A STUDY OF GENETIC HETEROGENEITY IN
ALBRIGHT HEREDITARY OSTEODYSTROPHY

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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September 1996
This thesis is dedicated

to my father and

to the memory of my mother
Abstract

A STUDY OF GENETIC HETEROGENEITY IN ALBRIGHT HEREDITARY OSTEODYSTROPHY

Monique Elisabeth Maria Oude Luttikhuis

Albright hereditary osteodystrophy (AHO) is a disorder characterised by short stature, obesity, subcutaneous calcifications, brachydactyly and mental retardation. AHO can occur with or without resistance to hormones acting via adenylyl cyclase, even within one kindred. Both phenotypic forms can result from identical heterozygous deactivating mutations in the gene encoding Gsa (GNAS1) leading to reduced Gsa activity. Parental origin of the mutation is suggested to play a role in the development of hormone resistance.

In this study, 27 GNAS1 mutations have been identified, of which 25 were unique, in a cohort of 58 individuals with characteristics of AHO. The mutations were localised throughout the gene although 7/27 (26%) were present in exon 1. All mutations were predicted to lead either to amino acid substitutions in functionally or structurally important domains or to truncated proteins. Parental origin of the mutations was established in 17 parent-to-child transmissions (14/17 maternal and 3/17 paternal transmissions) and supports a parental effect as mutations on the maternally derived allele always resulted in offspring with AHO and hormone resistance and mutations on the paternally derived allele in offspring with AHO and hormone responsiveness. Tissue specific imprinting of GNAS1 has been excluded as the mechanisms leading to the observed parental effect. Furthermore, a second locus for AHO on chromosome 2q37 has been identified by deletion mapping. The data did not reveal if AHO localised to this region of the genome results from a single gene defect or from a deletion of contiguous genes. Linkage of the gene for brachydactyly E, one of the typical characteristics of AHO, to 2q37 was excluded in one extensive kindred with isolated autosomal dominant brachydactyly type E.
Acknowledgements

This body of work could not have been accomplished without the efforts and cooperation of a large number of people to whom I offer my thanks. Foremost amongst those is Dr. Richard Trembath, my supervisor and the keystone of the project. In addition, I would like to thank Dr. Louise Wilson for providing samples and medical examinations of many of the patients studied, as well as performing Gsα bioactivity measurements and for being a friend. Furthermore, I thank Mr. Lee Turnpenny who screened exons 4 and 11 of GNAS1, providing me with the chance to put my feet up and play supervisor. Of course I am indebted for all the help and support I received from the members of the Department of Genetics.

Thank you Marcus, Steve and Yuri for being such good opponents during our weekly badminton games, always good for getting rid of an overload of aggression.

De "oprichting" van 'Neerlands hoop' was een fantastisch idee, al worden die vrijdagavonden wel erg laat (of moet ik zeggen die zaterdagochtenden wel erg vroeg).

For the moral support from my two new families and my two old families, I am extremely grateful.

A special thank you is to my father who always supports me wherever I go, and to Neil who now goes there with me.
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<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AHO</td>
<td>Albright hereditary osteodystrophy</td>
</tr>
<tr>
<td>BE</td>
<td>brachydactyly type E</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cyclic adenosine monophosphate</td>
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<td>complementary DNA</td>
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<td>CEPH</td>
<td>Centre d'Etude du Polymorphisme Humain</td>
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<td>cM</td>
<td>centimorgan</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<td>2'-deoxyadenosine 5'-triphosphate</td>
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<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
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<td>dGTP</td>
<td>2'-deoxyguanine 5'-triphosphate</td>
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<td>dNTP</td>
<td>equimolar solution of 2'-deoxynucleoside 5'-triphosphates</td>
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<tr>
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<td>2'-deoxythymidine 5'-triphosphate</td>
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<td>2'3'-dideoxyguanine 5'-triphosphate</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
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<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Animal Cell Cultures</td>
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<td>ethylenediamine tetracetic acid</td>
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<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
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<tr>
<td>EST</td>
<td>expressed sequence tag</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
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<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
</tr>
<tr>
<td>GDB</td>
<td>Genome Database</td>
</tr>
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<td>GDF</td>
<td>growth/differentiation factor</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<td>GNAS1</td>
<td>human Gsa gene</td>
</tr>
<tr>
<td>Gsa</td>
<td>α subunit of the stimulatory G protein</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HGMP-RC</td>
<td>Human Genome Mapping Project Resource Centre</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-indolyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase, a thousand base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase, a million base pairs</td>
</tr>
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<td>MIM</td>
<td>Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
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<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidyl inositol 4,5 biphosphate</td>
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<td>PHPI</td>
<td>pseudohyoparathyroidism type I</td>
</tr>
<tr>
<td>PHPIa</td>
<td>pseudohyoparathyroidism type Ia</td>
</tr>
<tr>
<td>PPHP</td>
<td>pseudopseudohyoparathyroidism</td>
</tr>
<tr>
<td>PIC</td>
<td>polymorphic information content</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>STS</td>
<td>sequence tagged site</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/Acetate/EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNN'N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactoside</td>
</tr>
</tbody>
</table>
Symbols for amino acids

A  Ala  Alanine
B  Asx  Asparagine or aspartic acid
C  Cys  Cysteine
D  Asp  Aspartic acid
E  Glu  Glutamic acid
F  Phe  Phenylalanine
G  Gly  Glycine
H  His  Histidine
I  Ile  Isoleucine
K  Lys  Lysine
L  Leu  Leucine
M  Met  Methionine
N  Asn  Asparagine
P  Pro  Proline
Q  Gln  Glutamine
R  Arg  Arginine
S  Ser  Serine
T  Thr  Threonine
V  Val  Valine
W  Trp  Tryptophan
Y  Tyr  Tyrosine
Z  Glx  Glutamine or glutamic acid
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M.E.M. Oude Luttikhuis et al. (1997) Molecular analysis of the Gsα gene (GNAS1) in Albright hereditary osteodystrophy identifies clustering of mutations in exon 1 and support for a parental effect (manuscript in preparation)

Posters

Chapter 1: Introduction

1.1 Clinical aspects of Albright hereditary osteodystrophy

1.1.1 History and terminology of Albright hereditary osteodystrophy

In 1942, Albright and colleagues (Albright et al., 1942) described three patients with the clinical features of hypoparathyroidism: depressed serum-calcium level (hypocalcaemia) and elevated serum-inorganic-phosphorus level (hyperphosphataemia). In an attempt to restore normal serum-calcium levels, bovine parathyroid extract was administered to these three patients as well as to a patient with primary hypoparathyroidism. The response to parathyroid extract was absent in Albright's three patients in contrast to the hypoparathyroid subject. Albright presumed that the patients' symptoms were caused by the failure of the end-organ to respond to parathyroid hormone (PTH) rather than by a lack of that hormone as seen in other causes of hypoparathyroidism (Heath, 1987). A diagnosis of pseudohypoparathyroidism (PHP) was made. In addition to the metabolic abnormalities, the three patients had unusual physical features including short stature, a stocky build, round face, soft tissue and subcutaneous calcification and short metacarpals (fig. 1.1). These physical features are referred to as Albright hereditary osteodystrophy (AHO). Moderate to severe mental retardation was also seen in two of the patients. A connection between the disturbed calcium metabolism and the physical features was not obvious.

Ten years later, Albright and colleagues (Albright et al., 1952) described a patient who had the physical features of PHP but who had normal levels of serum-calcium and serum-phosphate indicating she was responsive to PTH. To distinguish these findings from those previously reported for PHP, Albright coined the term pseudopseudo-hypoparathyroidism (PPHP).

Subsequent studies confirmed Albright's hypothesis of end-organ resistance as the basis of PHP. Individuals with untreated PHP were found to have normal or hyperplastic parathyroid tissue suggesting normal or increased production of PTH (Mann et al., 1962), immunologically and biologically active PTH was found in parathyroid glands confirming that the hormone is synthesised (Tashjian et al., 1966) and immunoreactive PTH in plasma was found to be raised (Chase et al., 1969) confirming that the hormone is being released into the circulation.

Since the condition was first described, insight has been gained into the mechanism of action of PTH. Stimulation by PTH leads to the formation of cyclic adenosine monophosphate (cAMP) in the kidney leading to reduced reabsorption of phosphate and increased reabsorption of calcium in the proximal tubules. The action of PTH is described in more detail in 1.1.3.1. Administration of PTH to normal rats (Chase & Aurbach, 1967) and humans (Chase et al., 1969) cause an increase in urinary excretion of cAMP and
Figure 1.1 X-ray of hands from an individual with AHO

X-ray showing the various bones in the hands and numbering of the digits. The 4th metacarpal on the left hand and the 4th and 5th on the right hand are abnormally short showing brachydactyly. Metatarsals are bones in the feet equivalent to metacarpals.
- distal phalange
- middle phalange
- proximal phalange
- metacarpal
phosphate through direct action of the hormone on the metabolism of the kidney. The response to exogenous PTH may vary between individuals with AHO and forms the basis of a biochemical classification. Individuals with PTH circulating in high concentrations, who fail to raise the urinary excretion of cAMP or phosphate after administration of PTH are classified as PHP type I (PHPI) (Chase et al., 1969) and those with normal cAMP but abnormal phosphate response as PHP type II (Drezner et al., 1973). The individuals with PHPI may also be resistant to other hormones that stimulate cAMP production such as thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Marx et al., 1971; Levine et al., 1983a). The majority of these individuals have a deficiency of the α subunit of the stimulatory guanine nucleotide binding protein (Gsa) and are classified as PHP type Iα (Levine et al., 1983a; Farfel et al., 1982). A deficiency of Gsa activation also occurs in individuals with PPHP (Levine et al., 1986) although they are not resistant to PTH (Chase et al., 1969). Both PHPIa and PPHP can occur within the same family although concordance of phenotypes is consistently observed between affected siblings (Fischer et al., 1983; Levine et al., 1986). Gsa deficiency does not occur in individuals with PHP type Ib (Farfel et al., 1982) or type Ic (Barrett et al., 1989). PHPIb patients are resistant to PTH only and do not have AHO (Silve et al., 1986) whilst PHPIc patients show multiple hormone resistance identical to PHPIa patients and have features of AHO (Barrett et al., 1989). For clarity, the characteristics of PHP subtypes and PPHP are summarised in Table 1.1.

1.1.2 The clinical spectrum of Albright hereditary osteodystrophy

A notable variation in the presentation of both physical and biochemical features in AHO is seen amongst individuals. Therefore an overview of features which may occur in AHO patients will be given.

Short stature and obesity are seen in many, but not all AHO patients (fig. 1.2). Individuals with PHPI are relatively shorter as adults than they are as children reaching puberty early and ceasing to grow (de Wijn & Steendijk, 1982a; de Wijn & Steendijk, 1982b). Advanced skeletal age which presents as premature fusion of the epiphyses, is thought to be a factor contributing to short stature (de Wijn & Steendijk, 1982a). The extremities are usually shortened (Goeminne, 1965), resulting in an arm span which is less than height. Obesity usually arises in early childhood (Fitch, 1982).

Round face and short neck are common in AHO, whilst other features include a flattened bridge of the nose, strabismus, widely spaced orbits and a high arched palate (Fitch, 1982).

Soft tissue calcification is common in AHO, especially in subcutaneous tissues and the brain. Subcutaneous calcifications may be observed within days of birth (Steinbach et al., 1965). The subcutaneous calcium deposits are associated with ectopic ossifications (bone formation) (Barranco, 1971). Calcification in the brain occurs most frequently in the
Table 1.1: The biochemical classification of pseudohypoparathyroidism

<table>
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<tr>
<th>subtype</th>
<th>cAMP response to PTH</th>
<th>phosphate response to PTH</th>
<th>AHO</th>
<th>multiple hormone resistance</th>
<th>Gsα levels</th>
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<td>blunted</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td>Gsα mutation</td>
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<tr>
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<td>blunted</td>
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<td>no</td>
<td>normal</td>
<td>PTH receptor ?†</td>
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<td>blunted</td>
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<td>yes</td>
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<td>no</td>
<td>no</td>
<td>normal</td>
<td>distal to adenyl cyclase ?</td>
</tr>
<tr>
<td>PPHP</td>
<td>normal</td>
<td>normal</td>
<td>yes</td>
<td>no</td>
<td></td>
<td>Gsα mutation</td>
</tr>
</tbody>
</table>

† means reduced when compared to normal controls

† PHPⅠb is not caused by mutations in the coding exons (and their splice junctions) of the parathyroid hormone receptor gene. If PHPⅠb is indeed caused by mutations in this gene, then they must be present in intronic or regulatory sequences (Schipani et al., 1995).
Figure 1.2 Appearance of a child with AHO

Typical appearance of a child with AHO showing short stature, round face and stocky build.
basal ganglia although other structures may also be involved and is uncommon in PPHP (Goeminne, 1965).

