpter} - \text{DXS43} - \text{DXS197} - (\text{DXS365} - \text{HYP}) - (\text{DXS274}, \text{DXS41}) - \text{Xcen}
\]

**3.3.4 Linkage analysis of the HYP families with a microsatellite marker derived from DXS365**

As marker DXS365 was not informative in many of the families screened, a microsatellite derived from DXS365 (Browne et al. 1992) (GDB:188646), was used to screen the families. Although a total of 9 alleles were reported with this marker, only 5 were observed in the twenty families in this study (Fifteen in the original screening, and the five additional kindreds which were screened at a later date). The nine alleles ranged in size between 201 to 217 bp, with a maximum heterozygosity 0.8190. The data derived from this marker was combined with that from DXS365 to increase the informativity of this marker.

When the data from both DXS365 and the microsatellite derived from DXS365 were combined, only families N, T and XH13 remained uninformative.
When family E was screened with this microsatellite marker derived from DXS365, the affected mother, I-2, had alleles 3 and 4, whilst the unaffected father had allele 2. The mother passed allele 3 onto her affected daughters, and allele 4 onto her unaffected daughter. The affected children of the affected mothers (III-1, III-2, III-5, III-6, and III-8), all inherited allele 3 from their mothers, and the unaffected daughters inherited allele 2. Therefore, the disease segregated with allele 3 throughout this family. As there was a recombination event in individual III-8 between marker DXS43 and HYP, this placed marker DXS365 proximal to DXS43 (figure 3.6).

![Figure 3.6: A section of family E showing key individuals, with the results of screening the family with markers DXS43 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, and marker DXS365 represented as 365), and the allele that each member had (either 1, 2, 3 or 4; or a combination of two for the females). The recombinant allele is shown in red.](image)

Family F was incompletely screened with this marker, since no DNA was available for individuals II-5, II-6, III-1, and III-3 to III-7. In this family the disease was found to
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segregate with allele 4, as the affected daughters for whom DNA was available (II-2, II-3 and III-2), had inherited allele 4 from their mother.

In family P the entire family for whom DNA was available was informative. The disease segregated with allele 3, as the affected mother (I-1) had alleles 1 and 3, and had passed allele 3 to her affected children, and allele 1 to her unaffected children. The affected children in the third generation also all inherited allele 3 from their affected mother, whilst the unaffected individual (III-2) inherited allele 5 from her mother. This therefore placed marker DXS365 proximal to marker DXS197, since individual II-10 was not recombinant; and distal to marker DXS41, as II-6 was also not recombinant with this marker, figure 3.7.

Figure 3.7: A section of family P showing key individuals, with the results of screening the family with markers DXS41, DXS197 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS41 represented as 41, marker DXS197 represented as 197 and marker DXS365 represented as 365), and the allele that each member had (either 1,2,3,4 or 5; or a combination of two for the females). The recombinant alleles are shown in red.

The father in family U had allele 5, which his three daughters all inherited from him. They had two affected daughters, III-1 and III-3 who had inherited allele 5, and one unaffected
daughter, who had inherited allele 3 from their mother. The disease therefore segregated with allele 5.

In family V the affected father, I-1, passed allele 3 onto both his daughters, II-2 and II-3, whose affected sons, III-2 and III-3 both inherited it. From this it was determined that the disease was segregating with allele 3.

In family W the disease segregated with allele 5. The affected female in the first generation, I-1, had alleles 3 and 5, and both her affected children inherited allele 5 from her. Her son, II-2 passed allele 5 onto all his daughters, and his daughter III-3 passed allele 5 onto her affected son. None of the unaffected children inherited allele 5 from their affected parents.

In family Z individual II-2 was affected, and his affected daughter inherited allele 1 from him. Individual II-5 had alleles 1 and 4, and passed allele 1 onto her affected children, and allele 4 onto her unaffected children. In this family there were two individuals that had been recombinant for marker DXS43, one of whom was also recombinant for marker DXS197. As these individuals were not recombinant with marker DXS365, this placed DXS365 as lying proximal to DXS43 and DXS197, figure 3.8.
Figure 3.8: A section of family Z showing key individuals, with the results of screening the family with markers DXS43, DXS197 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, marker DXS197 represented as 197 and marker DXS365 represented as 365), and the allele that each member had (either 1, 2 or 4; or a combination of two for the females). The recombinant alleles are shown in red.

The section of family XH02 that had DNA available for study was informative. The mother, I-2, was affected, and had alleles 2 and 3. She passed allele 3 onto her unaffected son, II-1, and daughter, II-2, and allele 2 onto her affected daughter. Therefore the disease segregated with allele 2 in this family.

XH04 was informative, the disease segregated with allele 2. The affected mother, II-2, had alleles 2 and 4, and passed allele 2 onto her affected children, III-1, III-3 and III-4, and allele 4 onto her unaffected daughter, III-2.
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In family XH05 the affected mother, II-2 had alleles 1 and 3, and passed allele 1 onto her four affected sons. This confirmed that DXS365 was located distal to markers DXS41 and DXS274, both of which were recombinant in individual III-1, figure 3.2.

With family XH06 the entire family was informative, the mother, I-1, had alleles 1 and 2, and passed allele 2 onto her three unaffected children, II-3, II-5 and II-6, and allele 1 onto her affected children. Individual II-2 also passed allele 1 onto her affected son, III-2. Her unaffected daughter, III-1, inherited allele 1 from her father, II-1, and allele 3 from her mother. The disease segregated with allele 1. Individual II-6 was not recombinant, which again supported the location of DXS365 proximal to markers DXS43 and DXS197, both of which were recombinant in this individual, figure 3.9.

![Figure 3.9: A section of family XH06 showing key individuals, with the results of screening the family with markers DXS43, DXS197 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, marker DXS197 represented as 197 and marker DXS365 represented as 365), and the allele that each member had (either 1, 2 or 3; or a combination of two for the females). The recombinant alleles are shown in red.](image-url)
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In family XH09 the HYP locus segregated with allele 3, as the mother (III-1) passed allele 3 onto her affected children, and allele 2 onto her unaffected son. Individual IV-3 was not recombinant, which he was with markers DXS43 and DXS197, demonstrating the proximal location of DXS365 with respect to these two markers, figure 3.10.

![Family XH09 diagram](image)

Figure 3.10: A section of family XH09 showing key individuals, with the results of screening the family with markers DXS43, DXS197 and DXS365. The numbers to the side of the individual family members refer to the marker (marker DXS43 represented as 43, marker DXS197 represented as 197 and marker DXS365 represented as 365), and the allele that each member had (either 1, 2, 3 or 4; or a combination of two for the females). The recombinant alleles are shown in red.

These data suggested that DXS365 was therefore potentially the closest marker to the HYP locus, with a $z_{max} = 18.1$ and a $\theta_{max} = 0.000$ (table 3.2).

3.3.5 Screening of an additional five kindreds with microsatellite marker DXS365

DNA from an additional five kindreds became available after the initial fifteen families had been screened with DXS365, and so these kindreds were analysed subsequent to this
initial study. The data generated was not used to calculate LOD scores, but were used to look for recombination events, and thus to place the markers. There was 1 recombination event found in these five families, and that was in XHYP06. Families XFG8 and XFG10 were not informative for this marker.

In family XHYP06 both members of the first generation had died, so DNA was not available for them. DNA was available from both of their offspring however. One of these daughters, II-1, had inherited alleles 3 and 4; her affected children inherited allele 4 from her, and her unaffected daughter that had DNA available for her had inherited allele 3 (as allele 2 must have been inherited from her father (II-2)). The other female of the second generation (II-3), who was also affected, had inherited alleles 2 and 3. As individual II-1 had alleles 3 and 4, and individual II-3 had alleles 2 and 3, both must have inherited allele 3 from their father. Therefore, daughter II-1 inherited allele 1 from her mother, and daughter II-3 inherited allele 3 from her mother. Both daughters were affected by the disease, as was their mother, so it can be inferred that daughter II-1 had inherited allele 1, which segregated with the disease, and daughter II-3 inherited allele 3 which also segregated with the disease, so one of this pair must have been recombinant for marker DXS365 against the HYP locus. However, it was not possible to identify which allele the disease segregated with in the mother, so it was not possible to say which of these individuals was recombinant; the phase could not be determined, figure 3.11.
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Figure 3.11. Key individuals in family XHYP06 for marker DXS365. The two parents in the first generation had died; there was no DNA available for either. The numbers to the side of the individual family members refer to the marker (marker DXS365 represented as 365), and the allele that each member had (either 2, 3 or 4; or a combination of two for the females). As both the first generation had died it was not possible to determine which individual was recombinant; the possible recombinant alleles are shown in blue.

Family XHYP01 was informative. The mother, II-2, had alleles 1 and 2, and passed allele 1 onto her affected children, III-2, III-4 and III-6. DNA was not available for her unaffected children. Her daughter, III-4, could have passed either allele 1 or 2 to her daughter; as DNA for the father (III-15) was not available, it was not possible to determine which allele the daughter, IV-4, had inherited from which parent. The affected son of II-2, III-6, passed allele 1 onto both his daughters. The HYP locus therefore segregated with allele 1 in this family.
With family XFG8 the disease segregated with allele 4, but as DNA was not available for the mother, I-2, this family was not informative.

XFG9 was informative, the mother, II-2, passed allele 4 onto her affected son, and allele 2 onto her unaffected children. The disease therefore segregated with allele 4.

3.3.6 Linkage analysis of affected families using the microsatellite marker DXS999

Details of marker DXS999 (AFMa234yf12), were supplied by Genethon (Weissenbach et al. 1992). Four alleles were reported with this marker.

Fourteen of the kindreds were screened with DXS999 (DNA for family Z was not available at the time of the screening), and two recombinants were found in these families. These were in families F (III-2) and T (II-1). T II-1 was recombinant with marker DXS43 (although uninformative with marker DXS197), whilst F III-2 was uninformative with both of these markers.

In family T the mother (I-1) had alleles 3 and 4, the father (I-2) had allele 1, but the status of the affected daughters II-2 and II-6 could not be determined because there was no DNA available for analysis. The unaffected daughter (II-4) had alleles 1 and 4 and the unaffected son (II-1) had allele 3. Whilst there was no data for the two affected daughters (II-2 and II-6), DNA was available for the sons of individual II-6 (III-4 and III-5). Both of
these had allele 3, confirming that allele 3 segregated with the disease in this family. Since the unaffected son, II-1, had also inherited allele 3 from his mother, I-2, this meant that he was recombinant for this marker against HYP, figure 3.12 and figure 3.13. This individual had also been found to be recombinant with marker DXS43, so therefore DXS999 segregated with DXS43.

Figure 3.12: A section of family T, showing key individuals for the recombination. 999 refers to marker DXS999 and 43 to DXS43, with the subsequent numbers referring to the allele that an individual had, either 1, 2, 3, 4, or a combination of two of these for the females. The recombinant alleles are shown in red. ? represents unknown alleles, as no DNA was available for individual II-6 when the family was screened with marker DXS999.

In family F individual III-2 was recombinant against HYP for DXS999. In the first generation, the affected female (I-2) had alleles 1 and 2 and the unaffected male (I-1) had allele 2. The three affected daughters (II-2, II-4, II-5) had inherited allele 2 from their father (I-1) and allele 1 from their mother (I-2). All the affected members of the third generation (III-1, III-4, III-5, III-6, III-7) had also inherited allele 1 from their mothers.
Figure 3.13. Family T screened with marker DXS999. A: is the family tree with the alleles indicated. B: is the actual gel showing the alleles. The family tree is shown in the order the alleles appear on the gel. Individual II-1 was recombinant with this marker against HYP.
apart from individual III-2, who had inherited allele 2 from her mother, and allele 1 from her father. She was therefore recombinant for this marker against the HYP locus, since the disease must have segregated with allele 2 in this individual, figure 3.14. This individual was uninformative for markers DXS43 and DXS197, so there was no information as to the placement of DXS999 with respect to those markers in this family.

In family E the disease segregated with allele 4. The mother, I-2, had alleles 1 and 4, passed allele 4 onto her affected daughters, II-2, II-5 and II-7; and passed allele 1 onto her unaffected daughter. The affected daughters in turn passed allele 4 onto their affected children, III-1, III-2, III-5, III-6 and III-8, and not to any of their unaffected children, III-3, III-4 and III-7. No recombinants were found, which, as DXS43 was recombinant in individual III-8, located DXS999 as lying proximal to DXS43. DXS197 was not informative with this family, and so the relationship between DXS999 and DXS197 could not be confirmed in this family.
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In family N the first generation female was uninformative, as she was homozygous for allele 1. Her two daughters had inherited alleles 1 and 3, and their affected children, III-1, III-2 and III-3 had all inherited allele 1 from their mother. Therefore the disease segregated with allele 1 in this family.