The shortening of metacarpals and/or metatarsals (fig. 1.1) is termed brachydactyly (Bell, 1951). This is frequently associated with shortening of the distal phalanges in AHO. The hand abnormalities appear to be identical for PHP and PPHP and are often asymmetric. The most frequently affected is the distal phalanx of the thumb, apparent as an increased ratio of the width to the length of the nail, and the fourth metacarpal (Poznanski et al., 1977). Metacarpal shortening can be recognised as dimpling over the knuckles of a clenched fist. Within one family, the shortened digits may vary between affected individuals. The typical shortening is a feature that develops with age. Steinbach and colleagues (Steinbach et al., 1965) described an individuals whose skeletal lesions were absent at nine months but had developed by the age of four years as a result of premature fusion of the epiphyses.

Individuals with AHO may have mental retardation varying from mild to moderate. Mental retardation has been reported more often in hypocalcaemic (70%) than in normocalcaemic individuals (30%) (Fitch, 1982). Hypocalcaemia, whilst associated with mental retardation, is unlikely to be the primary factor leading to mental retardation in AHO (Farfel & Friedman, 1986).

1.1.3 Endocrinological features

The action of PTH plays a central role in maintaining the phosphate and calcium homeostasis by controlling the flux of calcium and phosphate into and out of the kidney, the gastrointestinal tract and bone (fig. 1.3). PTH action can however not fully explain the phenotypic characteristics, such as metacarpal and/or metatarsal shortening and subcutaneous calcifications, observed in PHP type Ia and in PPHP, where PTH action is not disturbed.

1.1.3.1 Parathyroid hormone action and its control of calcium metabolism

PTH is synthesised in the parathyroid glands as pre-pro-PTH which is 115 amino acids long. Subsequent cleavages give rise to the 84 amino acid peptide PTH. Only the first 32 amino acids are required for biological activity (Heath, 1987). PTH is extremely sensitive to variations in an individual's basal calcium level. Secretion of PTH is increased after a fall in calcium level and decreased after a rise. The main sites of action of PTH are the kidney and bone.

PTH can activate target cells in two ways; by synthesis of cAMP through stimulation of adenyl cyclase or by a cAMP independent mechanism (Hosking & Kerr, 1988). Binding of PTH to its receptor will activate a guanine nucleotide binding protein which stimulates the action of adenyl cyclase whilst cAMP, an intracellular messenger, is formed (see 1.2.1). Alternatively PTH can bind to a receptor whose activation leads to the hydrolysis of phosphatidyl inositol 4,5 biphosphate (PIP2). The resulting products,
Figure 1.3 Diagram of calcium homeostasis maintenance

Diagram showing how calcium homeostasis is maintained through the action of PTH and vitamin D and its metabolites.

25(OH)D means 25-hydroxy vitamin D; 1,25(OH)₂D means 1,25-dihydroxy vitamin D; + indicates stimulation of a pathway.
diacylglycerol (DAG) and inositol triphosphate (IP\textsubscript{3}), act as intracellular messengers. DAG stimulates protein kinase C activity and IP\textsubscript{3} increases intracellular calcium.

Normal levels of calcium are maintained primarily by PTH action. Absence of PTH or resistance against the hormone will lead to hypocalcaemia. PTH raises the level of serum calcium by immobilisation of calcium from bone, reduction of calcium excretion by the kidney and increase in calcium absorption by the intestine. More than 90% of calcium in human bodies is present in bone and therefore bone is of major importance in the calcium homeostasis. Two independent processes regulated by PTH occur; a remodelling system (involved in removal and reabsorption of old bone and replacement by new tissue) and a homeostatic system (involved in transport of calcium between bone fluid and extracellular fluid). Both systems require one or more metabolites of vitamin D to function.

Reabsorption of phosphate by the kidney occurs by an active transport system which is inhibited by PTH. Increased concentration of PTH will lead to decreased phosphate reabsorption. Ultimately the levels of phosphate in urine will increase and serum levels will fall. Phosphate reabsorption occurs mainly in the proximal tubules.

Vitamin D is converted to 1,25-dihydroxy vitamin D (1,25(OH)\textsubscript{2}D) in the kidney. This metabolite is primarily responsible for vitamin D actions on the kidney, bone and intestine. When serum phosphate levels are low, PTH stimulates the formation of 1,25(OH)\textsubscript{2}D. By increasing the formation of 1,25(OH)\textsubscript{2}D, PTH acts indirectly on the gastrointestinal tract to increase calcium absorption. 1,25(OH)\textsubscript{2}D is also required for PTH to release calcium from bone. Loss of PTH action in the kidney impairs calcium homeostasis through the direct effects of PTH on phosphate and calcium clearance and through the indirect effects mediated by 1,25(OH)\textsubscript{2}D.

1.1.3.2 Endocrinological features of Albright hereditary osteodystrophy

The major symptoms of PHP are the consequence of reduced concentrations of ionised calcium in blood and extracellular fluid primarily caused by resistance to the action of PTH in the kidney. Hypocalcaemia leads to tetany, a condition characterised by increased neuromuscular excitability. Long periods of hypocalcaemia and hyperphosphataemia can lead to soft tissue calcifications in individuals with PHP. However, most individuals are hypocalcaemic for short periods of time implying that hypocalcaemia is not the primary cause of their soft tissue calcifications. In addition, soft tissue calcifications also occur in individuals with PPHP who are not hypocalcaemic.

Resistance to PTH is most widely studied in PHP1a. Of the other hormones to which these individuals show resistance, those with effects on the thyroid and the reproductive system are clinically most important.

Thyroid abnormalities are common, particularly hypothyroidism. A large proportion of individuals with PHP1 (85%) (Levine et al., 1983a) have high basal levels of TSH and an exaggerated TSH response to thyrotropin-releasing hormone.
Sexual immaturity, absence of menstruation or mensturation with a long, irregular time in between periods has been observed in 77% of PHPI patients (Levine et al., 1983a). A lack of male-to-male transmission in familial cases of AHO as well as a lack of affected males with children was noted (Cederbaum & Lippe, 1973), which lead to the suggestion of reduced male fertility. In a review of the literature (Davies & Hughes, 1993), 36 parent-to-child transmissions of AHO were noted. Of these, 33 were maternal and only three were paternal. This may reflect an ascertainment bias because affected children of mothers with AHO always appear to have the hormone resistant form (PHPIa) and therefore promptly come to the attention of clinicians.

1.1.4 Diagnosis

Hypocalcaemia and hyperphosphataemia, in the absence of renal failure, are indicative of a diagnosis of hypoparathyroidism (Heath, 1987). Assaying the levels of immunoreactive PTH will distinguish between the hormone-deficient and the hormone-resistant forms of hypoparathyroidism. Increased PTH levels are seen in patients with PHP. The response to exogenous PTH can confirm this diagnosis and distinguish between PHPI (blunted cAMP and phosphate response) and PHPII (normal cAMP and blunted phosphate response). The physical features of AHO can help to distinguish between PHPIa and Ic, and PHPIb (see table 1.1). The distinction between PHPIa and PHPIc can only be made after measurement of Gsα bioactivity levels (see 1.2.4) but this test is only performed in a few laboratories and mainly for research purposes.

Individuals with normocalcaemic AHO should not automatically be diagnosed with PPHP since periods of normocalcaemia are observed in PHPI despite proven PTH resistance (Levine et al., 1985; Schuster et al., 1994).

1.1.5 Differential diagnosis

Peripheral dysostosis is a malformation of bone predominantly characterised by short metacarpals, metatarsals and phalanges. Those features are similar to AHO but represent a distinctly different disorder. Soft tissue calcifications are absent in peripheral dysostosis, the phalanges and metacarpals are short but usually proportionate whilst in AHO they usually are disproportionate, and serum calcium and phosphate levels are normal (Steinbach & Young, 1966).

Peripheral dysostosis associated with nasal hypoplasia, short stature and mental retardation is called acrodysostosis (Maroteaux & Malamut, 1968). No endocrine abnormalities or soft tissue calcifications are described in this disorder. Short stature, shortening of metacarpals, metatarsals and phalanges, advanced bone age and mental retardation are common between acrodysostosis and AHO. The hands and feet in acrodysostosis are notably smaller than those in AHO, since the shortening of metacarpals and metatarsal appears to affect all digits (Poznanski et al., 1977). Nasal hypoplasia is seen
in AHO but is more severe in acrodysostosis. A family has been described in which acrodysostosis coincided with PHP and PPHP (Ablow et al., 1977).

Brachydactyly type E is defined as variable shortening of the metacarpals and/or metatarsals (Bell, 1951). Short stature and round faces are also seen in this disorder whilst subcutaneous calcifications and mental retardation do not occur. Brachydactyly E is radiologically indistinguishable from AHO (Poznanski et al., 1977). The two disorders have been reported to cosegregate in one family (Cherninkov & Cherninkova, 1989).

Turner syndrome and AHO have short stature, short fourth or fifth metacarpal and short metatarsals in common. Soft tissue calcifications and metabolic abnormalities are not seen in Turner syndrome. Abnormal sex chromosomes occur in Turner syndrome, nearly always in only one generation, whilst AHO is characterised by normal karyotypes and familial occurrence (Steinbach & Young, 1966).

1.2 Biochemistry of Albright hereditary osteodystrophy

Resistance to PTH and various other hormones is characteristic for PHPI. Evidence that cAMP acts as a second messenger for all hormones to which individuals with PHPI are resistant suggested that the observed endocrine abnormalities were due to defective cAMP synthesis. Since the various hormones each bind to their own specific receptor, it was thought that a general defect in the adenylly cyclase pathway, distal to the hormone receptor would cause defective cAMP synthesis (Farfel & Bourne, 1980).

1.2.1 The adenylly cyclase signalling pathway

The adenylly cyclase signalling pathway consists of three main components; receptors which respond to specific stimuli, a catalytic unit which converts ATP to cAMP, and a guanine nucleotide binding (G) protein that couples the receptor with the catalytic unit (fig. 1.4). G proteins form a functional link between plasma membrane receptors and the formation of intracellular messengers. Extracellular stimuli (hormones, neurotransmitters, odorants and photons of light) activate their specific receptors which bind to the G protein in its GDP-bound (inactive) state. The G protein subsequently exchanges GDP for GTP. Binding of GTP leads to dissociation of the G protein from the receptor and to dissociation of the α subunit from the βγ subunits. The GTP-bound (active) state of the α subunit interacts with and regulates its effector, adenylly cyclase. The intrinsic GTPase activity of the α subunit leads to hydrolysis of bound GTP to GDP causing dissociation of the α subunit from its effector and reassocation with the βγ subunits (Pennington, 1995).
1.2.2 The family of heterotrimeric G proteins

Heterotrimeric G proteins mediate signal transduction by coupling extracellular signals to various intracellular effectors, such as adenylyl cyclase, phospholipases, phosphodiesterase, kinases or ion channels. All heterotrimeric G proteins consist of α, β and γ subunits, each encoded by separate genes.

1.2.2.1 Gα subunits

At least 18 mammalian genes are known to encode the α subunits (Pennington, 1995). These are divided into four classes based on amino acid similarity; Gs, Gi/o/t, Gq and G12. The Gs class contains two α subunits; Gsα and Golfα, the Gi/o/t class nine, the Gq class five and the G12 class two α subunits. The molecular mass of the Gα subunits varies from 40 to 56 kDa.

The variety of Gα subunits is further increased by alternative splicing in a proportion of the Gα genes; Gsα (Kozasa et al., 1988), Golfα (Murtagh et al., 1994) and Gia2 (Montmayeur & Borrelli, 1994). The alternatively spliced form of Gia2 is preferentially located in the Golgi apparatus suggesting that alternative splicing may regulate the subcellular localisation of the G proteins (Montmayeur & Borrelli, 1994).

Investigations into the function of various G proteins are carried out as many more G proteins are discovered. Gs and Golf stimulate adenylyl cyclase in contrast to Gi which inhibits this effector. Gt (transducin) is involved in transduction of light signals, members of the Gq class mediate activation of phospholipase C, whilst the function of G12 is not well understood yet (Gilman, 1987; Wilkie et al., 1992). Considering that the α subunits function in a wide range of cellular processes, their sequences are remarkably conserved. Five regions (G-1 to G-5), involved in nucleotide binding and Mg^2+ coordination, are highly conserved amongst all members of the GTPase superfamily (Pennington, 1995).