In family U the disease segregated with allele 4 as the father, I-2, passed that allele onto his three daughters, II-1, II-2 and II-4. Their affected children, III-1 and III-3, inherited allele 4 from their mothers, and the unaffected child, III-2, inherited allele 1 from her mother.

In family V, the father, I-1, who was affected, had allele 3. He passed this allele onto his two daughters, both of whom were affected. Each of these had an affected son, III-2 and III-3 both of whom had inherited allele 3 from their mothers. Therefore the disease segregated with allele 3 in this family.

In family XH02 the disease segregated with allele 4. Individual I-2, who was affected, passed allele 1 onto her two unaffected children, and allele 4 onto her affected daughter.

In family XH04 the disease segregated with allele 1. The affected mother, II-2, passed allele 1 onto her affected children, III-1, III-3 and III-4, and allele 3 onto her unaffected daughter, III-2.
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In XH06 the disease was found to segregate with allele 1 when screened with marker DXS999. The mother, I-1, had alleles 1 and 3, and her two affected daughters, II-2 and II-4, inherited allele 1 from her, and her unaffected children inherited allele 3. Individual II-2 passed allele 1 onto her affected son, III-2, and allele 3 onto her unaffected daughter (III-1). As individual II-4 was recombinant with markers DXS197 and DXS43, this placed marker DXS999 as lying proximal to these markers.

In family XH09, which was informative for the 5 individuals that DNA was available for, the disease segregated with allele 2. The mother, III-1, passing allele 2 onto her affected children, IV-1 and IV-2, and allele 3 onto her unaffected son, IV-3. As individual IV-3 was recombinant with markers DXS43 and DXS197, this confirmed the proximal placing of DXS999 with respect to those markers as he was not recombinant with marker DXS999.

Four families; P, W, XH05, and XH13; were uninformative with this marker.

The additional families, XHYP01, XHYP06, XFG8, XFG9 and XFG10 were not screened with this marker, as the position of this marker, with respect to the other markers, was known, and it was not a close flanking marker to the HYP locus.
3.3.7 Linkage analysis of affected families with the microsatellite marker DXS443

Marker DXS443 (GDB:188656 ) (Browne et al. 1992) was a microsatellite marker with seven alleles, ranging from 204 to 210 bp, with a maximum heterozygosity of 0.6000. Fifteen families were screened with this marker, and seven different alleles were observed. No recombinants were found with this marker in any of the families screened.

Family E was informative for this marker, and the disease segregated with allele 4. The mother, I-2, had alleles 3 and 4, and passed allele 4 onto her three affected daughters, II-2, II-3 and II-5, and allele 3 onto her unaffected daughter, II-4. The affected children of the three affected females had all inherited allele 4, whilst none of the unaffected children had. Individual III-8 was not recombinant with this marker, as he had received allele 4 (which the disease segregated with) from his mother. This placed DXS443 as proximal to DXS43, as individual III-8 was recombinant for DXS43.

In family F the mother passed allele 4 to all her daughters, and they passed allele 4 onto all their affected children, and none of the unaffected children. Allele 4 therefore segregated with the disease in this family. Individual III-2 was not recombinant for DXS443, and so DXS443 was therefore placed centromeric to DXS999, and therefore DXS197 and DXS43. The other family (family T), that had an individual recombinant for DXS999 was not informative for DXS443.
In family N the first generation female, I-2, was homozygous for allele 3. The second generation was informative however; the affected females had alleles 2 and 3. Their affected children, III-1, III-2 and III-3 all inherited allele 3 from their affected mothers, demonstrating that the disease segregated with allele 3.

In family P, in which individual II-6 was recombinant for DXS41 against HYP, DXS443 was informative. The affected mother, I-1, had alleles 3 and 4. Her affected daughters, II-1, II-3, II-6 and II-10, all inherited allele 4 from her, and they in turn passed allele 4 onto their affected children, III-1, III-3, III-4, III-5 and III-6. None of the unaffected children inherited this allele from their mother. The disease therefore segregated with allele 3, and, as there were no recombinants, this placed DXS443 distal to DXS41. Also, individual II-10 was not recombinant, which she was with marker DXS197, which confirmed the proximal placing of DXS443 with respect to DXS197.

In family XH05 the family was informative, the mother had alleles 3 and 4, and she passed allele 3 onto her four affected sons, so the disease segregated with allele 3 in this family. Individual III-1 was not recombinant, which placed DXS443 distal to markers DXS274 and DXS41.

In family U the affected father, I-2, passed allele 4 onto his three daughters, and they in turn passed allele 4 onto their affected daughters, whilst the unaffected daughter, III-2, inherited allele 3 from her mother. The disease therefore segregated with allele 4 in this family.
Family V was also informative. The father, I-1, had allele 4, which he passed onto both of his daughters. They in turn passed allele 4 onto their affected daughters, III-2 and III-3, so the disease segregated with allele 4.

Family W was informative, the mother, I-1, had alleles 3 and 4, and passed allele 3 onto her children, who were affected. The son passed allele 3 onto his four daughters, of whom III-2 passed allele 3 onto her affected son, IV-1. None of the unaffected children of either generation III or IV inherited allele 3 from an affected parent.

In family XH02 the affected mother, I-2, had alleles 1 and 3, her unaffected daughter inherited allele 1, whilst her affected daughter inherited allele 3, so the disease segregated with allele 3.

In family XH04 the mother, II-2, had alleles 1 and 2, and passed allele 1 onto her affected children, and allele 2 onto her unaffected daughter. The disease therefore segregated with allele 1.

In family XH13 the mother (II-2), was heterozygous, having alleles 3 and 4. Her two affected sons both inherited allele 3, so the disease segregated with allele 3.

Families T, Z, XH06 and XH09 were uninformative with DXS443.
Of the additional five kindreds, XHYP01, XHYP06, XFG8, XFG9 and XFG10 only XHYP01 was screened with DXS443. The other families were not screened as the location of this marker with respect to the other marker loci was known when DNA for those four kindreds was available.

In family XHYP01 the disease segregated with allele 3. The mother (II-2) was heterozygous, as she had alleles 3 and 4, and all her affected children inherited allele 3 from her. For the children in the fourth generation, all the affected who had DNA available for study had inherited allele 3 from their affected parent, and the one unaffected male (IV-3) had inherited allele 2.

3.3.8 Linkage analysis of affected families using microsatellite marker DXS1052

Marker DXS1052 (AFMa163yh2) (GDB:189364), was used to screen fifteen of the families (Biancalana et al. 1994; Rowe et al. 1994b). Families E, V, Z, XH06 and XH13 were uninformative when screened with this marker.

Family F was partially informative, as the mother (I-2) had alleles 1 and 2, but her three daughters were all homozygous for allele 2, and so were not informative. This meant that individual III-2, who was recombinant with marker DXS999, was not informative, and so this family gave no information on the location of DXS1052 with respect to DXS999.
With family N the affected mother, I-2, had alleles 2 and 3, and her two daughters also
had inherited alleles 2 and 3. As there was no DNA available from the father for this study,
it was not possible to determine which allele they had inherited from their mother. Their
children, all of whom were affected, had all inherited allele 2, so the disease had
segregated with allele 2.

Family P was informative in the third generation, as the first generation female (I-1), who
was affected, was homozygous for allele 3. This meant that individual II-6, who was
recombinant with marker DXS41, was uninformative, as was individual II-10 who was
recombinant with marker DXS197. However, the disease segregated with allele 3 in those
individuals who were informative. This family therefore gave no information on the
placing of DXS1052 with respect to DXS41 or DXS197.

Family T was informative, and the disease segregated with allele 3. The mother had alleles
2 and 3, and passed allele 2 onto her unaffected children. No DNA was available for either
of her affected children. However, the sons of II-6 had inherited allele 3 from their mother,
which confirmed that the disease segregated with allele 3. Individual II-1 was not
recombinant, as he had been for markers DXS999 and DXS43, which placed DXS1052
proximal to these markers.

The father in family U passed allele 2 onto his three affected daughters, who then passed
allele 2 onto their affected daughters, but not their unaffected daughter (III-2), so the
disease had segregated with allele 2 in this family.
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With family W the mother, I-1 had alleles 1 and 2. She passed allele 1 onto her two affected children, and the affected son, II-2, passed allele 1 onto his 4 daughters. Of these daughters, III-2 passed allele 1 onto her affected son; none of the other members of the fourth generation were affected, and none of them had inherited allele 1. The disease segregated with allele 1 (figure 3.15).

The mother, I-2, in family XH02 had alleles 1 and 2, and her unaffected children, II-1 and II-2, had inherited allele 2 from her, whilst her affected daughter, II-3, had inherited allele 1. The disease therefore segregated with allele 1 in this family.

In family XH04 the disease segregated with allele 3. The mother, II-2, had alleles 2 and 3, and her three affected children, III-1, III-3 and III-4 had all inherited allele 3 from her, whilst her unaffected daughter, III-2, had inherited allele 2.

In XH05 the affected female, II-2, had alleles 1 and 3; her four affected sons had all inherited allele 3 from her. This placed marker DXS1052 as lying closer to the HYP locus than markers DXS41 and DXS274, as both of these markers were recombinant in this family for individual III-1.

The mother in family XH09, III-1, had alleles 2 and 3, her two affected children, IV-1 and IV-2, inherited allele 2 from her, whilst her unaffected child, IV-3, had inherited allele 3. Individual IV-3 was recombinant for markers DXS43 and DXS197 but not DXS1052, which confirmed the placing of DXS1052 proximal to these markers.
Figure 3.15. Family W screened with marker DXS1052. A: is the family tree with the alleles indicated. B: is the actual gel showing the alleles. The family tree is shown in the order the alleles appear on the gel. There were no recombinants against HYP with marker DXS1052 in this family.
3.3.9 Screening of an additional five kindreds with microsatellite marker DXS1052

The additional five kindreds were screened with DXS1052. XHYP06, XFG9 and XFG10 were all uninformative. In family XHYP01 the disease segregated with allele 1. The mother (II-2) had alleles 1 and 3, and her affected daughters also had alleles 1 and 3, so it was not possible to tell which allele had been inherited from their mother. The affected females of the fourth generation however had inherited allele 1 from their parents, whereas the unaffected male (IV-3) had inherited allele 3.

In family XFG8 the first generation female did not have DNA available for study. The father however did, he had allele 3. The three children all inherited allele 1 from their mother, so the disease must have segregated with this allele in this family.

3.3.10 LOD scores and position of markers DXS365, DXS999, DXS443 and DXS1052

Looking at the recombination events in this data derived from screening the families with markers DXS365, DXS999, DXS443 and DXS1052, the four markers were located closer to the HYP locus than any of the previously studied markers. However, only DXS999 had recombinants which were used to place the marker distal to the disease locus. The maximum LOD score for this marker was 9.6 at a $\theta_{\text{max}} = 0.04$. The remaining three
markers, DXS443, DXS365 and DXS1052 had maximum LOD scores of 16.0, 18.1 and 9.9 respectively, at a $\theta_{\text{max}} = 0.00$ (table 3.2). Thus, from analysis of the recombinants the following marker order was inferred:

Xpter - DXS43 - DXS197 - DXS999 - (DXS365, DXS1052, DXS443, HYP) - DXS274 - DXS41 - Xcen

Although the order of DXS365, DXS443 and DXS1052 could not be deduced by analysis from the HYP families screened in this study, data derived from screening the CEPH families (Rowe et al. 1994b), placed DXS443 distal to DXS365. To resolve the location of DXS1052, a large (approximately 1.2 Mb) YAC isolated from the CEPH library using marker DXS1052 was screened with markers DXS365, DXS443 and DXS274. Markers DXS274 and DXS1052 both gave amplified product when this YAC was screened by PCR, whilst markers DXS365 and DXS443 were both negative. This placed marker DXS1052 as lying centromeric to DXS365. From these data the following marker order was inferred:

Xpter - DXS43 - DXS197 - DXS999 - (DXS443-DXS365, HYP, DXS1052) - DXS274 - DXS41 - Xcen (Rowe et al. 1994b)

The markers used to screen the families were used to isolate a number of YACs from the region of the HYP locus (Francis et al. 1994). A number of YACs were isolated, of which three were overlapping non-chimaeric YACs that contained markers DXS365 to DXS274,
and which therefore contained the HYP locus as these were the flanking markers. A number of cosmids and P1 clones were also isolated from this YAC contig.

3.3.11 Isolation of microsatellite markers from P1 clones

A novel technique was developed to isolate new microsatellites to screen the families (Rowe et al. 1994a). P1 clones isolated from the minimal span contig were used to develop these new microsatellite markers, although DNA from YACs or cosmids could also have been used. In brief, DNA from the P1 clones was extracted (section 2.2.1) and digested with a variety of restriction enzymes that generate blunt ends. PCR linkers were ligated on to these DNA fragments and then amplified by PCR. The resulting amplicons were electrophoresed on an agarose gel and blotted onto a nylon membrane. The blots were then screened with a d(C.A)n -d(G.T)n heteropolymer. Positive bands were excised from a duplicate gel, purified and cloned into a TA cloning vector (Invitrogen). Recombinants were then selected, the insert amplified by PCR and the products electrophoresed on an agarose gel. This gel was blotted and screened with a d(C.A)n - d(G.T)n heteropolymer (section 2.7). Positive clones were then sequenced manually, using solid phase technology (section 2.8).

Two microsatellites were isolated using this technique, DXS7473 (Cap 32) was isolated from P1 10220 and DXS7474 (Cap 29) was isolated from P1 175D (Rowe et al. 1996a). These markers were used to screen the families, along with three other markers that were available for screening; DXS7475 (7v2), DXS1683 and DXS7101 (AFMa176zb1) (Rowe
et al. 1996a). DXS7475 was isolated by shotgun subcloning YAC 900E01138 into M13 subclones and screening for microsatellite repeats (Rowe et al. 1996a). DXS1683 (Econs et al. 1994a) was isolated from cosmid A0563, which mapped to YAC 900A0472 (Econs et al. 1994b). DXS7101 was supplied by Genethon.

3.3.12 Screening the families with microsatellite marker DXS7473

DXS7473 (GDB:593087), was isolated by the method described above and section 2.7. It had four alleles, in the range of 262 to 271 bp, and a maximum heterozygosity of 0.6115.

DXS7473 was used to screen the twenty families, and a single recombinant was found, in family XHYP06. In this family both members of the first generation had died, so there was no DNA available for them, although there was DNA available for their two daughters. One of these daughters (II-1) had alleles 1 and 3. Her affected children (III-1 and III-4) both inherited this allele from her, whilst her unaffected daughter for whom DNA was available (III-2) had inherited allele 3 from her mother (as allele 2 must have been inherited from her father (II-2)). The other daughter in the second generation (II-3) was homozygous for allele 3. Therefore, in the second generation one daughter II-1 had alleles 1 and 3, and daughter II-3 had allele 3. Each must have inherited allele 3 from their father. Therefore, daughter II-1 inherited allele 1 from her mother, and daughter II-3 inherited allele 3 from her mother. Both daughters were affected by the disease, as had their mother, so it was inferred that daughter II-1 had inherited allele 1 from her mother, which segregated with the disease, and daughter II-3 had inherited allele 3 which segregated with
the disease. Therefore, one of these sisters was recombinant with this marker against the HYP locus. However, it was not possible to identify which allele the disease segregated with in the mother, so therefore it is not possible to say which of these individuals was recombinant; phase could not be determined, figure 3.16.

No recombinants were found in any of the other families screened. In family E the first generation female was informative as she had alleles 2 and 3. However, all the affected daughters in the second generation were homozygous for allele 2, and so were not informative. There was a recombinant individual with marker DXS43 in this family (III-8)

Figure 3.16. Key individuals in family XHYP06 screened with marker DXS7473. The number to the side of the individuals refer to the marker (7473 is marker DXS7473), and the subsequent numbers refer to the allele that an individual had inherited, either alleles 1, 2, 3 or a combination of two alleles for the females. As both the first generation had died it was not possible to determine which individual was recombinant; the possible recombinant alleles are shown in blue.
but as he was uninformative for marker DXS7473 no there was no information with regards to the placing of DXS43 with DXS7473.

In family F the first generation mother (I-2) was homozygous for allele 2, and so was uninformative. In the second generation the daughters (all of whom were affected by the disease), had alleles 2 and 3. Their affected children all inherited allele 2 from their mothers, whilst the unaffected female (III-3), inherited allele 3. Therefore, the disease segregated with allele 2. Individual III-2 was recombinant for marker DXS999, so therefore DXS7473 was placed proximal to this marker.

In family N the entire family was informative, the female in the first generation had alleles 1 and 3, as did her two daughters. Their affected children inherited allele 3 from their mother, so the disease had segregated with allele 3 in this family.

The female in the first generation in family P had alleles 1 and 2, however, the second generation affected females were all homozygous for allele 1, so were not informative for this marker. Individual II-10, who was recombinant for marker DXS197 was not recombinant for marker DXS7473, so DXS7473 was placed proximal to DXS197. Also in this family, individual II-6 was not recombinant, which placed DXS7473 telomeric to DXS41.

Family U was informative; the disease segregated with allele 2. The second generation females who had offspring were both homozygous for allele 2, so were not informative.
Family V was informative throughout, and the disease had segregated with allele 3. The affected father, I-1, had allele 3, and his two daughters had both inherited this allele. Each of these had an affected son, who had inherited allele 3 from their mothers.

Family W was also informative, the first generation female had alleles 3 and 4, and passed allele 3 onto her children. Her son, II-2 passed allele 3 onto all his daughters. Daughter III-2 passed allele 3 onto her affected son, and allele 4 onto her unaffected daughter. The other affected female with children passed allele 4 onto them; they were both unaffected; so therefore the disease must have segregated with allele 3.

In XH02 the family was informative for those individuals for who DNA was available; the disease segregated with allele 2. The affected mother passed allele 2 onto her affected daughter, II-3, and allele 3 onto her unaffected children.

Family XH05 was informative, the disease segregated with allele 3. The mother in the second generation, II-2, had alleles 2 and 3, and her four sons, all of whom were affected, inherited allele 3. As III-1 was recombinant for markers DXS41 and DXS274, this placed DXS7473 telomeric to this pair of markers.

In family the disease segregated with allele 2. The mother, I-1, had alleles 2 and 3, and her affected daughters inherited allele 2 from her; whilst her unaffected children inherited allele 3. The affected son of II-2 also inherited allele 2 from his mother, whereas his sib had
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inherited allele 1. Individual II-4 was recombinant for markers DXS43 and DXS197, but not DXS7473. This located DXS7473 as lying proximal to these two markers.

In family XH13 the disease segregated with allele 3; the mother, II-2, had alleles 1 and 3, and her two affected sons had inherited allele 3.

In family XHYP01 the female, II-2, had alleles 2 and 3, and her affected children, III-2 and III-6 had inherited allele 2 from her. Their children also inherited allele 2 from them (although III-2 was homozygous for allele 2, and therefore uninformative).

In family XFG8 no DNA was available for the mother. However, from the offspring, all of who had allele 1 so the disease segregated with allele 1. However, as the genotype of the mother could not be determined it was not possible to determine whether she was informative or not. The remaining families, T, Z, XH04, XH09, XFG9 and XFG10 were all uninformative for marker DXS7473.

3.3.13 Screening the families with microsatellite marker DXS7474

Marker DXS7474 (GDB:593090) was isolated as described in section 2.7. It had three alleles, ranging from 463 to 467 bp, and a maximum heterozygosity of 0.5400. The twenty families were screened with this marker. There was one recombinant in these families with this marker, in XHYP06. Six of the families, T, Z, XH04, XH09, XH13, XFG8 and XFG9 were uninformative for this marker.
The recombinant in XHYP06 was one of the sisters in the second generation (either II-1 or II-3). The two parents of these had died, so there was no DNA available for the study. The first daughter (II-1) had alleles 1 and 3, of which she passed allele 1 onto her two affected children, III-3 and III-4, and allele 3 onto her unaffected daughter, III-2. Her sister, II-3, who was also affected, was homozygous for allele 3. Both daughters must have inherited the same allele (3) from their father, II-1 must have inherited allele 1 from her mother, and II-3 must have inherited allele 3. Therefore, one of them must have been recombinant, but it was not possible to tell which. This recombinant placed DXS7474 clustering with DXS365 and DXS7473, which were also recombinant for this marker, figure 3.17.

Figure 3.17. Key individuals in family XHYP06 screened with marker DXS7474. The number to the side of the individuals refer to the marker (7474 is marker DXS7474), and the subsequent numbers refer to the allele that an individual had inherited, either alleles 1, 2, 3 or a combination of two alleles for the females. As both the first generation had died it was not possible to determine which individual was recombinant; the possible recombinant alleles are shown in blue.
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In family E only the mother in the first generation was informative, she had alleles 2 and 3. All of her affected daughters were homozygous for allele 2, and therefore uninformative. There was a recombinant individual with marker DXS43 in this family (III-8) but as he was uninformative for marker DXS7474 no there was no information with regards to the placing of DXS43 with DXS7474.

In family F the first generation female was uninformative, as she was homozygous for allele 2, but her daughters were informative, as they had inherited allele 1 from their father, and allele 2 from their mother. All three of the affected daughters passed allele 3 onto their children, and the one unaffected child in the third generation inherited allele 1 from her mother. In this family the disease segregated with allele 2. As individual III-2 was not recombinant in this family, it placed marker DXS7474 as lying proximal to marker DXS999 which was recombinant in this individual.

In family N the disease had segregated with allele 3. The female in the first generation, I-2, had alleles 1 and 3, and her two daughters had also both had alleles 1 and 3. Their three children, all of whom were affected, had inherited allele 3 from their mother.

In family P the first generation was informative, the mother was heterozygous for alleles 2 and 3. Her affected children, II-1, II-3, II-6 and II-10 had all inherited allele 2 from her, whilst her unaffected children, II-5, II-8 and II-9 had inherited allele 3 from her. The disease therefore segregated with allele 2 in this family. As neither individuals II-6 or II-10

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were recombinant, which they had been with markers DXS41 and DXS197 respectively, DXS7474 was located as lying between them.

The affected father in family U passed allele 2 onto his daughters; however they were all homozygous for this allele and so were uninformative.

In family V the affected father had allele 3, which he passed onto daughter II-2. She in turn passed allele 3 onto her son. DNA was not available for individual II-3, however as her son had inherited allele 3, it was inferred that she had inherited allele 3 from her father, and passed this allele onto her son, as she must have inherited allele 2 from her mother, I-2, who was homozygous for this allele.

The disease segregated with allele 2 in family W. The mother in the first generation, I-1, passed this allele onto her two children. Her son, II-2, passed it onto his daughters, who passed it onto the one affected child in the fourth generation, IV-1, whilst the other three children in this generation all inherited allele 3 from their mothers.

Family XH02 was informative in the portion of the family that there was DNA available, and the disease segregated with allele 2. The affected mother passed allele 2 onto her affected daughter, and allele 3 onto her two unaffected children, as the father must have had allele 2.
In family XH05 the mother had alleles 2 and 3, and her four affected sons all inherited allele 3 from her. As individual III-1 was recombinant with markers DXS41 and DXS274, this confirmed the placing of marker DXS7474 telomeric to this pair of markers.

In family XH06 the mother had alleles 2 and 3, and she passed allele 3 onto her unaffected children, and allele 2 onto her affected children. Her daughter II-2, passed allele 2 onto her affected son, III-2 and allele 1 onto her unaffected daughter, III-1. In this family individual II-4 had been recombinant with markers DXS43 and DXS197, but was not recombinant with marker DXS7474, which placed this marker proximal to markers DXS43 and DXS197.

The first generation in XHYP01 had both died, as had the husband in generation two. Individual II-2 had alleles 2 and 3, and passed allele 2 onto her affected children. Individual III-7 passed allele 2 onto his two affected daughters. The disease therefore segregated with allele 2 in this family.

In family XFG10 the mother (I-2), was uninformative, but her daughter who had children was informative, having alleles 2 and 3. Her affected son inherited allele 2 from her, so therefore the disease segregated with allele 2 in this family.
3.3.14 Screening the families with microsatellite marker DXS7475

Marker DXS7475 (GDB:593109), was isolated by shotgun subcloning YAC 1138 into M13 subclones, and screening for CA repeats (Rowe et al. 1996a). It had a total of 5 alleles, ranging from 150 bp. There were no recombinants found in the families when they were screened with marker DXS7475.

In family E the first generation female was uninformative as she was homozygous for allele 2. Her daughters had all inherited alleles 2 and 3 from their parents, and the affected daughters passed allele 2 onto their affected children, but not onto their unaffected children. Individual III-8 had been recombinant with marker DXS43, but was not with this marker, which therefore placed DXS7475 as lying proximal to DXS43.