The expression of different α subunits varies from ubiquitous (Gsα, Gia2, Gqα, G11α, G12α, G13α), to partially restricted (Gia1, Gia3, Goc), to highly tissue specific (Gto) depending on their function (Weinstein & Shenker, 1993). G protein α subunits reside within the plasma membrane through covalent attachment of myristic acid and palmitic acid (Pennington, 1995) but may be released following activation of receptors (Milligan & Unson, 1989).

G protein α subunits can be adenosine diphosphate (ADP)-ribosylated by cholera and pertussis toxin from Vibrio cholerae and Bordetella pertussis, respectively (Gilman, 1987). Both enzymes covalently modify specific residues in the α subunit of different G proteins. Gsα is ADP-ribosylated by cholera toxin at the residue Arg201 resulting in the constitutive activation of adenylyl cyclase due to the accumulation of Gsα in the GTP-bound (active) state (Cassel & Selinger, 1977). Gia, Golfα and Gtα are ADP-ribosylated by pertussis toxin at a cysteine residue four amino acids from the carboxyl terminus resulting in inhibition of the hormone-activated pathway regulated by the G protein.
1.2.2.2 Gβ and Gγ subunits

At least 4 genes are known to encode β subunits and 7 genes encoding γ subunits. The diversity of α, β and γ subunits may lead to a large number of unique heterotrimers. For a long time the α subunit was thought to be responsible for the specificity of the signalling pathway. However, recent studies revealed that βγ subunits may also regulate effectors such as adenylyl cyclase (Tang & Gilman, 1991; Federman et al., 1992) and phospholipase Cβ (Camps et al., 1992; Katz et al., 1992). Further studies by Kleuss and colleagues have shown that β subunits determine G protein interaction with transmembrane receptors (Kleuss et al., 1992) and γ subunits determine selectivity in signal transduction (Kleuss et al., 1993).

1.2.3 The Gsα protein

Two Gsα proteins have been identified (molecular weight 45 and 52 kDa) and are referred to as Gsα-S and Gsα-L (Northup et al., 1980). Relative amounts of the two forms of Gsα vary amongst tissues. In one study (Mumby et al., 1986), the short form was most abundant in heart whilst the long form was most abundant in brain, adrenal medulla, adrenal cortex and tracheal smooth muscle. In another study, the expression of mRNAs encoding the short and long forms were measured in a variety of rat tissues (including cerebral cortex, cerebellum, liver, heart, adrenal gland, kidney, brown and white adipose tissue) (Granneman et al., 1990) and in all tissues examined Gsα-L was most abundant varying from 95% for brain tissue and 57% for brown adipose tissue. Changes in relative amounts of the two proteins have been observed during reticulocyte maturation (Larner & Ross, 1981). The concentration of both forms of Gsα decreased with maturation although a disproportionately greater reduction of the long form was noticed. The expression of the two forms of Gsα is thus regulated in a tissue specific, and perhaps developmental, manner. A difference in function between the two different sized proteins is unknown if indeed both proteins are functional.

Information about functional and structurally important domains in the Gsα subunit can be derived from the crystal structure of Gα (Lambright et al., 1996) and by comparison with other closely related Gα subunits. Both N- and C-terminus are involved in receptor binding (Conklin & Bourne, 1993) whilst the N-terminus is also involved in βγ interaction (Neer et al., 1988) as are exons 3 and 4 and part of exon 9 (Conklin & Bourne, 1993). Interaction with the effector adenylyl cyclase takes place in a region comprising residues 236-356 (exons 10, 11, 12) (Berlot & Bourne, 1992).

1.2.4 Measurements of Gsα bioactivity

The identification of the G protein coupling extracellular with intracellular signalling was facilitated by the availability of a mouse mutant lymphoma cell line, S49 cyc− (Bourne et al., 1975). No cAMP was formed when these cells were exposed to certain stimuli but complementation by a protein in a detergent extract from cells containing adenylyl cyclase
made the cyc− cells respond (Ross & Gilman, 1977; Johnson et al., 1978). The protein lacking from S49 cyc− was shown to be the G protein, also referred to as N protein (Bourne et al., 1981). Adenylyl cyclase and a G protein-coupled receptor were still present. Reconstitution of the adenylyl cyclase pathway in S49 cyc− cells was measured using a complementation assay. Extracts from membranes, which can be of any cell type, are treated with a detergent to solubilise the G protein from the membrane. The soluble fractions of the extracts are added to plasma membrane preparations of S49 cyc− together with adenylyl cyclase stimuli and adenylyl cyclase assayed at 30°C. During the reaction [α32P]−ATP is converted into [32P]−cAMP. Measurement of the amount of cAMP formed, involves separation of radioactive ATP from cAMP by chromatography (Salomon et al., 1974). Before addition of the mixture to the columns, [3H]−cAMP is added as an internal standard to measure the recovery of cAMP.

Gsα can also be assayed by measuring cholera toxin-catalysed incorporation of [32P]−ADP-ribose, from [32P]−nicotinamide adenine dinucleotide (NAD)+, into the Gsα unit, which is subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) and isolated from the gel (Northup et al., 1980).

1.2.5 Gsα bioactivity in individuals with Albright hereditary osteodystrophy

Resistance to multiple hormones acting via adenylyl cyclase was observed in many individuals with PHPI. As receptors to these hormones are tissue specific, and G proteins are common to all tissues, a defective G protein should lead to resistance to all hormones acting via adenylyl cyclase. To test this hypothesis, Gsα bioactivity was measured in erythrocytes of PHPI patients (Farfel et al., 1980; Levine et al., 1980; Farfel & Bourne, 1980; Farfel et al., 1981; Fischer et al., 1983; Levine et al., 1983a; Van Dop et al., 1984; Akita et al., 1985; Farfel & Friedman, 1986; Levine et al., 1986; Radeke et al., 1986). Erythrocytes do not contain adenylyl cyclase and may act as substrate for the cyc− complementation assay. In addition, erythrocytes are a readily accessible cell type. Erythrocyte Gsα bioactivity was reduced to around 50% in only a proportion of individuals with PHPI; those with reduced bioactivity were designated PHPIa and those with normal bioactivity were designated PHPIb (Farfel et al., 1982). A variety of other tissues, some of those hormone responsive tissues, were also tested for their Gsα bioactivity. A reduction was observed in platelets (Farfel & Bourne, 1980), cultured fibroblasts (Bourne et al., 1981; Levine et al., 1983b), transformed lymphoblasts (Farfel et al., 1982) and renal membranes (Downs et al., 1983) confirming that the defect in PHPIa was a generalised deficiency of a G protein. Furthermore, in vitro studies confirmed that the defect was maintained in cultured cells, implying a defect in the gene encoding Gsα (Bourne et al., 1981). A common genetic defect was considered in both PHPIa and PPHP as a similar reduction in Gsα bioactivity was observed in individuals with PHPIa and their first degree relatives with PPHP (Levine et al., 1986). The expression of the defect was thought to be modified by environmental or additional genetic influences. G protein
deficiency was alternatively measured by cholera toxin-catalysed labelling of the Gsa subunit (Farfel et al., 1981; Farfel et al., 1982; Downs et al., 1983; Levine et al., 1983b; Akita et al., 1985). The reduction in Gsa bioactivity may be associated with decreased levels of Gsa mRNA, as measured in cultured fibroblasts (Carter et al., 1987; Levine et al., 1988) although normal mRNA levels can be present despite reduced Gsa bioactivity (Levine et al., 1988).

1.3 The genetics of Albright hereditary osteodystrophy

1.3.1 The history of the genetics of Albright hereditary osteodystrophy

Very few instances of male-to-male transmission of AHO have been reported and females are affected twice as often as males, leading to the early hypothesis that AHO was an X-linked dominant disorder (Mann et al., 1962). Autosomal dominant inheritance of AHO was also proposed, based on male-to-male transmission in a family (Weinberg & Stone, 1971) as was autosomal recessive inheritance in a family with two affected sibs whose parents were clinically unaffected (Cederbaum & Lippe, 1973). Father to son transmission of decreased erythrocyte Gsa bioactivity (Van Dop et al., 1984), segregation of heterozygous mutations in the gene encoding Gsa (1.3.4) and mapping of this gene to chromosome 20 (1.3.2) support autosomal dominant transmission of AHO. The above mentioned family showing autosomal recessive inheritance was further studied. The two affected siblings had erythrocyte Gsa deficiency whilst both unaffected parents showed normal Gsa bioactivity (Farfel et al., 1981) implying autosomal recessive inheritance in this family, although gonadal mosaicism was not excluded.

1.3.2 The Gsa gene (GNAS1)

The first DNA clone encoding part of the a subunit of the stimulatory G protein (Gsa) was isolated from a bovine cDNA library by screening with an oligonucleotide probe derived from amino acid sequence common to the known G proteins transducin and Go (Harris et al., 1985). A synthetic peptide was generated of a region unique to this clone and subsequently polyclonal antibodies against this synthetic peptide were raised. The antiserum did not recognise the a subunits of bovine brain Go, bovine retinal transducin or rabbit liver Gi, nor did it recognise any proteins from an extract of S49 cyc- cells (which genetically lack Gsa). The antiserum recognised only Gsa from S49 wild-type cells. Northern blotting using the cDNA clone as a radioactively labelled probe revealed an RNA present in S49 wild-type cells and not in S49 cyc- cells. The immunological and RNA data confirmed that the isolated clone was indeed bovine Gsa. Subsequently mouse (Sullivan et al., 1986), rat (Itoh et al., 1986) and human (Bray et al., 1986) cDNA clones were isolated. Four types of human cDNA were cloned that differed in the region corresponding to codons 71-88. It was suggested that the four cDNA species were generated following
alternative splicing of a precursor RNA (Kozasa et al., 1988). Not long before Robishaw and colleagues (Robishaw et al., 1986) had reported the existence of two cDNAs corresponding to two distinct Gsα mRNAs. Expression of the two cDNAs yielded proteins with apparent molecular weights of 45 and 52 kDa, identical to the molecular weights observed for bovine Gsα (Northup et al., 1980), showing that the two cDNAs encoded the two forms of Gsα.

Gsα cDNA clones have been identified in different species and their respective genes given chromosomal assignments using panels of somatic cell hybrids. The mouse Gsα gene is located on chromosome 2 in the region 2E1-2H3 (Ashley et al., 1987) and the human Gsα gene is located on chromosome 20 (Blatt et al., 1988).

The human Gsα gene (GNAS1) has been isolated from human genomic libraries using rat Gsα cDNA as a probe (Kozasa et al., 1988). GNAS1 contains 13 exons spanning 20 kb. The promoter region has a high G+C content (85%) and 4 GC boxes. It lacks a typical TATA box or CAAT box. One copy of GNAS1 is present per human haploid genome. Comparison of the structure of the human Gsα gene with the four types of human cDNAs that have been described (Bray et al., 1986) revealed that alternative splicing of a single Gsα gene accounts for the generation of different mRNA species. Two long forms, Gsα-1 and Gsα-2, contain the complete sequence of exons 3 and 4 whilst the short forms, Gsα-3 and Gsα-4, lack exon 3. Gsα-2 and Gsα-4 have three additional nucleotides at the 5’ end of exon 4, encoding a serine residue (fig. 1.5).

A further Gsα mRNA species has been identified in canine and human. This mRNA encodes a truncated form of Gsα. It uses an alternative promoter, encodes a leading exon (termed exon 1’) and its tissue distribution is similar to that of the mRNA lacking exon 1’ (Ishikawa et al., 1990). Exon 1’ is located 2.5 kilo bases upstream of exon 1 and does not contain an in-frame ATG resulting in a truncated Gsα protein lacking the N-terminus. The region 5’ of exon 1’ has a high G+C content and absence of TATA or CAAT box in common with the region 5’ of exon 1. The function of the putative protein product using exon 1’ is still unclear.