The mother in the first generation of family F had alleles 2 and 3, and passed allele 2 onto her three affected daughters. They passed allele 2 onto all their affected children, but not onto the unaffected daughter, III-3, who inherited allele 1 from her mother. With marker DXS999 individual III-2 was recombinant. As she was not recombinant with marker DXS7475, this placed it lying proximal to DXS999.

Family P was uninformative in the first generation, as the female was homozygous for allele 4. Her affected daughters, all of whom had children, inherited alleles 2 and 4. They all passed allele 4 onto their affected children, but not to any of the unaffected children. The disease therefore segregated with allele 4 in this family. Individual II-10 was
recombinant with DXS999, but as I-1 was uninformative, it was not possible to tell if this individual was recombinant with DXS7475.

In family U the affected father passed allele 3 onto his three affected daughters, who passed allele 3 onto their affected daughters, but not onto the unaffected daughter III-2, who inherited allele 2 from her mother. The disease therefore segregated with allele 3 in this family.

The affected male in the first generation in family V passed allele 1 onto his two daughters, who passed this allele onto their affected sons, so the disease segregated with allele 1 in this family.

The affected female in the first generation of family W was uninformative, her children inherited allele 2 from her. Her son, II-2, passed allele 2 onto all his daughters, but the two daughters who had offspring, III-2 and III-5, were both homozygous for this allele, and so were uninformative.

In family Z the there was no DNA available for the parents in the first generation. The daughter for whom DNA was available, II-5, was homozygous for allele 1, and so that branch of the family were uninformative, so there was no information regarding the placement of DXS7475 with respect to markers DXS43 and DXS197, which were recombinant in individuals III-15 and III-20. In the branch of the family that were
informative, the affected daughters of the affected sons had all inherited allele 1 from their father. The disease therefore segregated with allele 1 in this family.

With family XH06 the mother in the first generation had alleles 1 and 3, and the father had allele 2. The affected daughters inherited allele 3 from the mother, and the unaffected children inherited allele 1. The disease therefore segregated with allele 3 in this family. As individual II-4 was recombinant with markers DXS43 and DXS197, but was not with this marker, these data placed marker DXS7475 proximal to this pair.

With family XHYP01 individual II-2 was informative, however her female children were not. With those members of the family who were informative the disease segregated with allele 2.

In family XHYP06 the disease segregated with allele 1; II-1 passed this allele onto her affected children, and not onto her unaffected children. One of the sisters, either II-1 or II-3 (as phase could not be determined), was recombinant with DXS365, DXS7473 and DXS7474. However, as both of the sisters had alleles 1 and 2, it was not possible to say whether they were nonrecombinant, or whether their mother was uninformative. So this family gave no information on the location of DXS7475 with respect to DXS365, DXS7473 and DXS7474. Families N, T, XH02, XH04, XH05, XH09, XH13, XFG8, XFG9, and XFG10 were all uninformative for this marker.
3.3.15 Screening the families with microsatellite marker DXS7101

Marker DXS7101 (GDB:453484), was supplied from Genethon, it is also known as AFMa176zb1. It had four alleles ranging from 156 bp, and a maximum heterozygosity of 0.4900.

With marker DXS7101 there was one recombination event, in family XHYP06. Individual II-1 was homozygous for allele 3. This means that she must have inherited one copy of allele 3 from her father, and one from her mother. Her sister (II-3), had alleles 1 and 3. She must have inherited allele 3 from her father, and therefore allele 1 from her mother. As both sibs were affected by the disease, but inherited different alleles from their mother, one of these must have been recombinant for the disease. As the mother had died, and no more information was available; the phase was unknown, figure 3.18.

Figure 3.18. Key individuals in family XHYP06 screened with marker DXS7101. The number to the side of the individuals refer to the marker (7101 is marker DXS7101), and the subsequent numbers refer to the allele that an individual had inherited, either alleles 1, 3 or a combination of two alleles for the females. As both the first generation had died it was not possible to determine which individual was recombinant; the possible recombinant alleles are shown in blue.
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There were no recombination events in the remaining families that were informative. In family F only the first generation was informative; the mother had alleles 2 and 3, and passed allele 3 onto her daughters, therefore the disease segregated with allele 3. Her three daughters (II-2, II-3 and II-5), were homozygous for allele 3, and therefore uninformative. Individual III-2 was recombinant for marker DXS999, but uninformative for marker DXS7475. Therefore no information was available to place DXS7475 with respect to DXS999 in this family.

In family N the first generation female was homozygous for allele 2. Her two daughters had alleles 2 and 3, and passed allele 2 onto their three affected offspring. The disease therefore segregated with allele 2.

In family P the first generation female was homozygous for allele 2. All of her daughters had inherited alleles 2 and 3. Their affected daughters had all inherited allele 2 from their mother, whilst none of the unaffected daughters had. The disease segregated with allele 2 in this family. As I-1 was uninformative it was not possible to tell if individuals II-6, who was recombinant with DXS41, or II-10, who was recombinant for marker DXS197, were recombinant for this marker.

In family T the first generation female was uninformative, and there was no DNA available for the two affected daughters, so there was very little information with this family. Individual II-1 was recombinant with markers DXS43 and DXS999, but as his mother was
homozygous with DXS7101 it was not possible to place this marker with respect to the others.

In family U the first generation male had allele 4, which he passed onto his two daughters. They in turn passed this allele onto their affected children, but not onto the unaffected daughter III-2, so the disease had segregated with allele 4 in this family.

Family V was also informative, with the disease segregating with allele 3. The father, I-1, had allele 3, which his daughters inherited. Their two affected sons, III-2 and III-3, both inherited this allele.

In family W the disease segregated with allele 2. The daughters of II-2, an affected male, all inherited allele 2 from him, and the affected son in the fourth generation also inherited this allele from his mother, whilst none of the unaffected individuals in this generation inherited allele 2 from their mother.

In family Z there was no DNA available for the first generation, but the three affected children for whom DNA was available, II-2, II-5 and II-7, all had allele 1. Allele 1 was inherited by all the affected children, and none of the unaffected children in this family. As there were recombinants with markers DXS43, individual III-20, and DXS43 and DXS197, individual III-15, marker DXS7101 was placed proximal to these.
In family XH06 the disease segregated with allele 3. The mother, I-1, had alleles 2 and 3, and her affected daughters inherited allele 3, whilst her unaffected children inherited allele 2. Individual II-4 was not recombinant for this marker, as she had been for markers DXS43 and DXS197, so marker DXS7101 was placed proximal to this pair of markers.

Individual II-2 had alleles 2 and 3 in family XH13. She passed allele 2 onto her two affected sons, so the disease segregated with allele 2 in this family.

In family XFG10 the disease segregated with allele 3. The affected mother had alleles 2 and 3, and passed allele 3 onto her three affected children. II-2 also passed this allele onto her affected son. Families E, XH02, XH04, XH05, XH09, XHYP01, XFG8 and XFG9 were all uninformative.

### 3.3.16 Screening the families with marker microsatellite DXS1683

Marker DXS1683 (GDB:269614), was derived from cosmid clone AO563, which mapped to YAC 900AO472 and which was absent from YAC 900EO1138 (Econs et al. 1994a; Econs et al. 1994b; Rowe et al. 1996a). It had three alleles in the range of 142 to 174 bp, and a maximum heterozygosity of 0.6700. When the families were screened with marker DXS1683 families U, V, XH04, XH06, XH09, XH13, XHYP01, XHYP06, XFG8 and XFG10 were all uninformative.
There was one recombinant found, in family E. In this family the first generation female (I-2) had alleles 1 and 2, and the male (I-1) had allele 2. The unaffected daughter had inherited allele 1 from her mother, and the affected daughters II-2 and II-5 had inherited allele 2 from their mother. II-7 however, was affected, but had inherited allele 1 from her mother, and so was recombinant for this marker against HYP. This individual had not been recombinant with markers DXS7473, DXS7474 or DXS365, but had been uninformative with markers DXS274, DXS1052, and DXS41, so the distal placement of this marker with regards to HYP was not confirmed, figure 3.19.

With family F only the first generation female was informative, she had alleles 1 and 2. Her daughters all inherited allele 2 from her, and so were uninformative. Therefore there was no information with this marker in individual III-2, who had been recombinant with DXS999.
Family N was informative, the mother, I-2, had alleles 1 and 2. Her two daughters were also heterozygous, they had alleles 1 and 2. Their three affected children had all inherited allele 2, so the disease had segregated with allele 2 in this family.

In family P only the first generation was informative, the mother had alleles 2 and 3. All of her affected daughters had inherited allele 3 from her, and her unaffected children had all inherited allele 2, so the disease segregated with allele 3. Individuals II-6 and II-10 were not recombinant in this family for this marker, which they had been with markers DXS41 (II-6) and DXS197 (II-10), so therefore marker DXS1683 was placed as lying between these markers.

Family T was informative, and the disease segregated with allele 1. The mother in the first generation, I-1, had alleles 1 and 2, and passed allele 2 onto her two unaffected children. There was no DNA available for either of her affected daughter, II-2 and II-6, but the two affected sons of II-6 had inherited allele 1 from their mother, and so the disease must have segregated with this allele in this family. As individual II-1 was recombinant for markers DXS43 and DXS999, marker DXS1683 was placed as lying proximal to these.

Family W was informative; the disease segregated with allele 2. The mother, I-1, had alleles 1 and 2, and passed allele 2 onto her children, who were both affected. Her son, II-2, passed allele 2 onto his daughters, and individual III-2, passed allele 2 onto her affected son. None of the unaffected children inherited allele 2 from an affected parent.
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In family Z there was no DNA available for the first generation, but the three affected children for whom DNA was available, II-2, II-5 and II-7, all had allele 1; the females were heterozygous, having alleles 1 and 2. Allele 1 was inherited by all the affected children, and none of the unaffected children in this family. As there was a recombinant with marker DXS43; individual III-20; and also with DXS43 and DXS197; individual III-15; marker DXS1683 was placed proximal to these.

The mother in family XH02 had alleles 1 and 2. Her two unaffected children, II-1 and II-2 inherited allele 2 from her, whilst her affected daughter inherited allele 1. The disease therefore segregated with allele 1.

In XH05 the disease segregated with allele 2. The mother, II-2, had alleles 1 and 2, and her four affected sons had all inherited allele 2 from her. Individual III-1 was recombinant with markers DXS41 and DXS274, which placed DXS1683 as lying telomeric to these two markers.

With XFG9 the disease segregated with allele 1. The mother, I-2, had alleles 1 and 3, as did her affected daughter, II-2. II-2 passed allele 1 onto her affected son, and allele 3 onto her unaffected children.
3.3.17 Confirmation of the location of marker DXS1683 by screening family E with the proximal marker DXS451

To confirm the proximal location of marker DXS1683 family E was screened with marker DXS451 (GDB:188659) which had previously been shown to lie proximal to markers DXS274 and DXS41 (Browne et al. 1992; Econs et al. 1994b). It had nine alleles in a range of 182 to 204 bp, and a maximum heterozygosity of 0.8000.

With this marker the first generation female had alleles 1 and 2, whilst the male had allele 1. Two of the affected daughters (II-2 and II-5) had alleles 1 and 2, and the unaffected daughter (II-4), had allele 1 only. The disease segregated with allele 2. However individual II-7, who was affected, was homozygous for allele 1, which meant that she was recombinant, as she had inherited allele 1 from her mother, figure 3.20.

Figure 3.20. Part of family E showing key recombinants for marker DXS451. Numbers to the side of each individual represent the marker (451 represents DXS451), and the subsequent numbers show the alleles that the person has, either 1, 2 or 1 and 2. The recombinant allele is represented in red.
As individual II-7 in family E was recombinant for markers DXS451 and DXS1683, although uninformative for markers DXS41, DXS274 and DXS1052, marker DXS1683 was placed centromeric to the HYP locus.

3.3.18 LOD scores, and marker order, for markers DXS7473, DXS7474, DXS7475 and DXS7101

From analysis of these data these five markers were thus placed as being the closest markers to the gene locus. No recombinants were found with marker DXS7475, which had a maximum LOD score \( (z_{\text{max}}) \) of 12.9 at \( \theta_{\text{max}} \) of 0.00. Markers DXS7473, DXS7473 and DXS7101 all had a recombination in family XHYP06, with maximum LOD scores of 12.02, 11.93 and 11.09 with \( \theta_{\text{max}} \) of 0.015, 0.018 and 0.02 respectively. These data gave the following most likely locus order:

Xpter - DXS43 - DXS197 - DXS999 - (DXS7475, DXS365, DXS443, DXS1052
[DXS7473, DXS7474, DXS7101], HYP - DXS1683 - DXS274 - DXS41 - DXS451 - Xcen
3.3.19 Screening the YAC contig with the markers used to screen the families, to help to finely position the markers, thus narrowing the region where the HYP locus was located

From the analysis of recombinants it was not possible to order all the markers studied with respect to each other, or with respect to the HYP locus. There was however a YAC contig available for screening (Francis et al. 1994). As well as the YAC contig, various cosmids, P1 clones and PACs from this region of the X chromosome were also available, figure 3.21. DNA from these clones was amplified using the primers for the markers that were used to screen the families. The minimal YAC contig consisted of three YACs, E01138, A0472, and 80G04. Marker DXS443 did not amplify any of the three YACs, and so therefore lay outside of this contig. This placed DXS443 distal to DXS365, as DXS365 was located on YAC E01138.

DXS7475 was located on YAC E01138, from which it was derived (Rowe et al. 1996a), and not located on either of the other two YACs. Markers DXS365 and DXS7101 were also located only on YAC E01138. Marker DXS7473 was derived from P1 clone 10220, and marker DXS7474 was derived from P1 clone 175D. Both of these markers were found on both YACs E01138 and A0472, which placed them centromeric to the DXS365, DXS7475 and DXS7101 cluster. DXS1683, which was derived from cosmid A0563 (Econs et al. 1994a), was found only on YAC A0472. DXS1052 was located on YAC 80G04. This thus gave a more detailed map of the markers surrounding the HYP locus:
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Xptel - DXS43 - DXS197 - DXS999 - DXS443 - (DXS365, DXS7475, DXS7101) -
DXS7474 - DXS7473 - HYP - DXS1683 - DXS1052 - DXS274 - DXS41 - Xcen

Figure 3.21. The YAC contig, showing the minimal span YACs, YAC E01138, A0472 and 80G04. The approximate locations of markers DXS7473, DXS7474 and DXS1683, the closest flanking markers to the HYP locus are shown. P1 clones 1022 and 705 are also shown, which were the clones that DXS7473 and DXS7474 were isolated from. Cosmids 177, 611, 1005, 5610 and 421 are also shown. These were cosmids that overlapped YAC A0472. The site of the deletion found in TK11 is also shown (section 3.3.21).

The crossovers in family XHYP06 (individuals II-1 or II-3) placed the HYP locus centromeric to DXS7473/DXS7474 and the crossover in family E (individual II-7) located the HYP locus as lying distal to marker DXS1683. As both of these markers were located on YAC A0472 the HYP locus was positioned within a 200-300 kb region on this YAC (Rowe et al. 1996a).
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3.3.20 Screening YACs and cosmids with a vitamin D response element

A radiolabelled osteocalcin construct containing a radiolabelled osteocalcin vitamin D response element (VDRE), and vector, were used to screen South-western blots containing immobilised vitamin D receptor (VDR). Only the osteocalcin containing construct hybridised to the band co-migrating with the VDR protein. Using this as a positive control, radiolabelled YACs were used to screen the VDR blots. YAC A0472 gave a strong band that corresponded in size to the VDR. Weak signals were seen with YACs E01138 and 80G04, and all the other YACs tested were negative, including one that did not map to the XP22.1-Xp22.2 region of the chromosome. YAC A0472 thus contained a putative VDRE, which was hypothesised to be involved with the HYP gene (Rowe et al. 1996a).

Using the same technique as described above for the YACs, cosmids that overlapped with YAC A0472 were also used to screen the VDR blots. The bands observed with the cosmids were much fainter than the bands seen with the YACs, but a faint band was observed with cosmid 611 (figure 3.22).

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Figure 3.22. South-western blots containing immobilised vitamin D receptor (VDR) screened with radiolabelled cosmids. Cosmid 2 (cos 611) gave a positive signal, three other cosmids were negative. V is the negative control, Os is the positive control.
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3.3.21 Screening patient DNA with radiolabelled cosmids to identify insertions or deletions in the HYP gene

Patient DNA that had been digested with a variety of restriction enzymes (single digests) was screened with cos 611. The cosmid was radiolabelled and pre-hybridised with total human placental DNA (section 2.6.3). Southern blots of the patient DNA that had been digested with different restriction enzymes was thus screened, and two individuals TK11 (figure 3.23) and DK378 showed band shifts, indicating a difference in the DNA of these patients with respect to the other patients and the control DNA used. Two cosmids cos 611 and cos 177 had band differences in both of these patients. These results demonstrated a large deletion in these patients, and, as the bands were of different mobilities in these two individuals, the deletions were of a different size.

This information was shared with the rest of the HYP consortium, and a further three deletions were found using cosmids cos 611 and cos 177. One deletion in a HYP patient was found by one of these groups that did not overlap any of the other deletions found in any of the other patients (The HYP consortium 1995). The two cosmids used to identify the deletions were used to isolate cDNA clones from an enriched cDNA library produced from three different tissue sources. Five clones were isolated.

Database searches using these cDNA clones detected homologies at amino acid level to a family of endopeptidases which include neutral endopeptidase (NEP, CALLA), endothelin converting enzyme-1 (ECE-1) and the Kell antigen. 5' RACE (Rapid Isolation of cDNA
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Ends) isolated one further exon that contains hydrophobic residues and is likely to be a transmembrane domain.
Figure 3.23. Southern blots of digested DNA from patient TK11. Five different restriction enzymes were used (DraI, PstI, HindIII, TaqI and PvuII). P is patient TK11 and N is control DNA. Sizes are shown in kilobases.
3.4 Summary

The gene which is affected in X-linked hypophosphataemic rickets was first localised to a region which was flanked by markers DXS41 and DXS43 (Machler et al. 1986). The region has since been extensively mapped, and a number of new polymorphic markers isolated to aid this (Rowe et al. 1994b; Rowe et al. 1993; Rowe et al. 1996a; Rowe et al. 1992).

This study screened 260 individuals in twenty kindreds with 10 new polymorphic markers. Two of these markers, DXS7473 and DXS7474, were developed using a novel technique, which proved to be far more rapid than previous methods used for isolating markers (Rowe et al. 1994a).

Out of the twenty kindreds that were analysed, there were twelve recombinant individuals in nine of the families that were used to position the markers, and refine the region where the HYP gene was located. The markers were also used to screen a YAC contig of the region, and thus the positions were more closely defined. The most likely consensus map was:

Xptel- DXS207 - DXS43 - DXS197 - (DXS999 - DXS257) - DXS443 - (DXS365 - DXS7475 - DXS7101) - DXS7474 - DXS7473 - HYP - DXS1683 - DXS1052 - DXS274 - DXS41 - Xcen
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The gene was thus found to lie between markers DXS7473 / DXS7474 and marker DXS1683, on a single YAC (YAC A0472, figure 3.21) with a possible maximum span of 300-400 kb.

Vitamin D metabolism is indirectly affected in HYP patients, so it was postulated that a Vitamin D response element (VDRE) may have a role in the regulation of the gene. The YAC contig, and additional YACs from around the region, as well as a YAC that was not mapped to this region of the X chromosome, were therefore screened for the presence of a VDRE. YAC A0472 was the only YAC screened which gave a strong signal, indicating a putative VDRE on this YAC. This provided further evidence for the presence of the gene on this YAC. Cosmids which map to this YAC were also screened for the presence of a VDRE and one, cos 611, gave a positive signal. The DNA of patients was screened with this cosmid, and two patients were found that had band shifts compared to control DNA, suggesting that these patients had deletions in the gene. When this information was shared with the other groups working on the HYP gene two of the groups also found deletions in three of their patients using the same cosmids. Two of these deletions overlapped the deletions found in TK11 and DK378, but one other did not. These cosmids were then used to screen a cDNA library. Five cDNA clones were thus identified. These cDNAs were used to screen a database, and homologies were found to a family of endopeptidase genes.
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YNKMNISELSAMIPQFDWLGYYIK
881 tac aac aaa att aag att tct gaa ctg agt agt att ccc cag ttc gac tgg ctc ggc tac atc aag
Exon 9 (SSCP4)

KVIDTRLYPHLKDISPSENVVV
950 aag gtc att gac acc aga ctc tac ccc cat ctg aaa gac atc aag ccc tcc gagaat gta gtt gtc
Exon 10

RVQPYQKDLFRILGSEKRKTID
1016 cgc gtc ccc cag tac ttt aaa gag att tat tta ggg tct gag aga aag aag acc att gac
Exon 10 (SSCP13)

NYLWRMVYSIRPNSRFRQYRRL
1082 aac tat tgt gtg tgg aga atg gtt tat tcc aga att cca aac ctg agc agg cgc ttg cag tat aga tgg ctg

EFSTRVIQGTTLLPQRDKCLNF
1154 gag ttc tca agg gta atc cag ggg acc aca act tgt ctg ctt cca agg gag aca tgg gta att
Exon 11 (SSCP12)

IESALPYVVVGMKFVDVFQEDKK
1220 att gag atg gcc ctc ctt cta tgt gtt gga gaa att tgt gat gtc tac tgt cag gaa gat gaa aag
Exon 11 (SSCP13)

QIELSALPYVVVGMKFVDVFQEDKK
1289 gag atg atg gag gaa tgt ggt gag ggc tgt cgc tgg gcc tgt att gac atg cta gag aca aag aat
Exon 12 (SSCP10)

EMMEELVGVRWAFLDMLKEN
1355 gag tgt atg gat gca gga agc aag aag gcc aca gaa gaa gac pgc aga gct gtt tgt gca aca
Exon 12 (SSCP11)

VGYPEFIIMNDTHVNEDELKAIKFS
1418 gtt ggc tat cca gag ttt atg aat gat act ctt gtt aat gaa gac ctc aca ggc ggt cta atc aag gtt
Exon 13 (SSCP14)

EADYFGNLQTRKYLAQSDFFW
1487 gaa gcc gcc gac tac ttt tgt gcc aac gtc tca caa act cgc aag tat tta gca cag tct gat tgt gcc cta
Exon 14 (SSCP15)

RKAVPKTEWFNTPTTVNAFYSA
1556 aca aca gcc ctt csa aca gac ctg ggg ttc aat ccc agc act gcc ttc agt gcc
Exon 15 (SSCP16)

STNQIRFPAGELQKPPFWSGTTEY
1622 tcc acc aac cag atc gga ttt cca gca gga gac tgt cag aag cct ttc ttt ttg gga aac gaa tat
Exon 16 (SSCP17)

PRSLSYGAIGVIVGHEFTGFDN
1688 ctc gca tct cag atg tgt gct atg atag gcc gta att gtc gga cat gaa ttt aca cat gga ttt gat aat
Exon 17 (SSCP18)

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Figure 3.24. The PEX gene sequence, both DNA and amino acid for exons 1-22. The 3’ end of the gene had not been identified and so is not included in the sequence. The zinc binding domain is underlined.
Figures 3.25 to 3.45 show the family trees for the twenty families used in the study described in this chapter. Each figure is split into two parts. The first part is a tree showing the relationships, males are shown as squares and females as circles. Individuals who are affected by HYP are shown as filled shapes, unaffected individuals are shown by open shapes. The numbering refers to generation (roman numerals) and then the position in that generation. The second part of the figure is a schematic representation of the chromosome flanking the HYP locus, with the markers positioned in order of position on the chromosome. The numbers in the chromosome represent the allele(s) each individual had. A ? represents an unknown allele. Recombinant alleles are shown in red, with the approximate position of the crossover shown as a X. H represents the HYP locus. Markers in grey were uninformative in that particular family. Where DNA was not available for an individual the whole chromosome is shown in grey. When a family was not screened with a particular marker, that marker is shown in green.

Family Z (figures 3.33 and 3.34) was a large family, and so was split into two figures. The family tree is shown in entirety on both figures. Those individuals whose chromosome are not represented on the figure are shown in light blue. Their chromosome are shown in the alternate figure.