1.3.3 Linkage to chromosome 20q13

Refinement of mapping by linkage studies using a polymorphism in exon 5 of the Gsα gene detected by denaturing gradient gel electrophoresis (DGGE) showed that GNAS1 was localised on the distal long arm of chromosome 20 (Gejman et al., 1991). In situ hybridisation with a [3H]-labelled probe from rat cDNA assigned GNAS1 to the chromosomal region 20q12-q13.2 (Gopal Rao et al., 1991) and with a [3H]-labelled probe from human cDNA to 20q13.2-13.3 (Levine et al., 1991). Mutations in GNAS1 in patients with AHO have been found (1.3.4) and therefore AHO can be assigned to the chromosome 20q12-13.3 region of the genome.
Figure 1.5 Alternative splicing of Gsα RNA

Four different cDNA species may be generated by alternative splicing of Gsα RNA.
1.3.4 GNAS1 mutations in Albright hereditary osteodystrophy

The first reported GNAS1 mutation in two related individuals with AHO was an A to G substitution at position +1 in the initiation codon (Patten et al., 1990). Normal and abnormal Gsα protein lacking the N-terminus were detected in erythrocytes from a mother (with PPHP) and her son (with PHPla) using C-terminal and N-terminal antibodies. Both patients had reduced Gsα bioactivity. The conclusion was made that at least in some patients with AHO, the disease was caused by inherited mutations in GNAS1. Gross deletions or rearrangements of GNAS1 were not detected in individuals with AHO (Levine et al., 1988; Patten et al., 1990; Miric et al., 1993) and therefore the search for GNAS1 mutations was focused on identification of small (point) mutations. Thus far, 18 other heterozygous GNAS1 mutations in exons 2-13 are reported. These mutations comprise five single base pair substitutions in exons 4 and 6 (Miric et al., 1993) and exon 13 (Schwindinger & Levine, 1992; Iri et al., 1994), two deletions of 1 bp in exons 5 (Shapira et al., 1996) and 10 (Weinstein et al., 1990), 7 deletions of 4 bp in exons 7 (Nakamoto et al., 1992; Weinstein et al., 1992; Yu et al., 1995) and 8 (Miric et al., 1993), a deletion of 43 bp comprising the 3' end of exon 4 and part of the following intron (Oude Luttikhuis et al., 1994), an insertion of 1 bp in exon 10 (Shapira et al., 1996) and two single base pair substitutions affecting splice donor sites of exons 5 (Wilson et al., 1994) and 10 (Weinstein et al., 1990). All mutations were unique except for the 4-bp deletions in exon 7 which occurred in six independent kindreds and a single base pair substitution in exon 13 which occurred in two unrelated boys with testotoxicosis and PHPla. Mutations occurred in individuals with PPHP as well as in individuals with PHPla. Related individuals with or without hormone resistance were shown to carry identical GNAS1 mutations (Patten et al., 1990; Nakamoto et al., 1992; Weinstein et al., 1990; Miric et al., 1993; Yu et al., 1995) implying that for most kindreds the Gsα deficiency may be necessary but not sufficient for the expression of hormone resistance.

Two exonic polymorphisms in GNAS1 have been described. Both involve a C to T transition in the recognition site of the restriction endonuclease FokI. The first polymorphism is located in exon 5 and was used for genetic mapping of GNAS1 (Gejman et al., 1991) and the second polymorphism is located in exon 13 (Waltman et al., 1994).

1.3.5 GNAS1 mutations in other disorders

GNAS1 mutations can result in loss or gain of Gsα protein function. Loss of function because of deactivating germline mutations account for both hormone responsive and hormone resistant forms of AHO (PPHP and PHPla). Activating somatic mutations leading to constitutively active Gsα protein are found in a subset of human endocrine tumours and in McCune-Albright syndrome.
Constitutive activation of Gsa was reported in a subset of growth hormone (GH)-secreting human pituitary tumours (Vallar et al., 1987). A distinct group of tumours was identified with elevated basal adenylyl cyclase activity (and therefore elevated cAMP levels) and poor or no response to stimulation. Gsa activity was normal in these tumours. Mutations in GNAS1 were thought to eliminate the requirement for stimulation in order to activate adenylyl cyclase in the tumour and thereby contribute to its growth. Four mutations were identified in GH-releasing pituitary tumours with proven elevated adenylyl cyclase activity (Landis et al., 1989). Mutations in three tumours replaced Arg201 with Cys or His and in one tumour Gln227 was replaced with Arg (designated R201C, R201H, and Q227R, respectively). The mutations inhibited the intrinsic GTPase activity of Gsa so that the active GTP-bound conformation was permanently maintained.

The mutant Gsa proteins found in this group of tumours shared the following characteristics with oncogene proteins; mutant proteins mimic the effect of an extracellular growth factor, mutations stabilise the active conformation of a signalling protein that normally stimulates growth of the cell type carrying the putative oncogene, the mutations are found in tumour DNA but not in DNA from other cells and the tumour genome contains mutant and non-mutant copies of the oncogene. Landis and colleagues suggested that the oncogene is called gsp, for Gs protein (Landis et al., 1989). Subsequently more gsp mutations were identified in GH-releasing pituitary tumours and thyroid tumours (Lyons et al., 1990; Suarez et al., 1991). These results show that gsp mutations can be found in tissues where elevated cAMP levels are a growth stimulatory signal. However, activating mutations of Gsa have not been reported in melanomas or adrenal cortex even though these tissues contain cell types in which cAMP is a positive growth stimulus. Instead, mutations in a second oncogene (gip2), encoded by the G protein gene Gia-2 were found in tumours of the adrenal cortex (as well as in endocrine tumours of the ovary) leading to the suggestion that oncogenic mutations in various G protein α chain genes result in human tumours (Lyons et al., 1990).

McCune-Albright syndrome

The McCune-Albright syndrome is characterised by abnormalities in three systems; skeleton (polyostotic fibrous dysplasia), skin (areas of hyperpigmentation) and endocrine system (precocious puberty, thyrotoxicosis, pituitary gigantism) (Albright et al., 1937; McCune & Bruch, 1937). The symptoms are very variable and incomplete forms of the syndrome may occur. Because cAMP is known to stimulate growth and/or function in gonads, thyroid, melanocytes and osteoblasts, it was assumed that overactivity of the Gs signalling pathway may lead to the autonomous endocrine hyperfunction as well as the skin and bone lesions associated with McCune-Albright syndrome. Mutations in GNAS1 that result in constitutive activation of Gsa (gsp mutations R201C and R201H) have been identified in affected tissues from McCune-Albright patients (Weinstein et al., 1991;
Schwindinger et al., 1992; Shenker et al., 1993) whereas the mutation was absent in unaffected tissues. These findings can be explained by the model originally proposed by Happle (Happle, 1986) whereby a somatic mutation early in embryogenesis results in a mosaic of normal and mutant cells. The constellation of abnormalities in each patient is dependent on the distribution of the cells carrying the mutation.

1.4 Parental effect of GNAS1 mutations in Albright hereditary osteodystrophy

A recent review of 31 published familial cases of AHO suggested that the phenotypic expression of AHO (with or without hormone resistance) was dependent upon the sex of the transmitting parent (Davies & Hughes, 1993). AHO with hormone resistance was present in 60/60 offspring who had inherited the disease from their mothers whilst the hormone responsive form was present in 6/6 offspring who had inherited the disease from their fathers. Involvement of genomic imprinting in the expression of AHO, to explain the observed parental effect, was suggested.

1.4.1 Genomic imprinting

Mendelian inheritance of a trait is characterised by an equal contribution of the paternally and maternally derived alleles. Genes which are differentially expressed, depending whether an allele has come from the father or the mother, are said to be subject to genomic imprinting. Evidence for genomic imprinting came from studies on mice and humans (Hall, 1990).

(a) Pronuclear transplantation in mice showed that zygotes with only paternally derived chromosomes have relatively normal development of membranes and placenta but very poor development of embryonic structures. Zygotes with exclusively maternally derived chromosomes had a reverse phenotype suggesting that both paternally and maternally derived chromosomes were necessary for embryonic development and that they are complementary to each other.

(b) Human triploids have two chromosomes from one parent and one chromosome from the other parent. They are phenotypically different dependent on the parental origin of the extra chromosome.

(c) Uniparental disomy, whereby both members of a chromosome pair are inherited from one parent, has been observed in almost all segments of the mouse genome. Mice carrying balanced translocations still carry all genetic material but both copies of a whole or part of a chromosome may have been derived from one parent. Studies on mice with reciprocal parental contributions had opposite phenotypes (e.g. hyperactive versus inactive) and revealed regions of the mouse genome involved in imprinting (Cattanach & Kirk, 1985).
(d) Deletions of certain regions in the human genome produce a different phenotype depending on the origin of the deletion. Prader-Willi syndrome is associated with deletions of the paternally derived chromosome region 15q12 and Angelman syndrome with deletions of the same region on the maternally derived chromosome (Knoll et al., 1989).

(e) Expression of a gene, inserted in the genome of a mouse, depends in subsequent generations on the sex of the transmitting parent (Reik et al., 1987).

(f) The phenotypic expression of a number of human disorders can differ depending on the sex of the transmitting parent. For example, the age of onset in Huntington chorea is higher when the disorder is inherited from the mother (Myers et al., 1985).

The role of genomic imprinting is still unclear. However, suggestions include a maintenance of the requirement for genetic material from both parents thereby contributing to genetic health and diversity of future generations (Haig, 1992) and a role for genomic imprinting in the control of intrauterine embryonic growth (Barlow, 1995).

Genomic imprinting requires that parental alleles are distinguished in every embryonic cell and that this imprint is established after gametogenesis. A removal of imprinting occurs in the germline and imprinting is re-established late in gametogenesis (Szabo & Mann, 1995). Three markers of imprinting have been described so far. DNA methylation controls differential expression of paternal and maternal alleles (Li et al., 1993) and hence maintenance of monoallelic expression. Transcription is inhibited by DNA methylation via binding of a methyl-CpG binding protein (Boyes & Bird, 1991) suggesting that transcription of an imprinted gene only takes place from the un- (or hypo-) methylated allele. Regions of the genome containing imprinted genes show asynchronous replication in somatic cells, suggesting that the chromatin structure may correlate with imprinting status (Kitsberg et al., 1993). Yet another observed difference between paternal and maternal alleles is the sex-specific meiotic recombination frequencies of imprinted regions (Paldi et al., 1995).

At least sixteen imprinted genes have been identified (Barlow, 1995); twelve in human (WT1 (Wilms' tumor suppressor), INS (insulin), IGF2 (insulin-like growth factor type 2), H19 (non-open reading frame RNA), p57KIP2 (cyclin-dependent kinase inhibitor), SNRPN (small nuclear riboprotein particle SmN), ZNF127 (zinc finger protein), PAR1 and PAR5 (anonymous transcripts in the Prader-Willi consensus region), IPW (non-open reading frame RNA), IGF2R (insulin-like growth factor type 2 receptor) and XIST (X chromosome-inactive specific transcript)) and thirteen in mouse (Wt1, Ins, Igf2, H19, p57Kip2, Mash2 (helix-loop-helix transcription factor), Snrpn, Znf127, Igf2r, Mas (cell surface receptor), XIST, Peg1/Mest (mesoderm-specific transcript) and Sp2 (related to U2 small nuclear ribonucleoprotein auxiliary factor)). Their expression is paternal or maternal and can be general or tissue specific. For example, the paternal allele of the mouse Igf2 gene is expressed in embryos with the exception of the choroid plexus and leptomeninges, where paternal and maternal alleles are both expressed (DeChiara et al., 1991). The human homologue, IGF2, shows monoallelic expression in various tissues except adult liver.
Furthermore, mouse and human homologues of certain genes differ in their imprinting status. Only the maternal allele of the mouse Igf2r is expressed (Barlow et al., 1991) whilst the human homologue, IGF2R is not imprinted (Kalscheuer et al., 1993).

1.4.2 Genomic imprinting in Albright hereditary osteodystrophy

The observed parental effect in the phenotypic expression of AHO (maternal transmission leading to hormone resistance (PHPiA) and paternal transmission to hormone responsiveness (PPHP)) has been suggested to be a result of genomic imprinting of GNAS1 (Davies & Hughes, 1993). Additional support for imprinting of GNAS1 came from studies of the mouse homologue Gnas. Gnas is localised on mouse chromosome 2, in a region which is syntenic to human chromosome 20q (Lyon & Kirby, 1993), where GNAS1 is localised. More specifically, Gnas is localised to region 2E1-2H3 of mouse chromosome 2 (Blatt et al., 1988), a region known to show imprinting effects (Cattanach & Kirk, 1985). Hall was the first to suggest genomic imprinting being involved in the pathogenesis of AHO, based on the location of the mouse homologue of GNAS1 (Hall, 1990).

Imprinting of GNAS1 in all cell types cannot explain the difference in hormone responsiveness between PHPiA and PPHP. An imprinting mechanism involving only cells responsive to PTH and other hormones acting via adenylyl cyclase would offer an explanation. A model of such tissue specific imprinting of GNAS1 is shown in figure 1.6. In cells which are not responsive to the above mentioned hormones, such as lymphocytes, both alleles will be expressed so a mutation on either the paternally or maternally derived allele would lead to a 50% reduction in Gsa expression. However, in hormone responsive cells, such as proximal tubules, only the maternally derived allele will be expressed. A mutation on this allele would lead to no Gsa expression, resulting in hormone resistance and PHPiA. A mutation on the non-expressed paternally derived allele would have no effect on these cells and therefore lead to no hormone resistance and PPHP. Such a process of tissue specific imprinting has been described for the insulin-like growth factor 2 in mice (DeChiara et al., 1991) and humans (Kalscheuer et al., 1993), insulin 1 and 2 in mice (Giddings et al., 1994) and for the Wilms' tumour suppressor gene in humans (Jinno et al., 1994).