Family XHYP06 had two individuals, one of whom was recombinant. However, as their parents had died it was not possible to determine which of the two was recombinant. Their alleles are shown in blue.
Figure 3.25. Family tree of family E. Individual II-7 was recombinant with markers DXS1683 and DXS451. Individual III-8 was recombinant with marker DXS43. Markers DXS197, DXS7101 and DXS1052 were uninformative.
Figure 3.26. Family tree of family F. Individual III-2 was recombinant for marker DXS999. Markers DXS43, DXS197, DXS274 and DXS41 were uninformative with this family. This family was not screened with marker DXS451.
Figure 3.27. Family tree of family N. There were no recombinants in this family. Markers DXS197, DXS365, DXS7475 and DXS41 were uninformative. This family was not screened with marker DXS451.
Figure 3.28. Family tree of family P. Individual II-6 was recombinant with marker DXS41 and individual II-10 was recombinant with marker DXS197. Markers DXS43 and DXS999 were uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.29. Family tree of family T. Individual II-1 was recombinant with markers DXS43 and DXS999. Markers DXS197, DXS365, DXS7475, DXS7474 and DXS7473 were not informative in this family. This family was not screened with marker DXS451.
Figure 3.30. Family tree of family U. There were no recombinant individuals in this family. Markers DXS43, DXS197, DXS1683 and DXS41 were not informative in this family. Marker DXS451 was not used to screen family U.
Figure 3.31. Family tree of family V. There were no recombinants in this family. Markers DXS1683, DXS1052 and DXS41 were uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.32. Family tree of family W. There were no recombinants in this family. Markers DXS43, DXS197 and DXS999 were uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.33. Family tree of family Z, part 1. This family was too large to be displayed in a single figure. The individuals whose data is displayed in this figure, are shown in black. Those whose data are shown in part 2 are shown in cyan. There were no recombinants in this section of the family. Marker DXS7474 was uninformative in this family. This family was not screened with markers DXS999 or DXS451.
Figure 3.34. Family tree of family Z, part 2. This family was too large to be displayed in a single figure. The individuals whose data is displayed in this figure, are shown in black. Those whose data are shown in part 1 are shown in cyan. Individual III-15 was recombinant with markers DXS43 and DXS197, and individual III-20 was recombinant with marker DXS43. Marker DXS7474 was uninformative in this family. This family was not screened with markers DXS999 of DXS451.
Figure 3.35. Family tree of family XH02. There were no recombinants in this family. Markers DXS43, DXS197, DXS7475, DXS7101 and DXS1683 were all uninformative. Marker DXS451 was not used to screen this family.
Figure 3.36. Family tree of family XH04. There were no recombinants in this family. Markers DXS43, DXS197, DXS999, DXS443, DXS365, DXS7475, DXS7101, DXS7474, DXS7473, DXS1683, DXS1052, DXS274, DXS41, and DXS451 were all uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.37. Family tree of family XH05. Individual III-1 was recombinant with markers DXS274 and DXS41. Markers DXS999, DXS7475 and DXS7101 were not informative in this family. Marker DXS451 was not used to screen this family.
Figure 3.38. Family tree of family XH06. Individual II-4 was recombinant with markers DXS43 and DXS197. Markers DXS443, DXS1683 and DXS1052 were uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.39. Family tree of family XH09. Individual IV-3 was recombinant with markers DXS43 and DXS197. Markers DXS443, DXS7475, DXS7101, DXS7474, DXS7473, DXS1683 and DXS41 were all uninformative. Marker DXS451 was not used to screen this family.
Figure 3.40. Family tree of family XH13. There were no recombinants in this family. Markers DXS197, DXS999, DXS365, DXS7475, DXS7474, DXS1683 and DXS1052 were all uninformative in this family. Marker DXS451 was not used to screen this family.
Figure 3.41. Family tree of family XHYP01. There were no recombinants in this family. Markers DXS7101, DXS1683 and DXS274 were uninformative. This family was not screened with markers DXS43, DXS197, DXS999, DXS41 or DXS451.
Figure 3.42. Family tree of family XHYP06. Individual II-1 or II-3 was recombinant, but as their parents had died it was not possible to determine phase. Markers DXS1683, DXS1052 and DXS274 were uninformative. This family was not screened with markers DXS43, DXS197, DXS999, DXS443, DXS41 or DXS451.
Figure 3.43. Family tree of family XFG8. There were no recombinants in this family. Markers DXS7475, DXS7101 and DXS1683 were not informative in this family. The family was not screened with markers DXS43, DXS197, DXS999, DXS443, DXS41 or DXS451.
Figure 3.44. Family tree of family XFG9. There were no recombinants in this family. Markers DXS7475, DXS7101, DXS7474, DXS7473 and DXS1052 were uninformative. The family was not screened with markers DXS43, DXS197, DXS999, DXS443, DXS41 or DXS451.
Figure 3.45. Family tree of family XFG10. There were no recombinants in this family. Markers DXS7475, DXS7473, DXS1683 and DXS1052 were uninformative. This family was not screened with markers DXS43, DXS197, DXS999, DXS443, DXS41 or DXS451.
Chapter 4

Mutation analysis of the PHEX gene
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4.1 Introduction

The gene implicated in X-linked hypophosphataemic rickets was isolated as described in chapter 3, and consisted of twenty two exons. It showed homology at the amino acid level to a family of zinc metalloendopeptidases; which include neprilysin (NEP), endothelin converting enzyme-1 (ECE-1). The gene was originally named PEX: phosphate regulating gene with homologies to endopeptidases on the X chromosome (The HYP Consortium, 1995), but has since been re-named PHEX to avoid confusion with a similarly named gene (Rowe, 1998). This family of genes that include PHEX have a short cytoplasmic N-terminal region, a single transmembrane domain and a large extracellular C-terminal domain with a zinc binding motif. A clustal alignment of PHEX, NEP and ECE-1 is shown in figure 4.1. This shows the level of amino acid conservation between the three proteins. There is high homology throughout the three proteins, in particular the 10 cysteine residues (shown in yellow) which are fully conserved in all of them, and also the zinc binding domain (HExxH).
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Figure 4.1 Clustal alignment of PHEX, Neprilysin (NEP) and Endothelin converting enzyme-1 (ECE-1).

Cysteine (C) is shown in yellow, Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I), Methionine (M) and Proline (P) are shown in light green, Phenylalanine (F), Tyrosine (Y), Tryptophan (W) and Histidine (H) are shown in dark green. Aspartic acid (D), Glutamic acid (E), Asparagine (N) and Glutamine (Q) are shown in blue. Lysine (K) and Arginine (R) are shown in red. Serine (S) and Threonine (T) are shown in cyan. Matched residues showing no sequence similarity are shown in black. Gaps are shown by a "-".
Chapter 4: Mutation analysis of the PHEX gene

To confirm that PHEX is the gene involved in HYP, patient DNA was analysed to search for mutations. There are numerous techniques available for this, but the one selected for this study was Single Stranded Conformational Polymorphism (SSCP).

SSCP (section 2.10) is a technique that is sensitive enough to detect single point mutations. The process is rapid and identification of mutations is relatively easy. Its disadvantage is that it only about 80% of mutations are expected to be able to be identified this way (Clark et al., 1995; Grompe, 1993; Sheffield et al., 1993), possibly due to mutations lying in untranslated regions of the genes, or due to an inherent lack of sensitivity of the technique.

The technique can be summarised as follows: the DNA to be analysed is amplified by PCR together with a control sample, both samples are denatured and the single stranded products thus produced are electrophoresed on a non-denaturing polyacrylamide gel. The single stranded DNA (ssDNA) form secondary structures which differ depending upon the base composition. These ssDNAs migrate at different speeds depending upon this secondary structure (figure 4.2). Thus, single base differences can be identified depending upon the resulting band pattern by comparing the sample with the control sample. Changes in the DNA composition are seen as bandshifts on the gels (figure 4.3). The amplicons that gave these bandshifts can then be sequenced to identify the mutation if needed. When the expected mutations are known, the bandshift pattern seen with SSCP is sufficient to identify the mutation and sequencing is not needed. However, in this case, with the PHEX gene, no mutations were known and it was not known if there would be a small number of
mutations in the majority of the patients or whether the majority of patients would have
different mutations. Therefore sequencing of the DNA that showed a bandshift was
necessary.

The reason why only 80% of mutations are usually found by this technique is not clear. It
may lie in an inherent insensitivity of the technique, or it may be that the mutations occur
outside of the coding region, in the promoter or other control regions. Also, the
temperature at which the bandshifts representing the mutations are detected is important,
and so ideally the gels should be run at a range of temperatures. However, due to time
constraints, this was not possible for this study, and a single temperature was used (section
2.10)
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The mutation in (b) alters the single-stranded conformation.

Migration is dependent on confirmation of a single-stranded DNA.

Figure 4.2. SSCP. Single stranded DNA molecules are electrophoresed on a polyacrylamide gel.

a: control DNA, b: DNA with a point mutation (represented by the circle).

The strands form secondary structures, which differ depending on the base sequence. These strands electrophorese at different rates on the gel giving a bandshift in the mutant when compared to the control.
Figure 4.3. SSCP gel of patients screened for mutations in exon 7. M: Marker. W: negative control. + positive PCR control (samples not denatured). C: control samples. A: Patient XH007. D: Patient XH013. B: Patient B10. X: Patient XH015. The lane next to D was a false negative; the PCR failed in this sample.


Chapter 4 : Mutation analysis of the PHEX gene

4.2 Materials and Methods

DNA from eighty-one unrelated patients were analysed by SSCP, as described in section 2.10. The genomic sequence was supplied by Dr F Francis. Primers were designed to amplify the exons from the patients DNA using PCR, and the amplicons were then analysed using SSCP. When an individual showed a bandshift and therefore a potential mutation in a particular exon, that exon was sequenced to confirm and identify the mutation. For the sequencing, the PCR product was cloned into a PCR cloning vector and the insert sequenced using internal primers either side of the insert. For each PCR product thus cloned, three different colonies were selected and sequenced in order to verify that the changes were not due to PCR or cloning artefacts. Due to time constraints, only exons 1 to 17 were screened by SSCP.

4.2.1 Primer Design

In this study primers amplifying exons 2 and 3 were designed by Dr T Strom, and primers amplifying exons 6, 7 and 8 were designed by Dr C Oudet; all other primers were designed by myself. For exon 4, two sets of primers were designed, as the first pair gave poor amplification. Exons 18-22 were not screened in this study.
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4.3 Results

DNA from eighty-one affected, unrelated patients were examined in this study. For each of seventeen exons of the PHEX gene, PCR amplicons from these patients were examined by SSCP. A number of mutations were seen, both bandshifts and deletions (figure 4.2) as detailed below.

4.3.1 Deletions

Deletions in the patients were identified by PCR by either the lack of an amplicon, or, in the case of MW54 and MW180, by shorter than expected PCR products. Thirteen patients were identified who had deletions in the gene (table 4.1). These included two deletions previously characterised by Southern blot analysis, TK11 and DK378 (section 3.3.21). The deletion in TK11 spanned exons 2 to 5, whilst DK378 had a deletion that spanned exons 1 to 5. At the time of this study the exon preceding exon 1 had not been identified, which meant that in DK378 the 5' end of the deletion was not identified. The remaining eleven patients all had a single exon, or part of an exon deleted. These deletions were not clustered in a specific region of the gene but were found throughout the seventeen exons studied. Two individuals had exon 2 deleted, patients E and XH003. Individual RS311 had exon 4 deleted; individuals MW54 and MW180 had part of exon 5 deleted (section 4.3.7); individuals XH007 and XH013 had exon 7 deleted; TP185 had exon 8 deleted; individual H had exon 10 deleted; XH005 had exon 13 deleted and SB168 had exon 13 deleted (Rowe et al., 1997).
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Table 4.1: Deletions found in HYP patients: + exon present when amplified by PCR, - exon not amplified by PCR

<table>
<thead>
<tr>
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Table 4.2 Individuals that had apparent bandshifts on an SSCP gel, but without a mutation being confirmed. Individuals in blue had bandshifts or deletions in other exons.

<table>
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<tr>
<th>Individual code</th>
<th>Exon No</th>
<th>SSCP No</th>
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<td>MK132</td>
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<tr>
<td>RS311</td>
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4.3.2 Point mutations

A bandshift was observed in thirty-four out of the eighty-one families screened using SSCP. Fourteen of these bandshifts were not sequenced due to time constraints, so it is not certain if these bandshifts arose due to active mutations, were silent mutations, or possibly arose due to a polymorphism in the intronic sequence (table 4.2).

Exon 14 of individual SB168 was sequenced (using both automated technology and manual sequencing) and the sequence showed vector sequence, primer sequence and then unrelated (junk) sequence (i.e. not the sequence expected for this exon). To verify whether this was a cloning artefact or not, the genomic sequence was amplified by PCR using the SSCP15 primers, electrophoresed on an agarose gel, the band excised from the gel, purified by GeneClean (section 2.3) and sequenced on a PE Biosystems automated sequencer using the SSCP15 primers as sequencing primers. The sequence was again unrelated to the expected sequence of exon 14. It was possible that these results may have arisen due to a deletion in this individual, with the SSCP15 primers annealing non-specifically to the genomic DNA. To determine whether this was the case, DNA from SB168 was amplified by PCR under more stringent conditions. A hot start was incorporated into the PCR (section 2.4.1), the number of PCR cycles were reduced from 35 to 25 and the annealing temperature was increased from 59°C to 64°C. When the PCR product was electrophoresed on an agarose gel, a single band was seen with the female control DNA and 2 bands were seen with SB168 (figure 4.4). Therefore it appeared that the second, larger band was the one that had been sequenced, and that these primers were annealing non-specifically in this individual.
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Figure 4.4. DNA from patient SB168 amplified using SSCP15 primers under stringent PCR conditions (see text above). Lane 1: F1 control DNA. Lane 2: DNA from SB168. Two bands were observed, suggesting non specific annealing in the case of this patient. Lane 6: negative control. Lane 7: marker.