1.5 Albright hereditary osteodystrophy-like syndrome

The primary molecular defect appeared to be resolved in individuals with the hormone resistant form of AHO (PHPiA) and the hormone responsive form (PPHP), namely mutations in the gene encoding the α subunit of the stimulatory G protein. In 1993, two unrelated individuals with the physical features of AHO and small terminal deletions
Figure 1.6 Possible mechanism for cell or tissue specific imprinting of Gsα

Possible mechanism for cell or tissue specific imprinting of Gsα. Biallelic expression will occur in cells not responsive to PTH and other hormones acting via adenyl cyclase. A deactivating mutation on either allele will lead to a 50% reduction in Gsα. Expression solely from the maternally derived allele will occur in cells responsive to those hormones. A deactivating mutation on the only expressed allele will lead to no Gsα expression whilst an identical mutation on the non-expressed paternally derived allele will not have an effect and will express 100% Gsα compared to normal hormone responsive cells.

M/P means maternally/paternally derived allele; the thicker bar indicates the allele which is not expressed.
Non-hormone responsive cells

Maternally derived mutation

- 50% Gsα

Hormone responsive cells

Paternally derived mutation

- 100% Gsα
- no hormone resistance
- PHPla
of chromosome 2 were described (Phelan et al., 1993). Both individuals had mental retardation/developmental delay, brachydactyly, obesity, short nose with depressed nasal bridge, round face and normal serum calcium suggestive of PPHP. Chromosome analysis was performed in one individual to exclude the diagnosis of Prader-Willi syndrome (obesity, mental retardation, cytogenetic deletion at chromosome 15q11-13 in 50-60% of patients). Her karyotype was 46,XX,del(2)(q37) and the karyotype of the other individual was 46,XY,del(2)(q37). The finding of a small terminal deletion in two unrelated individuals with a similar phenotype suggests that a gene (or genes) in the 2q37 region may be involved in the pathogenesis of AHO (fig. 1.7). AHO due to a possible defect on chromosome 2 will be referred to as AHO-like syndrome.

1.5.1 Identification of disease genes

Disease genes with unknown function, whose subchromosomal localisation is known (either by linkage analysis, cytogenetically visible deletions or translocations) can be identified using the strategy of positional cloning. The initial localisation usually defines a relatively large candidate region of several million base pairs (Mb) which could contain over 50 genes, based on an average gene size of 40 thousand base pairs (kb) (Schmickel, 1986).

The search for the disease gene takes place in several stages and will be described for identification of genes through deletions of a region of the genome. First, polymorphic markers are identified of which one allele is lacking in individuals with the disease. This allows definition of an interval, ideally several hundred kb or less, containing the disease gene. The extent of the deletions is compared between individuals in order to find a minimal region that is deleted in all individuals. The interval can be reduced in size by using a higher density of polymorphic markers or by screening more individuals. Once the markers flanking the critical region have been characterised, they are used to identify DNA clones covering this critical region. Expressed sequences from these clones are then identified and must be tested for evidence that they are involved in the disease. Conclusive evidence is the segregation of a mutation in individuals affected with the disease. An example of a gene isolated by the positional cloning approach, in an interval characterised by overlapping deletions, is WT1 in Wilms' tumour (Call et al., 1990).

Another strategy to identify disease genes is the investigation of candidate genes. These genes encode proteins whose properties may be consistent with involvement in the pathogenesis of the disease. Alternatively, they encode homologues of genes implicated in an animal model of the disease or show homology or functional relatedness to a gene involved in a similar disease phenotype. When the chromosomal localisation of the disease gene is known, the number of candidate genes is reduced to those localised to that chromosomal region.
Figure 1.7 Idiogram of chromosome 2

Idiogram of chromosome 2 showing the position of the cytogenetically visible deletion in two unrelated individuals with AHO (Phelan et al., 1993).
deleted region
1.5.2 Contiguous deletion syndromes

Syndromes resulting from involvement of adjacent genes are defined as contiguous gene syndromes (Schmickel, 1986), alternatively as microdeletion syndromes indicating the deletion event responsible for their pathogenesis. Ballabio (Ballabio, 1991) considers 'contiguous deletion syndrome' to be a more appropriate term referring to a deletion of more than one contiguous gene. Criteria for the identification of contiguous deletion syndromes include a correlation between the extent of the deletion and the phenotype, mapping of more than one disease gene in the deleted region and molecular dissection of components of the phenotype by mapping and cloning of each disease gene involved. The last criterion represents the evidence of a contiguous deletion syndrome. The occurrence of autosomal contiguous deletion syndromes is usually sporadic. The mechanism underlying those syndromes is probably haploinsufficiency; a reduction of 50% in the dosage of genes located in the deleted region as only one member of a pair of genes is present. Examples of contiguous deletion syndromes include WAGR syndrome (Wilms' tumour, aniridia, genitourinary dysplasia and mental retardation) on chromosome 11p13 (Call et al., 1990), Miller-Dieker syndrome on 17p13 (Reiner et al., 1993), Williams syndrome on 7q11 (Ewart et al., 1993) and DEFECT11 syndrome on 11p11 (Bartsch et al., 1996). For all these at least one gene has been isolated, responsible for part of the syndrome. Frequent findings in contiguous deletion syndromes are mental and growth retardation. AHO-like syndrome which is characterised by brachydactyly and mental retardation, has been identified with cytogenetic deletions of the terminal region of chromosome 2q (Phelan et al., 1993) and therefore appears a good candidate for a contiguous deletion syndrome.

1.6 Aims of this study

The aim of this study is to investigate the genetic heterogeneity underlying AHO. Three separate aspects of this heterogeneity will be investigated to dissect the molecular basis underlying this syndrome.

A cohort of 58 families with at least one individual affected with AHO was screened for GNAS1 mutations. Identification and characterisation of GNAS1 mutations may identify regions in the protein which are crucial for normal Gsa function and enables comparison between the nature or position of the mutation and the severity of the phenotype. In addition, the parental origin of the mutations, in familial and sporadic cases of AHO, was determined to find molecular support for the observed parental effect whereby maternal transmission of AHO leads to the hormone resistant form PHP1a and paternal transmission to the hormone responsive form PPHP.
A model of tissue specific genomic imprinting was investigated with the aim to establish tissue specific imprinting as a mechanism to explain the difference in hormone responsiveness in PHP1a and PPHP.

Finally, a second locus for AHO on distal chromosome 2q, was investigated by screening individuals with AHO (who have no Gsec defect), individuals with brachydactyly and mental retardation and individuals with cytogenetically visible deletions of 2q37 for deletions using polymorphic markers. Furthermore, the possibility of AHO-like syndrome being a contiguous deletion syndrome was examined. A family with isolated autosomal dominant brachydactyly type E was studied to find evidence that a gene for brachydactyly was amongst the genes deleted in individuals with AHO who had a defect on distal chromosome 2q.
Chapter 2: Patients, materials and methods

2.1 Patients

2.1.1 Patients with Albright hereditary osteodystrophy

A total of 58 families was referred in which at least one individual was affected with the physical characteristics of AHO (table 2.1). The total number of affected individuals was 88; 52 of whom had typical AHO, 20 had atypical AHO whilst insufficient data were available for the remainder. Of those 58 families, 31 were seen by Dr. L.C. Wilson (Departments of Genetics and Medicine, University of Leicester and Institute of Child Health, London). The following clinicians were involved in obtaining DNA or blood samples from individuals with AHO and where possible family members; J. Sills (Alderhey Children's Hospital, Liverpool), S. Davies (Institute of Medical Genetics, University Hospital of Wales, Cardiff), J. Clayton-Smith (Department of Clinical Genetics, St. Mary's Hospital, Manchester), E. Maher, A. Green (Addenbrooke's Hospital, Cambridge), J. Leonard, M. Dillon, D. Grant, P. Clayton (Great Ormond Street Hospital, London), I. Young (Queens Medical Centre, Nottingham), J.L.H. O'Riordan (Middlesex Hospital, London), D. Brenton (University College Hospital, London), P. Turnpenny (Royal Devon and Exeter Hospital, Exeter), K. McDermott (Royal Free Hospital, London), M. Whiteford, A. Trainer (Yorkhill Hospital, Glasgow), P. Swift (Leicester Royal Infirmary, Leicester), J.S. Bevon (Aberdeen Royal Infirmary, Aberdeen), J. Fischer (Klinik Balgrist, Zürich, Switzerland), J. Saraiva (Hospital Pediátrico de Coimbra, Coimbra, Portugal), C. van der Burgt (Clinical Genetics Center Nijmegen, Nijmegen, The Netherlands) and M. Parker (Radcliffe Hospital, Oxford).

2.1.2 Patients with cytogenetically visible deletions of 2q

In seven patients with cytogenetically detectable deletions of 2q, who had (amongst other characteristics) brachydactyly type E and mental retardation, the extent of the deletions was determined. Blood samples or DNA from those patients, and where possible their parents, were provided by Drs. M. Barrow (Clinical Genetics Department, Leicester Royal Infirmary, Leicester), K. Temple (Wessex Clinical Genetics Service, The Princess Anne Hospital, Southampton), C. Garrett (North West Thames Regional Genetics Service, Northwich Park Hospital, Harrow), A. Kidd (Clinical Genetics Service, Aberdeen Royal Infirmary, Aberdeen) and W.B. Dobyns (Division of Pediatric Neurology, University of Minnesota Medical School, Minneapolis (USA)).
Table 2.1 Overview of cohort screened for GNAS1 mutations

<table>
<thead>
<tr>
<th>number of affected individuals per family</th>
<th>number of families</th>
<th>families with known Gsα bioactivity values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>&gt;3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>total</td>
<td>58</td>
<td>41</td>
</tr>
</tbody>
</table>
2.1.3 Patients with brachydactyly type E and mental retardation

Seven patients with a brachydactyly type E and mental retardation phenotype, who had normal karyotypes, were screened for the presence of microdeletions in the terminal region of the long arm of chromosome 2. DNA from those patients, and where possible their parents, were provided by Drs. M. Barrow, D. Williams and R. Trembath (Clinical Genetics Department, Leicester Royal Infirmary, Leicester), R. Newbury-Ecob (Human Genetics Centre, Sheffield), C. Aalfs, E. Bijlsma and R. Hennekam (Institute of Human Genetics, Department of Clinical Genetics, University of Amsterdam, Amsterdam, The Netherlands) and B. Kerr (Paediatric Genetics Unit, Regional Genetics Service, Royal Manchester Children’s Hospital, Manchester).

2.1.4 Patients with brachydactyly type E

A large pedigree with multiple members affected with brachydactyly type E was reported in 1945 (Brailsford, 1945). Dr. D. Williams (Clinical Genetics Department, Leicester Royal Infirmary, Leicester) recontacted surviving members of this family. Molecular analysis of chromosome 20q13 and 2q37 was performed in eight available members of the family.

2.2 Human cells and tissues

2.2.1 Peripheral leucocytes

Control blood samples for isolation of DNA and RNA from peripheral leucocytes were obtained from individuals in the laboratory. Ten ml blood was collected in ethylenediamine tetracetic acid (EDTA)-coated plastic tubes to prevent clotting.

2.2.2 Immortalised leucocytes

Lymphoblastoid cell lines of AHO patients were established at the European Collection of Animal Cell Cultures (ECACC), Centre for Applied Microbiology & Research, Porton Down, Salisbury. Leucocytes were immortalised by transformation with the Epstein-Barr virus.

2.2.3 Renal cortex

Three samples of human renal cortex were kindly provided by Drs. G. Hawksworth and V. Rodilla, Department of Medicine and Therapeutics, Aberdeen Royal Infirmary, Aberdeen. The patients had renal carcinomas which were well defined with no metastases present. Tissue for this study was taken from the opposite pole of the kidney. The samples were frozen in liquid nitrogen, transported on dry ice and stored at -80°C until used.
2.2.4 **Renal proximal tubules**

Approximately 400,000 cultured cells consisting of over 95% human proximal tubules (which had gone through 1 passage) were kindly provided by Drs. G. Hawksworth and V. Rodilla, Department of Medicine and Therapeutics, Aberdeen Royal Infirmary, Aberdeen. The tubules cells were obtained from the same patients whose renal cortex samples were used in this study. The cultured cells were responsive to parathyroid hormone. The cells were resuspended in tissue culture medium, frozen in liquid nitrogen, transported on dry ice and stored at -80°C until used.

2.2.5 **Thyroid**

Two samples of human thyroid tissue, removed because of non-toxic goitre elsewhere in the gland, were kindly provided by Mr. M. Galea, Department of Surgery, Derby Royal Infirmary, Derby. The samples were frozen in liquid nitrogen, transported on dry ice and stored at -80°C until used.

2.2.6 **Osteoblasts**

Drs. B. Fraser and C.A. Walsh, Department of Human Anatomy and Cell Biology, The University of Liverpool, Liverpool kindly provided cDNA samples prepared from RNA isolated from cultured osteoblasts. Bone samples were obtained from five individuals (three females and two males) during surgery. The bone was broken up, washed in phosphate buffered saline (PBS) and cultured in the appropriate medium supplemented with serum. After approximately three weeks, RNA was extracted from a confluent monolayer and cDNA was prepared using oligo(dT) primers.

2.3 **Procedures involving bacterial cell cultures**

2.3.1 **Growing bacterial cell cultures**

A single colony of bacterial cells was inoculated in 10 ml Luria-Bertani (LB) medium (10 g/l trypton (Oxoid), 5 g/l yeast extract (Oxoid), 10 g/l NaCl, pH 7.0) and incubated, with shaking, at 37°C for at least 16 hours. The obtained culture is referred to as an overnight culture. If the bacterial cells were transfected with a plasmid or cosmid conferring ampicillin resistance for selection purposes, ampicillin (Sigma) was added to the growth medium at a final concentration of 100 μg/ml.

2.3.2 **Preparing competent bacterial cells**

A fifty-fold dilution of 0.6 ml of an overnight culture in LB medium was made and incubated, with shaking, at 37°C until the optical density at 550 nm reached 3.0, as measured with a spectrophotometer (CECIL, CE2040). Cells were collected by centrifugation for 1 min. at 11,600 g and resuspended in 0.5x the original volume of
freshly made, ice-cold 10 mM CaCl$_2$. The cells were incubated on ice for 20 min. after which the cells were collected as before and resuspended in 0.1x the original volume of freshly made, ice-cold 10 mM CaCl$_2$. The cells were then ready for transformation and stored on ice until required.

2.3.3 Transformation of bacterial cells

A maximum of 100 ng of circular DNA (usually an insert ligated into a vector) was added to 150 µl of competent cells and incubated on ice for at least 40 min. The mixture was incubated at 42°C for 2 min. and immediately put on ice for 5 min. One ml LB medium was added and incubation for 1 hour at 37°C followed. Cells were collected by centrifugation as described before (2.3.2) and resuspended in 200 µl LB medium.

2.3.4 Growing transfected bacterial cells

Resuspended cells were spread onto LB agar plates (15 g agar (Oxoid) per litre LB medium) containing ampicillin to a final concentration of 100 µg/ml. If the cells were transfected with a plasmid or cosmid containing an insert carrying the ß-galactosidase gene for selection purposes, 625 µg isopropyl-beta-indolyl-beta-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal; both Sigma) were spread on the top of the agar plate prior to spreading the cells. The plates were inverted to avoid contamination and incubated for at least 16 hours at 37°C until colonies were about 1 mm in diameter.

2.4 Preparation of nucleic acids

2.4.1 DNA isolation from human peripheral leucocytes

A total of 5-10 ml blood was frozen at -80°C for at least 24 hours. After thawing, the samples were transferred to a 50 ml tube and ice cold water was added to a total volume of 45 ml. The leucocytes were collected by centrifugation for 20 minutes at 1200 g and 4°C and washed once in 35 ml 0.1% Nonidet P-40 (BDH Chemicals Ltd.). The cells were disrupted in 7 ml 6 M guanidinium chloride and after 0.5 ml 7.5 M ammonium acetate, 0.5 ml 20% sarkosyl and 1.5 mg proteinase K (Sigma) were added, incubated at 60°C for 90 minutes. Seventeen ml ethanol was added to precipitate the DNA which was then spooled out and dissolved in 1 ml 25 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 10 mM EDTA (TE buffer). The DNA was recovered by adding 100 µl 3M sodium acetate pH 5.5 and 2.2 ml ice cold ethanol, spooled out and subsequently dissolved in 1 ml TE buffer.
2.4.2 DNA isolation from human thyroid, renal cortex and tubules

Small samples of thyroid and renal cortex (~1x5x5mm) were dissolved in 700 µl 6 M guanidinium chloride, 50 µl 7.5 M ammonium acetate, 50 µl 20% sarkosyl and 150 µg proteinase K and incubated at 60°C for at least 16 hours. The mixture was centrifuged for 1 min. at 11,600 g and DNA was recovered from the supernatant by precipitation with 1.7 ml ethanol, spooled out and dissolved in 200 µl TE buffer. Proximal tubules were collected by centrifugation and resuspended in 1 ml PBS. DNA was isolated from ~133,000 cells using the above method.

2.4.3 RNA isolation from human peripheral leucocytes

Total RNA was isolated from blood within 3 days of collection in EDTA-coated tubes (Chomczynski & Sacchi, 1987). Ten ml blood was carefully layered on top of 15 ml Histopaque (Sigma, H1077) in a 50 ml tube. After centrifugation for 20 minutes at 400 g and 20°C, the leucocytes were recovered from the yellow interphase and washed once in 20 ml PBS. The cells were resuspended in 500 µl GUT-buffer (4 M guanidinium thiocyanate, 0.04 M sodium citrate, 0.04 M N-lauroyl sarcosine, 20 mM β-mercaptoethanol), transferred to a 1.5 ml tube, incubated on ice for at least 2 hours after which 50 µl 2 M sodium acetate (pH 4.5) was added. The solution was extracted three times with 550 µl phenol:chloroform:isoamyl alcohol (25:24:1) (Maniatis et al., 1989), then 500 µl isopropanol was added and the mixture was stored at -20°C for at least 16 hours. The pellet obtained after centrifugation for 30 minutes at 11,600 g was dissolved in 250 µl GUT-buffer, 250 µl isopropanol was added and the mixture was stored at -20°C for at least 4 hours. After centrifugation under the same conditions as above, the pellet was dissolved in 20 µl H2O treated with diethyl pyrocarbonate (DEPC) (Sigma, D5758) (Maniatis et al., 1989).

2.4.4 RNA isolation from human immortalised leucocytes, thyroid, renal cortex and proximal tubules

Total RNA was isolated from human immortalised peripheral leucocytes, from approximately 1 gram of human thyroid and renal cortex and from ~133,000 tubules cells. The cells were collected by centrifugation and resuspended in 500 µl GUT-buffer and RNA was isolated according to the above procedure (2.4.3) starting from the first incubation on ice. Tissue was homogenised in a Dounce tissue homogeniser in 10 ml GUT-buffer and RNA was isolated according to the above procedure (2.4.3), adjusted proportionally for the larger volume, starting from the first incubation on ice. The pellet obtained after the first precipitation with isopropanol was dissolved in 500 µl GUT-buffer and 500 µl isopropanol was added. The recovered RNA was dissolved in 60 µl H2O treated with DEPC.
2.4.5 cDNA synthesis

Two different systems were used to synthesise cDNA. Initially a first strand cDNA synthesis kit (Amersham, RPN 2275) was used for immortalised human leucocytes, according to the manufacturers recommendations. Briefly, 4 μl 1st strand buffer, 1 μl sodium pyrophosphate solution, 1 μl human placental ribonuclease inhibitor, 2 μl deoxynucleoside triphosphate mix, 1 μl oligo-dT primer, 4 μl RNA (preboiled for 2 min. and incubated on ice for 5 min.), 6 μl H2O and 1 μl reverse transcriptase were combined and incubated at 42°C for 2 hours. The completed first-strand reaction was now ready for amplification by the polymerase chain reaction (PCR) (2.5.3). For the renal cortex, tubules, thyroid and leucocytes used in the GNAS1 expression study (4.3) a Ready-To-Go T-primed First Strand kit (Pharmacia, 27-9263-01) was used as recommended. The RNA sample was made up to a volume of 33 μl with DEPC-treated H2O, incubated at 65°C for 5 min. and subsequently at 37°C for 5 min.. At the same time, First-Strand reaction mix (dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAguard, bovine serum albumin (BSA), NotI-d(T)18 primer) was placed at 37°C for 5 min. after which the RNA solution was added to the First-Strand reaction mix without mixing. Subsequently, the mixture was incubated at 37°C for 5 min., the content of the tube was gently mixed and incubation at 37°C followed for 1 hour. The completed first-strand reaction was now ready for PCR amplification (2.5.3).

2.4.6 DNA isolation from bacterial cells

Cells from 1.5 ml of an overnight bacterial cell culture were collected by centrifugation at 11,600 g. The pellet was resuspended in 150 μl 50 mM glucose, 25 mM EDTA, 25 mM Tris-HCl (pH 8.0), incubated at 37°C for 10 min.. Then 300 μl 0.2 M NaOH, 1% sodium dodecyl sulphate (SDS) was added and a 5 min. incubation on ice followed. Subsequently 225 μl 5 M KAc:glacial acetic acid (10:7) was added followed by a 5 min. incubation on ice. After centrifugation at 11,600 g for 7 min. the supernatant was extracted once with phenol:chloroform:isoamylalcohol (25:24:1) (Maniatis et al., 1989) and precipitated by adding 1 ml ethanol (-20°C). The mixture was incubated for 2 min. and centrifuged for 6 min. at 11,600 g. The pellet was washed once in 70% ethanol, dried and resuspended in 20 μl TE buffer containing 0.02 μg/ml RNase A(Sigma).

2.4.7 Quantification of DNA

Genomic DNA was quantitated using a spectrophotometer (CECIL, CE2040). DNA concentrations of PCR fragments were determined by running those out on agarose gels (2.6.1) and comparing the intensity of the band with a known quantity of ΦX/HaeIII or λ/HindIII ladder. The quantity of DNA present can be estimated from the fraction of DNA that is present in a given band of the ladder as bands of equal intensity will contain similar amounts of DNA.
2.5 Polymerase chain reaction (PCR)

PCR is a technique for rapid exponential amplification of a defined sequence present in the template DNA (Saiki et al., 1988). Prior knowledge of the sequence on either side of the target region is required to allow synthesis of two oligonucleotide primers which should be complementary to sites on opposite strands on either side of the target region. Template DNA is denatured, the two primers are annealed to the single strands of DNA. A thermostable DNA (Taq) polymerase then extents the primers in the presence of a mixture of all four deoxyribonucleotides. The reaction mixture can be denatured again, followed by annealing and DNA synthesis. This process can be repeated for a number of cycles thereby increasing the amount of target sequence produced.

2.5.1 Oligonucleotides

Oligonucleotide primers were synthesised by Dr. J. Kyte at the University of Nottingham or by the Protein & Nucleic Acid Chemistry Laboratory at the University of Leicester on an Applied Biosystems (model 394-08) DNA synthesiser. Primers were prepared for use in PCR as follows. The final stage in the preparation of an oligonucleotide involves detaching the oligonucleotide from a matrix support using a weak ammonia solution. To 350 μl of the resulting primer solution, 35 μl 3 M Na-acetate (pH 5.2) and 800 μl ethanol were added. After 30 min. at -80°C and centrifugation for 30 min. at 11,600 g, the pellet was washed once with 70% ethanol, dried and resuspended in 300 μl H₂O. The primers were quantitated using a spectrophotometer. The concentration of the primer stock solution in pmol/μl was calculated by multiplying the optical density at 260 nm by 40 and 1000 and then dividing this by the molecular weight of the primer. Primer stock solutions were diluted with H₂O to working concentrations of 50 pmol/μl. Aliquots of primers for amplification of dinucleotide repeat markers (D2S336, D2S331, D2S206, D2S345, D2S140 and D2S125) were kindly provided by Prof. A. Read, Department of Medical Genetics, St.Mary’s Hospital, Manchester. Details of all the primers used in this study are summarised in table 2.2.