One individual, in family U, had a conservative change; the mutation did not alter the coding of the amino acid (The HYP Consortium, 1995). Six individuals, RK321; TS111; RS311; L; XFG6; and XH016 showed what appeared to be an intronic polymorphism.

Twelve patients had active mutations identified (Rowe et al., 1997). Two individuals, XH10 and XH006 had apparent bandshifts in exon 1 and 14 respectively. However, when they were sequenced, there was no change in the sequence from the wildtype (table 4.3).

<table>
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<tr>
<td>XH006</td>
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<td>15</td>
<td>No sequence change found</td>
</tr>
</tbody>
</table>

Table 4.3 Individuals with bandshifts that were not due to mutations.
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The remaining twelve individuals were all sequenced and a mutation identified (table 4.4).

<table>
<thead>
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<th>Individual Code</th>
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<th>SSCP No</th>
<th>Mutation</th>
</tr>
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<td>S</td>
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<td>17</td>
<td>18</td>
<td>GLY-ARG missense</td>
</tr>
</tbody>
</table>

Table 4.4 Individuals with sequenced mutations in the PHEX gene.

4.3.3 Splice acceptor/donor mutations

Of the twelve patients for whom point mutations were confirmed by sequencing, there were two patients who showed mutations in the splice acceptor site of an intron, and two patients who showed mutations in the splice donor site of an intron.

The two splice acceptor mutations were found at the same exon/intron boundary. Patients B10 and XHO15 both had mutations in the exon/intron boundary of exon 7. The wildtype sequence for this boundary was tacagTATCG. In patient B10 there was a transition mutation where the terminal base of the intron mutated from a G to an A, so the sequence was tacaaTATCG; and for patient XHO15 the mutation was a G to C transversion, the sequence being tacacTATCG (intronic sequence is shown lower case, exonic in uppercase). For patient B10, RNA was available, so the PHEX DNA was amplified from this patient and sequenced. A deletion was observed when the amplicons were
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electrophoresed on an agarose gel (figure 4.5). The amplicons were cloned into a vector and sequenced to confirm the deletion (figure 4.6). The three samples sequenced were a male and a female control and patient B10. The sequence shows CDR751, the primer used for amplification of the DNA, then, in the male and female controls a short leader sequence then exon 7, followed by exon 8. In the patient exon 7 is absent, the leader sequence is followed by exon 8, marked by an arrow in the figure.

The two splice donor mutations were found in individuals XH011 and XHYP018. The mutation in individual XH011 was a splice donor mutation in exon 6. The wild type sequence was AGTCTgtaag, and the sequence for XH011 was AGTCTttaag; this individual had a G to T transversion. The other splice donor mutation was found in individual XHYP018, exon 15. The wildtype sequence was GATCCgtgag, and the sequence for XHYP018 was GATCCatgag, a G to A transition mutation.
Figure 4.5. Amplicons from RT-PCR. C: control samples; P: Patient B10; W: negative control, M: Marker. Patient B10 has exon 7 deleted.
Figure 4.6. Partial sequence of PHEX gene, spanning exons 7 and 8, demonstrating exon skipping in patient B10. Primer CDR751 was used to amplify product. In the male and female controls the primer is followed by exon 7, then exon 8. In patient B10 the primer is followed directly by exon 8.
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4.3.4 Nonsense mutations

Two unrelated individuals were found who showed a premature stop, or nonsense, mutation in exon 1, position 50. These were individuals TH241 and S. In both of these there was a C to T transition mutation, which changed an arginine residue to an opal stop codon. The wildtype sequence was GGC ACT CGA ATT, whereas in these two individuals the sequence was GGC ACT TGA ATT.

4.3.5 Frameshift mutations

Of the twelve identified mutations, there were three frameshift mutations which were identified in individuals RG175, XHYP07 and XHYP018. Individual RG175 had a 5 bp deletion in exon 3, position 193. The wild type sequence was ATC TTA AGT AAA GTA, whilst the sequence for RG175 was ATC TTA AGT A. The individual in family XHYP07 had an 8 bp insertion in exon 9, position 954. The wildtype sequence was AGA AGG TCA TTG, whilst the sequence for XHYP07 was AGA AGG GAG TTA CTT CAT TG. In individual XHYP09 there was an additional C in a run of five. As with individual XHYP07, this mutation was in exon 9, though at position 973. The wildtype sequence was ACT CTA CCC CCA, and the sequence of individual XHYP09 was ACT CTA CCC CCC A.


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4.3.6 Missense mutations

One missense mutation was identified which occurred in individual LS40. Here there was a single base change in exon 17 at position 1729, a G to A transversion. The wildtype sequence was GTC GGA CAT which, in individual LS40, mutated to GTC AGA CAT. This mutation would change the glycine residue to an arginine at this position.

4.3.7 Microdeletion

Two individuals MW180 and MW54 (table 4.1 and figure 4.7) had a microdeletion of 103 bp in exon 5, which encompassed both exonic and intronic sequence. There were 54 bases deleted of the exon and 49 bases of the intron. The primer sequence for the amplification of exon 5 lay outside the deleted region, so the exact limits of the deletion in these two individuals were identified.
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AAAGCATCCA ATGAACATAT CTTGAAGgta taatgaggac ccattcatct
----------- ----------------------- -----------------------
tctttgctca gtcttagatt agcctttttg ggtgccatcc tggggaaga 
----------- -------- ttgg ggtgccatcc tggggaaga

gacctcatgct gaa
gacctcatgct gaa

Figure 4.7. Deletion in individuals MW54 and MW180 showing exonic sequence (uppercase) and intronic sequence (lower case). The wildtype sequence is shown in the upper row, and the mutant sequence below. The deletion spans from 173 bases into the exon, to 49 bases into the intron. Primer sequence is shown in magenta.
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4.4 Discussion

To confirm that the sequenced gene PHEX was the gene involved in X-linked hypophosphataemic rickets, patient DNA was examined for the presence of mutations by SSCP analysis.

A total of eighty-one unrelated patients were analysed using SSCP. Twenty-three patients with mutations were identified and there were a further twelve patients who had a bandshift on SSCP gels but which were not sequenced to confirm the mutation. Of these twelve bandshifts, four of them were in individuals who had mutations already identified in other exons, and one individual had a bandshift in two different exons. It is unlikely that an individual would have more than one mutation in a gene so it seems unlikely that these bandshifts were due to mutations. It is more probable that these were either silent mutations or polymorphisms.

4.4.1 Deletions

Thirteen of the patients (16.0%) had deletions in the gene, ranging from microdeletions of part of an exon, through single exon deletions, to large deletions spanning a number of exons. These large deletions were initially identified on Southern blots, and confirmed by SSCP (figure 4.3). The remaining single exon and microdeletions were all identified by SSCP. The microdeletions in individuals MW54 and MW180 were identical. Both of these patients were from Poland, and so it is possible that the mutation is the same in these two individuals due to a founder effect, and that if their ancestry was traced, a common
ancestor would be found. It could also be the case that this is a common mutation, and that when more patients are studied this mutation would be identified more frequently.

Deletions which do not contain an integral number of codons will also cause a frameshift mutation in addition to the exon deletion. This occurred in four of the patients (TK11, MW54, MW180, H). The deletions in MW54 and MW180 spanned the exon/intron boundary, meaning that the splice donor site was also deleted, which would lead to exon skipping (section 4.4.2).

4.4.2 Exon skipping

4.4.2.1 RNA splicing

RNA splicing occurs to remove the intron from transcripts of genes. It involves the breaking of the phosphodiester bonds at the intron/exon boundaries, and the formation of a bond between the two exons.

In RNA splicing two features stand out:

- There is no extensive homology or complementarity between the two ends of the intron. Therefore the formation of secondary structures, which would link the ends of the intron directly together, cannot be the initial step in splicing.

- Junctions have well conserved short consensus sequences.
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Examining the homology of intron exon boundaries, a consensus sequence is found:

\[ A_{64} G_{73} - G_{100} T_{100} A_{62} A_{68} G_{84} T_{83} \ldots . \ 6P Y_{74.87} N C_{65} A_{100} G_{100} - N \]

It can be seen that an intron starts with the dinucleotide GT and ends with the dinucleotide AG. These are known as the splice donor (GT) and splice acceptor (AG) sites. Mutations in these sites would lead to a non-recognition of the intron boundaries and so lead to an exon being spliced out (exon skipping). In many cases, exon skipping would be expected to lead to codon frameshifts and therefore premature stop codons.

In RNA splicing, the left junction of the intron is cut and the intron then forms a lariat where the left end of the intron is joined by a 5'-2' bond to a site near the right end of the intron. The lariat is then excised, the exon ends are joined, and the lariat is degraded (Figure 4.8) (Lewin, 1990).

![Diagram of RNA splicing](image)

Figure 4.8 Exon splicing. The intron is cut at the GT splice donor site, and loops round to form a lariat. The intron is then cut again at the AG splice acceptor site, and the two ends of the exons are joined, whilst the lariat is degraded.
4.4.3 Mutations in the splice acceptor and donor sites

Four unrelated mutations in splice acceptor and donor sites were identified. The most common result of a splice site mutation is the exclusion of the adjacent exon from the mRNA. For splice acceptor mutations, that is the downstream exon and for splice donor mutations it is the upstream exon. In mammalian genes, splice mutations occur at these frequencies: exon skipping, 51%; splicing at a nearby site, 32%; creation of a pseudo exon in an intron, 11%; intron retention 6%. In in vitro studies, mutations in the donor site affect splicing of both the previous intron and the intron in which they occur (O'Neill et al., 1998).

Splice acceptor mutations were found in two individuals. Both were found in intron 6, although the mutations were different. Mutations in the splice acceptor site of an intron would be expected to lead to the deletion of the subsequent exon, in these individuals this was the deletion of exon 7. It is possible that there could have been a cryptic splice site in exon 7, which would have led to part of, rather than the entire exon being deleted.

However, from analysing the sequence of the exon, this does not appear to have been the case. Exon 7 has an integral number of codons (39, 117 bp) so skipping of this exon would not have led to a frameshift mutation, so these two individuals would only have had exon 7 missing. The mutation in B10 was confirmed to be a deletion of exon 7 by rtPCR and sequencing (figures 4.5 and 4.6).
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Two splice donor mutations were found in the HYP patients, in introns 6 and 15. These mutations would have most probably led to exons 6 and 15 being deleted. Both exon 6 and exon 15 have an integral number of codons, and so neither of these mutations would lead to a frameshift.

Mutations in splice donor and acceptor sites are well documented in the literature. In Becker muscular dystrophy (BMD), Hagiwara et al. (Hagiwara et al., 1994) described a G to T transversion mutation in the splice donor site of intron 12. When cDNA from the patient was amplified and electrophoresed on an agarose gel a 430 bp amplicon was found, contrasting with the 552 bp control amplicon. Upon sequencing, it was found that exon 12 was joined directly to exon 14, with exon 13 totally absent. Sequence analysis of the patient’s genome revealed that the 3’ terminal nucleotide of exon 13 was mutated. This nucleotide is part of the consensus sequence, and so was deemed likely to have been the cause of the inactivation of the splice donor site of intron 13 and thus the skipping of exon 13. In this patient the skipping of exon 13 did not cause a frameshift, as the exon contained an integral number of codons. In another case of BMD a splice donor mutation was found which was predicted to give a truncated protein more consistent with a Duchenne muscular dystrophy (DMD) phenotype. They found however that small quantities of mRNA were also transcribed, sufficient to produce a reduced amount of normal dystrophin protein, and thus the milder Becker phenotype (Bartolo et al., 1996).
4.4.4 Nonsense mutations

A nonsense mutations was found in two unrelated families. The mutation was identical in both of these individuals, but there was no indication that they were related, and so this may be a mutational hotspot rather than due to a founder effect. The mutations were found in exon 1, so would give an extremely truncated protein, lacking exons 2-22. A mutation of this type and position would abolish all protein function.