2.5.2 PCR from genomic DNA

All PCR reactions were performed on a Biometra TRIO-Thermoblock thermal cycling apparatus. For each reaction 200-500 ng genomic DNA template, 10-50 pmol of primers, 1-2 units Taq polymerase (Applied Biotechnologies) were used in a volume of 20 or 50 μl. Reactions were carried out either in PCR buffer A with a variable amount of MgCl₂ and 200 μM dNTP or in PCR buffer B. PCR buffer A consists of 50 mM KCl and 10 mM Tris-HCl (pH 7.5) and buffer B consists of 45 mM Tris-HCl (pH 8.8), 11.1 mM ammonium sulphate, 4.5 mM MgCl₂, 6.7 mM β-mercaptoethanol, 4.5 μM EDTA (pH 8.0), 1.0 mM each of dATP, dCTP, dGTP and dTTP and 113 μg/ml BSA (Pharmacia). All PCR reactions were overlayed with two drops of liquid paraffin to avoid evaporation. Each


Table 2.2 Oligonucleotide sequences

a) Intronic primers for the Gsa gene

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'→ 3')</th>
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<tr>
<td>DV-157</td>
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<td>Patten et al., 1990</td>
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<td>MET1R</td>
<td>AGAGACATGACCGGCGGAG</td>
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<td>Gsa2FGC</td>
<td>CL1 + TGCCCAAAGTGTTAAAGTGCCT</td>
<td>L.S. Weinstein*</td>
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<td>Gsa2F</td>
<td>GTACGTACTGCCCAAAAATGTGTTAAAGGCCT</td>
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<td>Gsa2R</td>
<td>AAACTGCAATGAAAAGTCGTCTCTCTAC</td>
<td>L.S. Weinstein*</td>
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<td>L.S. Weinstein*</td>
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<td>L.S. Weinstein*</td>
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<td>L.S. Weinstein*</td>
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<td>L.S. Weinstein*</td>
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<td>Gsa13FGC</td>
<td>CL1 + CGAGGGCTGCTACGACAACTCC</td>
<td>L.S. Weinstein*</td>
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Gsa13F: GTACGTACCGAGGGTGTCACTGACAAGTCC
Gsa13AR: TAAATTTGGGGGTTCCCTTC
3'UTR-R: GTACGTACCATCATTCTTTGCTCACATTTA
GNAS1-A: ACCTCTAGCTGTTGGATTAAG
GNAS1-B: AGACCTAGAGTGGTTACCTG

Granqvist et al., 1992

CL1: GGC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
* primer sequences were kindly provided by Dr. L.S. Weinstein, Molecular Pathophysiology Branch, National Institute of Health, Bethesda, Maryland, USA

b) Exonic primers for the Gsa gene

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c) Primers for polymorphic markers on chromosome 2q

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<td>GGAAGCCACCAGAAT</td>
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AFM288vblm  AGATCAACAGACATAACCCA  D2S345  Gyapay et al., 1994  
AFM276zf5a  AGAGCCCAAAACAAAACTTCC  D2S338  Gyapay et al., 1994  
AFM276zf5m  TGGAATTTTGATTTTCAGATTTG  D2S338  Gyapay et al., 1994  
AFMb314yg9a  AGGACCACCTCGTTGC  Dib eta/., 1996  
AFMb314yg9m  ATGGCTGTGAATGCCTG  Dib eta/., 1996  
AFMb037ydla  CATTTCTGATGTAAACACAAGTTGG  D2S2253  Dib eta/., 1996  
AFMb037ydlm  CTGGCAAGGTTGAGGCTG  D2S2253  Dib eta/., 1996  
AFM112yd4a  GCAACAGAGTGAGACCCTGA  Gyapay et al., 1994  
AFM112yd4m  TTCTGAGAACCAGATTGTGATTG  Gyapay et al., 1994  
AFM356te5a  ATTTAATTTGCTTTGCCACC  Gyapay et al., 1994  
AFM356te5m  AGCTACCATCTCAGGCAG  Gyapay et al., 1994  
AFM142xh6a  GCAACTTTTCTGTCAATCCA  D6S273  Gyapay et al., 1994  
AFM142xh6m  ACCAAAACCTCAAAATTTTCC  D6S273  Gyapay et al., 1994  
AFM077xela  AACACTTCGATGTTCCTTCC  D11S903  Gyapay et al., 1994  
AFM077xelm  AGCTGAGAGCGCATGTATAA  D11S903  Gyapay et al., 1994  
AFM044xg3a  GAGTCTCCTAAATGCTGGGG  D17S784  Gyapay et al., 1994  
AFM044xg3m  AGCTCCTGCACAGTCTTCTTGAAT  D17S784  Gyapay et al., 1994  
RM109.A  TTCTGATCTGCTACCATGTA  D20S93  Melis et al., 1993  
RM109.B  CAGCCTGGGGTCAGAGAA  D20S93  Melis et al., 1993  
ELN.17A  AGTGAAGCTGTCACCGGTTAT  ELN  Foster et al., 1993  
ELN.Exon18  GGAGATCCAGGGTCGGTT  ELN  Foster et al., 1993  

**d) Various other primers**

<table>
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<td>D11S903</td>
<td>Gyapay et al., 1994</td>
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<td>Melis et al., 1993</td>
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<td>RM109.B</td>
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<td>ELN.17A</td>
<td>AGTGAAGCTGTCACCGGTTAT</td>
<td>ELN</td>
<td>Foster et al., 1993</td>
</tr>
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<td>ELN.Exon18</td>
<td>GGAGATCCAGGGTCGGTT</td>
<td>ELN</td>
<td>Foster et al., 1993</td>
</tr>
</tbody>
</table>
cycling program consisted of denaturing for 5 min. at 94°C, thirty five cycles of 94°C for 30 sec. or 1 min., 53.5-68°C for 30 sec. and 72°C for 30 sec. or 1 min. followed by a 3 min. extension at 72°C. PCR amplification of exon 1 was carried out in buffer B supplemented with 15% glycerol under the following conditions; thirty five cycles at 96°C for 30 sec., 66-68°C for 30 sec. and 72°C for 1 min. followed by a 3 min. extension at 72°C.

2.5.3 PCR from cDNA

All PCR reactions were performed on a Perkin Elmer DNA Thermal Cycler 480 or a Hybaid OmniGene apparatus. Reactions were carried out in a volume of 20 μl using 25-50 pmol of primers, 200 μM dNTP, buffer A with 1.5 mM MgCl₂, 2 units Taq polymerase (Applied Biotechnologies). When first strand cDNA was synthesised using the Amersham kit (2.4.5), 2 μl cDNA was used as template and cycling conditions were 94°C for 30 sec., 65°C for 30 sec. and 72°C for 1 min. for 35-40 cycles. When first strand cDNA was synthesised using the Pharmacia kit (2.4.5), 3.3 μl cDNA was used as template and cycling conditions were 94°C for 30 sec., 65-68°C for 30 sec. and 72°C for 1 min. for 40 cycles. An aliquot of 5 μl of this PCR reaction was used in a subsequent nested PCR reaction for 25 cycles.

2.5.4 Radioactive labelling of PCR fragments

PCR fragments were radioactively labelled either by incorporation of [α³²P]-dCTP (Amersham, 370 MBq/ml) or by end-labelling one of the oligonucleotides with [γ³²P]-ATP or [γ³³P]-ATP (both Amersham, 370 MBq/ml). In the case of labelling by incorporation, the PCR reaction was carried out as usual in the presence of 2 μM dCTP and 9 kBq of [α³²P]-dCTP. When using end-labelled primers, the standard PCR reaction was performed using one unlabelled and one radioactively labelled primer. End-labelling of 10 pmol primer was carried out in a 20 μl reaction containing 0.5 M Tris-Cl (pH 7.5), 0.1 M MgCl₂, 1 mM spermidine, 1 mM EDTA, 1.1 MBq [γ³²P]-ATP or [γ³³P]-ATP and 5 units T4 polynucleotide kinase (Gibco BRL). The mixture was incubated at 37°C for 1 hour followed by 20 min. at 65°C.

2.5.5 Purification of PCR products

PCR fragments were subjected to electrophoresis on an ethidium bromide containing agarose gel (2.6.1) and visualised by ultraviolet (UV) light. After visualisation, the band of the correct size was excised from the gel and cut into small pieces. The gel pieces were put in a 0.5 ml tube with a hole in the bottom, containing a small piece of 3MM paper. The tube was subsequently put in a 1.5 ml tube and after adding 20 μl TE buffer, centrifuged at 11,600 g for 10 minutes. The DNA was present in the solution in the 1.5 ml tube. Alternatively, the PCR fragments were purified using QIAquick PCR purification spin columns (Qiagen) according to the manufacturer's instructions. Briefly, the PCR reaction
was mixed with 5 volumes of PB buffer (supplied by Qiagen), applied to a QIAquick column, centrifuged for 1 min. at 11,600 g. The flow-through was discarded and 750 μl PE buffer was applied followed by centrifugation as above. Again the flow-through was discarded and a centrifugation followed. The DNA was eluted from the column by adding 50 μl Tris-HCl (pH 7.5) followed by centrifugation for 1 min.. Alternatively, 30 μl Tris-HCl (pH 7.5) was added, followed by incubation for 1 min. and centrifugation for 1 min..

2.6 Gel electrophoresis

2.6.1 Agarose gel electrophoresis

Agarose (Seakem HGT, Flowgen) and 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) were combined in the right proportions, brought to boiling to dissolve the agarose powder and ethidium bromide was added to a final concentration of 0.4 μg/ml. The mixture was casted into a gel tray after cooling to about 50°C and was left to set. PCR products were mixed with sucrose loading dye (1 M sucrose, 3.5x TBE) to a final concentration of 1x TBE and loaded onto gels which were run horizontally in 1x TBE buffer at 110 V. DNA fragments were visualised using UV light. PCR fragments were analysed on 2% agarose gels unless stated otherwise.

2.6.2 Non-denaturing polyacrylamide gel electrophoresis

A mixture of 12.5 % polyacrylamide (Ultrapure Accugel™, 40% (19:1) acrylamide:bisacrylamide, National Diagnostics) in 1x TBE buffer was prepared. Prior to pouring the polyacrylamide mixture between two glass plates (82x102mm, Hoefer Scientific Instruments), 100 μl 10% ammonium persulphate and 5 μl NNN'N'-tetramethylethylenediamine (TEMED) was added per 10 ml mixture allowing it to polymerise. DNA fragments were mixed with sucrose loading dye as above and subjected to electrophoresis on the 1 mm thick gels which were run vertically in 1x TBE buffer at 110 V. Gels were stained in 0.5 μg/ml ethidium bromide and DNA was visualised using UV light. Restriction digestion analysis of PCR products resulting in fragments smaller than 150 bp was performed on this type of gel.

2.6.3 Denaturing polyacrylamide gel electrophoresis

A mixture of 6 or 8% polyacrylamide, 1x TBE buffer and 7 M urea was prepared and polymerised between 82 glass plates separated by 0.2 mm thick spacers (Gibco BRL) as described above. Radioactively labelled DNA fragments were mixed with an equal volume of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% (w:v) xylene cyanol, 0.05% (w:v) bromophenol blue), heat-denatured and run on the gels in 1x TBE at 60W. After electrophoresis, the glass plates were separated and the gel was transferred to a sheet of 3MM paper. After drying the gel for 2 hours at 80°C on a gel dryer (Biorad, model
583), it was transferred to an autoradiography cassette and an X-ray film (Fuji medical X-ray film (RX)) was put on top of the gel. Gels containing \([\alpha^{32}\text{P}]-\text{dCTP}\) or \([\gamma^{32}\text{P}]-\text{ATP}\) were then placed at -80°C whilst gels containing \([\alpha^{35}\text{S}]-\text{dATP}\) or \([\gamma^{33}\text{P}]-\text{ATP}\) were kept at room temperature. Films were developed after at least 24 hours exposure.

2.6.4 Denaturing gradient gel electrophoresis (DGGE)

Mixtures of 8% polyacrylamide in 1x TAE (40 mM Tris-acetate, 2 mM EDTA, (pH 7.4)) were prepared containing 0 and 80% denaturant (100% denaturant is 7 M urea, 40% (v:v) formamide, deionised using Mixed Bed Resin (BioRad Laboratories)). Gels with linearly increasing gradients were poured between 160x180mm glass plates (Hoefer Scientific Instruments) using a SG100 gradient former (Hoefer Scientific Instruments) and were polymerised as described above. Prior to loading the PCR products, heteroduplex DNA was formed by denaturing for 5 min. at 96°C and subsequent cooling down for 1 hour at 65°C and 1 hour at 37°C. PCR products were mixed with sucrose loading dye to a final concentration of 1x TAE. Gels were preheated to 60°C and before loading the samples, the wells were rinsed twice with 1x TAE. Electrophoresis took place in a tank containing 1x TAE buffer whereby buffer from the lower compartment was pumped into the upper buffer chamber at a rate of 600 ml/min. using a peristaltic pump (Flowgen Instruments Ltd., type 328-S6). The temperature of the buffer was kept at 60°C by a thermomixer (Braun Diessel Biotech, Thermomix® ME, type 852 112/3) during electrophoresis at 150V. The 0.75 mm thick gels were stained in 0.5 μg/ml ethidium bromide and DNA was visualised using UV light.