4.4.5 Frameshift Mutations

Frameshift mutations occur when an insertion or deletion occurs that is more or less than a 3 bp (or multiple of 3 bp) insertion or deletion. The reading frame of the gene is thus altered, and the subsequent amino acids are changed dramatically. It is likely that the protein will contain a stop codon, giving a truncated protein. Three such frameshift mutations were found in the HYP patients, in exons 9 and 3. Two individuals had mutations identified in exon 9. One had a 8 bp insertion and the other a single base insertion. The mutation in exon 3 was a 5 bp deletion. All three of these mutations would have altered the reading frames, leading to a vastly altered protein.

4.4.6 Missense mutations

One missense mutation was identified in the families, which led to a change from a glycine to an arginine residue at position 576 in the PHEX gene, which is one amino acid downstream from the zinc binding motif.
The zinc binding motif HExxH has been found to be conserved in all of the zinc metallopeptidases. Jongeneel et al. (Jongeneel et al., 1989) screened the Swiss-Prot database (V8) for the occurrence of this HExxH motif, and found it ninety-two times in eighty-three sequences. Of these only fifteen were known to be zinc dependent proteases, indicating that this pattern does not define a primary structure pattern unique to zinc metallopeptidases. The search was refined to align the sequence of all the known zinc metallopeptidases containing this motif. The alignment showed that the similarity between the zinc metallopeptidases extends beyond this HExxH region. From the enzymes examined, the zinc signature could be redefined as (uncharged) - (uncharged) - H - E - (uncharged) - (uncharged) - H - (uncharged) - (hydrophobic). The PHEX sequence follows this motif, as do NEP and ECE-1 (figure 4.1). The mutation found in individual LS40 changed the amino acid immediately preceding the HExxH motif from a glycine (uncharged), to an arginine (positively charged). It seems probable that this mutation would interfere with the zinc binding capabilities of PHEX in this family.

Further evidence for the importance of zinc in rickets was supplied by Leek et al. (Leek et al., 1988; Leek et al., 1984). In this study rhesus monkeys were fed a zinc deficient diet and developed bone abnormalities associated with rickets.

**4.4.7 Summary of the mutations found in the PHEX gene**

Of the eighty-one patients examined by SSCP, twenty-three (28.4%) had mutations that were identified and confirmed; with a further nine (11.1%) that had an identified bandshift, but were not sequenced to confirm and identify a mutation.
In total 39.5\% of the HYP individuals had (or potentially had) mutations identified by SSCP. This is a considerably lower number than other reports on other genes, where mutations were identified in up to 85\% of the patients examined (Clark \textit{et al.}, 1995). However, in this study only exons 1 to 17 were examined by SSCP, so six exons were not screened. It therefore appears probable that more mutations would have been found in the exons not studied. Also some of the individuals who were screened in this study were female. If the mutation was due to a deletion in these individuals, since they have two copies of the PHEX gene, a deletion mutation would be masked by the presence of the non-mutant form.

4.4.8 PHEX mutations found by other groups

Mutations in PHEX have been reported by other groups. Rowe \textit{et al.} (Rowe \textit{et al.}, 1997) reported the mutations listed above and also described a study of a further 26 individuals who were screened for mutations in all of the 22 exons. In that study, a further three deletions, seven nonsense mutations, six splice site mutations and five missense mutations were described. So, for that study of 26 individuals, 80\% of the patients had mutations identified by SSCP, which is approximately the number expected from studies on other genes (Clark \textit{et al.}, 1995; Grompe, 1993; Sheffield \textit{et al.}, 1993). This supports the theory that a large number of the mutations not identified in the study described in this chapter lie in the exons not investigated by SSCP.
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There have been other studies on mutations in the PHEX gene in HYP patients. In a study by Holm et al. (Holm et al., 1997), nine mutations of the PHEX gene were found in twenty-two HYP patients (41%), consisting of three nonsense mutations, one frameshift, one splice site and four missense. Only 17 of the 22 exons were studied in that investigation, which gave a similar percentage of mutations found in the study described in this chapter. In another study, 60% of the patients screened by SSCP analysis showed mutations (Dixon et al., 1998) including 7 nonsense, 6 deletions, 2 deletional insertions, 1 duplication, 2 insertions, 4 splice site, 8 missense and a single mutation which lay within the 5' untranslated region. A study by Francis et al. (Francis et al., 1997) found mutations in 86% of familial cases and 57% of sporadic cases: 16 of these were frameshift or stop mutations, 8 were missense and there were 2 splice mutations. One of the stop mutations was only two residues from the end of the coding sequence, in a residue that has been shown in NEP to be involved in substrate specificity. Tyynismaa et al. (Tyynismaa et al., 2000) looked at 20 Finnish HYP patients, 15 of whom were sporadic cases, and 5 who had familial HYP. They identified 17 mutations (4 nonsense, 5 splice site, 5 missense and 3 small deletions) in all but one of the patients studied (although one mutation was identified by sequencing the entire gene). One of the mutations was present in two unrelated individuals, but as it had been described before (Rowe et al., 1997) they said it was more likely to be a mutational hotspot than a Finnish founder mutation. Fifteen of the mutations described were novel and had not been previously described. Filisetti et al. (Filisetti et al., 1999) described thirty newly detected mutations, of which 16% were deletions, 8% were insertions, 34% were missense mutations, 27% were nonsense mutations and 15% were splice site mutations.
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The mutations described in all of these studies were found throughout the length of the PHEX gene, and although there are a small number of the same mutations found in more than one unrelated family, it does not appear that there are mutation hotspots in the PHEX gene.

4.4.9 Murine Phex

The murine homologue to the human PHEX gene was identified by Du et al. (Du et al., 1996). Since osteoblasts have been shown to be an important target cell for both HYP and the mouse model, Hyp, RT-PCR was used to amplify transcripts from mouse bone cells. Using primers derived from the human PHEX sequence this produced a PCR product of 1.3 kb. The 5' end of the murine Phex was identified using inverse PCR on circularised double stranded cDNA generated from bone cells. The murine Phex gene was found to have a 91% identity with the published human PHEX data at the nucleotide level, and 95% identity at the amino acid level. The mouse Phex cDNA was used to screen a multiple tissue northern blot. Expression was found in bone cells, but not in brain, heart, kidney, lung, skeletal muscle, spleen or testis. The liver and kidney, both potential sites for the Hyp mutation were screened using RT-PCR, but were found to be negative. A cDNA clone, identified from rat incisors (Matsuki et al., 1995) was found to have high sequence homology with Phex, suggesting that Phex is expressed in teeth, as well as bone cells. In Hyp mutant mice (homozygous females) Phex RNA was not detectable by northern blot, but was amplified using RT-PCR, indicating a low level of expression. The coding region of Phex was screened for mutations, but sequence analysis revealed none. This suggested that the mutation lay in the untranslated or the promoter region of the gene, thereby
implying that the mutation was regulatory in nature, which is consistent with the reduced expression in Hyp mice.

Strom et al. (Strom et al., 1997) described the mutations in both the Hyp and Gy mouse. Both mutations were found to be due to non-overlapping deletions. Gy mice had a deletion which spanned the first three exons of the Phex gene, including upstream sequences, whilst the Hyp mouse had the last 7 or 8 exons deleted, including downstream sequences. It was predicted that both of these deletions would fully inactivate the Phex gene.

4.4.10 Adult onset Vitamin-D resistant hypophosphataemic osteomalacia

As described in chapter 1 (section 1.4.7) adult onset Vitamin-D resistant hypophosphataemic osteomalacia was originally thought to be a second form of dominant X-linked hypophosphataemic rickets. Econs et al. (Econs et al., 1998) described a family with this disease, who had a missense mutation in the PHEX gene. This C to T transition in exon 16 would result in an amino acid change from a leucine to a proline at position 555. This leucine residue is conserved in other zinc metallopeptidases, including NEP and ECE-1 (figure 4.1) and could lead to a change in secondary structure of the protein. It is possible that this mutation gives a milder or atypical phenotype to other PHEX mutations, and that a milder phenotype results in adult onset Vitamin-D resistant hypophosphataemic osteomalacia rather than HYP.
Chapter 5

Summary
5.1 Summary of linkage analysis

The gene implicated in X-linked Hypophosphataemic Rickets was localised to the Xp22.1-Xp22.2 region of the chromosome, flanked by markers DXS41 and DXS43. This study involved refining the region surrounding the HYP locus by screening the DNA of affected families with markers. This study also described a new, faster and easier way of generating microsatellite markers from cosmid, P1 or YAC DNA.

The area of the X chromosome where the gene was located was localised to a 300-400 kb region through linkage analysis. A cosmid which appeared to have a VDRE present was selected to screen southern blots of the patient DNA. Two patients were found with band patterns which differed from the patterns of the control DNAs. This suggested that these patients had either an insertion or deletion in this region of the genome.

This information was shared with collaborating groups, enabling the gene (originally named PEX and later renamed PHEX), to be sequenced. The two patients with mutant band patterns were shown to have deletions in the 3’ end of the gene. Additional patients with large deletions in this region were also identified by other collaborating groups.
5.2 Summary of mutation analysis

The DNA of eighty-one unrelated HYP patients were screened for possible mutations using SSCP. Mutations were confirmed in 39.5% of these patients. This figure is lower than those found in other similar studies for a number of reasons: 1) patients in the study had bandshifts identified by SSCP that were not subsequently sequenced to confirm the presence of a mutation. 2) a number of the patients in the study were female, and deletions in females would not be identified by SSCP, since with two copies of the PHEX gene, the presence of the normal gene masks the presence of a deletion. 3) only 16 out of the 22 exons were screened by SSCP, so additional mutations would be expected to be found in the remaining exons. 4) all the SSCP experiments were conducted at a single temperature. Some of the bandshifts may not have been expressed at this temperature, and so would not have been identified.

The mutations identified in this study included 11 deletions, 2 microdeletions, 2 nonsense mutations, 3 frameshift mutations, 4 splice mutations (2 in the splice acceptor site and 2 in the splice donor site) and 1 missense mutation. These mutations were not found to lie in any particular region of the PHEX gene, but were present throughout the length of the gene studied. There were no mutation hotspots, nor a particular mutation that was found with a high frequency in unrelated families. This suggests that the entire length of the PHEX gene is important for protein function.
Other mutations confirmed that PHEX was defective in HYP patients, (Dixon et al., 1998; Filisetti et al., 1999; Francis et al., 1997; Holm et al., 1997; Tyynismaa et al., 2000). These studies described mainly novel mutations.

5.3 The PHEX gene

The PHEX gene was found to have a high degree of homology with members of the neutral endopeptidase family, the zinc metalloendopeptidases. These are type II integral membrane glycoproteins, which include NEP, ECE-1 and KELL antigen. These proteins all have a short cytoplasmic N-terminal region, a single transmembrane domain and a large extracellular C-terminal domain with a zinc binding motif. These proteins all have a large number of small exons (22 in PHEX), a highly conserved zinc binding motif (HExxH), 10 conserved cysteine residues and conservation of the amino acids asparagine, alanine, histidine, glutamic acid, histidine, glutamic acid, aspartic acid, histidine and arginine at positions 538, 539, 580, 581, 584, 642, 646, 710 and 747 respectively (figure 3.24 and 4.1). These residues are important for sequestering the zinc atom, substrate specificity, stabilisation of the transition state and catalysis (Rowe, 1998).

This suggested that the role of PHEX is proteolytic processing of an as yet undiscovered bioeffector molecule, for example a hormone, signal transduction factor or receptor. NEP, which has a high degree of homology with PHEX, downregulates induced responses to several hormones, including enkaphalin, chemotactic peptide, substance P, oxotocin, neurotensin, bradykinin, gastrin releasing peptide, neuromedin C and angiotensins I and II (Rowe, 1997; Shipp et al., 1990; Shipp et al., 1991). ECE-1 activates big endothelin by cleavage,
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releasing a vasoactive peptide (Xu et al., 1994; Yorimitsu et al., 1995). Recent work by Rowe et al. (Rowe et al., In Press) has identified a protein (MEPE) which is expressed at high levels in oncogenic hypophosphataemic osteomalacia (OHO) tumours. Addition of a truncated form of recombinant MEPE (rec-MEPE) to a human renal cell line (CL8) resulted in altered phosphate-uptake and 24-hydroxylase mRNA expression. It is proposed that MEPE is the substrate which the PHEX gene affects.

With the identification of the PHEX gene, and more recently the substrate upon which it acts, the mechanism by which HYP occurs is much closer to elucidation. It is to be hoped that in the near future, treatments for HYP can be developed to prevent the debilitating effects of this disease. Treatments which will improve the quality of life for familial rickets patients, and potentially those suffering other bone mineral loss disorders.
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