2.7 Restriction digestion analysis

DNA was digested with restriction endonucleases (Gibco BRL, New England Biolabs, Boehringer Mannheim) in the appropriate buffers supplied by the manufacturers of the enzymes. Genomic DNA was digested for at least 16 hours in a 30 μl reaction and PCR fragments were digested for 2 hours in 20 μl at temperatures recommended by the manufacturers. Analysis of restriction digests was performed on agarose or non-denaturing polyacrylamide gels.

2.8 Southern analysis

2.8.1 Transfer of DNA

DNA fragments were size-separated on an agarose gel. After electrophoresis the gel was incubated in 0.25 M HCl twice for 15 min. to facilitate transfer of large (> 8 kb) fragments (only if the Southern blot was to be hybridised with the probe D2S90), in 0.5 M NaOH, 1.5 M NaCl for 30 min. followed by two incubations of 30 min. in 1.5 M NaCl, 0.5
M Tris-HCl (pH 7.5). The gel was then transferred to a southern blotting tank and blotted as described in "Molecular cloning, a laboratory manual" (Maniatis et al., 1989). Blotting took place for at least 16 hours using 20x standard saline citrate (SSC) (3 M NaCl, 0.3 M sodium citrate) as transfer buffer. The DNA was transferred onto Hybond N+ membranes (Amersham) which were baked for 2 hours at 80°C to bind the DNA irreversibly to the membranes.

2.8.2 Generation of probe DNA

The probes EX4, EX9 and EX12 were generated by PCR amplification across exons 4, 9 and 12 respectively of GNAS1. The amplification products were purified by excision from an agarose gel (2.5.5) and quantified by comparison to a known quantity of ladder (2.4.7).

The D2S90 probe was kindly provided by Dr. G. Vergnaud (Laboratoire de Génétique des Expèces, Institut de Biologie, 9, Quai Moncousu, 44035 Nantes Cédex 01, France) and prepared by Mr. N. Periam (Department of Genetics, University of Leicester). Briefly, the insert of the clone was removed from the vector by restriction digestion and then used as a probe. The insert was excised from a low-melting point agarose on which the restriction fragments were separated and diluted to a concentration of 10 ng/μl.

2.8.3 Radioactive labelling of probe DNA

Fifty ng of probe DNA in a volume of 11 μl was boiled for 2 min. and after 5 min. on ice, 11.4 μl labelling solution (242 mM Tris-HCl (pH 8.0), 24.2 mM MgCl₂, 48 mM β-mercaptoethanol, 97 μM dATP, 97 μM dTTP, 97 μM dGTP, 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (pH 6.6; HEPES), 0.3 OD260 units of hexadeoxyribonucleotides (adapted from Feinberg & Vogelstein, 1984)), 1 μl BSA (10 mg/ml, Promega), 3 μl [α32p]-dCTP and 5 units DNA polymerase I, Klenow fragment (Gibco BRL) were added, followed by incubation at room temperature for 3 hours.

2.8.4 Hybridisation of a probe

The membranes were put in a hybridisation bottle with 25 ml Denhardt's prehybridisation buffer, and incubated at 65°C in a hybridisation oven (Hybaid) for 3 hours. Denhardt's prehybridisation buffer is prepared by mixing 20 ml 100x Denhardt's (2% (w:v) each of Ficoll 400, polyvinylpyrrolidone, BSA (fraction V) in 3x SSC (Denhardt, 1966)), 40 ml 20x SSC, 118 ml H₂O, 2 ml (10 mg/ml) salmon sperm DNA and 20 ml 10% SDS. The radioactive probe was boiled for 2 min., added to the prehybridisation buffer and hybridised for at least 16 hours at 65°C. Excess probe was washed off using solutions of 2x SSC, 0.1% SDS at 65°C. More stringent washing was performed by lowering the salt concentration in the washing solution. Filters were wrapped in 'Saran' plastic wrap and put in an autoradiography cassette. X-ray autoradiography was performed as described previously (2.6.3).
2.8.5 Removal of a radioactive probe from a filter

Radioactive probe was removed from a filter by incubation at 45°C in 0.4 M NaOH for 30 min. followed by incubation at 45°C in 0.1x SSC, 0.1% SDS, 0.2 M Tris-HCl (pH 7.5) for 30 min. The filter was now ready for use with another probe and stored in 'Saran' plastic wrap until further use.

2.9 DNA sequencing

2.9.1 Cycle sequencing

PCR reactions were performed as described previously (2.5.2 and 2.5.3). Gel purified fragments were dissolved in 10 μl H2O and sequenced using a cycle sequencing method. Asymmetric PCR was performed using 1 μl of DNA template, 75 μM dNTP and 1 μM forward or reverse primer for fifteen cycles identical to the cycling program used to generate the initial PCR fragment. The product was precipitated, redissolved in 5 μl H2O and cycle sequenced using primers endlabelled with [γ32P]-ATP (Amersham) and 1 unit Taq polymerase. The products were analysed on denaturing polyacrylamide gels and visualised by X-ray autoradiography as described before (2.6.3).

2.9.2 Direct sequencing of double-stranded PCR fragments

In a 10 μl reaction, 100 ng double-stranded PCR product, 1 μl dimethyl sulphoxide (DMSO), 1 μg primer, 2 μl sequenase reaction buffer (200 mM Tris-HCl (pH 7.5), 100 mM MgCl2, 250 mM NaCl) and H2O were combined, boiled for 2 min. and snap-frozen on dry ice/ethanol. To each reaction, 1 μl 0.1 M dithiothreitol (DTT), 2 μl 1:15 diluted labmix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP), 0.5 μl [α35S]-dATP (Amersham) and 4.3 units Sequenase™ version 2.0 (United States Biochemicals) were added. Of the total mixture 3.2 μl was added to each preheated tube with 2 μl termination mix (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 8 μM ddGTP (or ddATP, ddTTP, ddCTP), 50 mM NaCl). After 10 min. incubation at 37°C, 4 μl formamide loading dye was added. The products were analysed on denaturing polyacrylamide gels and visualised by X-ray autoradiography as described before (2.6.3).

2.9.3 Sequencing of cloned PCR products

Gel-purified PCR products were cloned in a pGEM®-T vector (Promega, A3600) according to the manufacturers instructions. For ligation 1 μl 10x ligase buffer (300 mM Tris-HCl (pH 8.0), 100 mM MgCl2, 100 mM DTT, 10 mM ATP), 50 ng vector (3 kb), variable amounts of PCR insert (depending on size), 1 μl (3 units) T4 ligase and H2O to 10 μl were combined and incubated at 15°C for 3 hours. The molar ratio of vector:insert was 3:1. Subsequently, competent cells (2.3.2) were transformed with 2-5 μl ligation mix.
(2.3.3), plated out on agar plates containing ampicillin, IPTG and X-gal and incubated at 37°C (2.3.4). Overnight cultures, inoculated from white colonies obtained after incubation, were prepared (2.3.1), DNA was isolated as described before (2.4.6) and sequencing of the insert was performed as follows. To 5 µl double-stranded DNA (3-5 µg), 20 µl 0.2 M NaOH, 0.2 mM EDTA was added. After a 5 min. incubation at 20°C, 2 µl 2 M ammonium acetate (pH 4.5), 1 µl sequencing primer (0.5 pmol), 1 µl H_{2}O and 60 µl ethanol were added and incubation at -80°C for 15 min. followed. The DNA was collected by centrifugation for 15 min. at 11,600 g, washed once with 70% ethanol and after drying, dissolved in 2 µl sequenase reaction buffer (2.9.2) and 8 µl H_{2}O. To the redissolved DNA, 1 µl 0.1 M DTT, 2 µl 1:5 diluted labmix (2.9.2), 0.5 µl 0.5 µl [α^{35}S]-dATP (Amersham) and 3.25 units Sequenase™ version 2.0 (United States Biochemicals) were added. After a 4 min. incubation at 20°C, 3.5 µl of the total mixture was added to each preheated tube with 2.5 µl termination mix (2.9.2). Then, after a 5 min. incubation at 37°C, 4 µl formamide loading dye was added. The products were analysed on denaturing polyacrylamide gels and visualised by X-ray autoradiography as described before (2.6.3).

2.10 Computer software

2.10.1 Computing facilities at the UK Human Genome Mapping Project

The UK Human Genome Mapping Project Resource Centre (HGMP-RC) allows access to software by members of the scientific community. The programs listed below (2.10.1.1 to 2.10.1.4) were accessed at the HGMP-RC.

2.10.1.1 Online Mendelian Inheritance in Man

Online Mendelian Inheritance in Man (OMIM) is a weekly updated database from the John Hopkins University Medical Library, Baltimore, USA, maintained by Victor McKusick. OMIM contains the text of the latest printed edition of Mendelian Inheritance in Man by V. McKusick and information obtained after its publication. Each disorder or trait is given a unique MIM number and title and for each entry, information is provided concerning clinical observations, observed inheritance patterns, linkage information, references, chromosomal localisation, identified allelic variants, known defective gene products and molecular genetics. In addition, OMIM contains the McKusick Human Gene Map and Molecular Defects List.

2.10.1.2 Human Genome Database

The Human Genome Database (GDB) is a collaboration project between the Howard Hughes Medical Institute, the John Hopkins School of Medicine and the William H. Welch Medical Library and contains information relevant to human genome mapping. It was established to collect, organise, store and distribute data generated by scientists. The
information in GDB is organised by map location and comprises locus name, chromosomal localisation, citations, MIM number, polymorphic description, probes, maps, mutations, populations and cell lines. Information about polymorphic markers used in this study, such as size and frequency of certain alleles, was retrieved from GDB.

2.10.1.3 PRIMER

PRIMER is a computer program that can be used for automatically selecting PCR primers. Information regarding the sequence of the region to be amplified should be given, as well as information regarding the desired size and GC-content of the PCR fragment and length, melting temperature and GC-content of the primers. Version 0.5 May 1991 was used to design primers for amplification of certain exons of GNAS1.

2.10.1.4 Genetics Computer Group package

The Genetics Computer Group package (GCG) comprises programs for nucleic acid and protein sequence analysis. The program 'fetch' was used to retrieve genomic DNA sequences of GNAS1 from the European Molecular Biology Laboratory (EMBL) nucleotide sequence database. The program 'map' was used to find restriction endonuclease recognition sites. Once mutations in GNAS1 were determined, the program 'seqed' was used to edit sequences so that restriction enzyme cutting sites of the mutated sequence could be determined using 'map'.

2.10.2 MacMelt Software

The MacMelt™ Software, version 1.0 was obtained from Biorad Laboratories. The program is a simplified version of the original Melt87 software (developed by Lerman & Silverstein, 1987) and is used for predicting the melting behaviour of a specified DNA sequence in a denaturing gel. To use the program, a sequence is entered, the melting pattern is determined and the data are graphed (for an example see fig. 3.1). The graph is called a melt map and ideally should show one high peak and a lower melting plateau. In first instance PCR primer pairs were designed using PRIMER (2.10.1.3), then the pair was tested for the melting pattern of the resulting PCR fragment with a 40-bp GC-clamp on the 5' end as well as with a clamp on the 3' end. The melt maps were determined for each fragment that was analysed by denaturing gradient gel electrophoresis (DGGE).
Chapter 3: Detection and analysis of mutations in GNAS1 in Albright hereditary osteodystrophy

3.1 Preface

Deactivating mutations in GNAS1 have been identified in AHO patients with or without hormone resistance. At the time this study started, six such mutations had been reported (Patten et al., 1990; Weinstein et al., 1990; Nakamoto et al., 1992; Schwindinger & Levine, 1992; Weinstein et al., 1992). One mutation was in the initiation codon, two mutations were in exon 7, two in exon 10 and one in exon 13. A GNAS1 mutation screen in a cohort of AHO patients was initiated to identify regions in the protein critical for normal Gsα function and to determine any relationship between the nature or position of the mutation and the severity of the phenotype.

A total of 58 families with at least one member who had features of AHO, was screened for mutations in the Gsα gene. Exons 2 to 13 and their flanking regions were screened using DGGE. Exon 1 was impossible to screen by DGGE because of the highly GC-rich sequence surrounding this exon, and therefore direct sequencing of two overlapping PCR products encompassing exon 1 was used to identify mutations. The effect of three different mutations on the expression of GNAS1 was also studied.

GNAS1 mutations have been observed in individuals with PPHP and PHP1a, both sporadically and in the same kindred, suggesting that the Gsα deficiency may be necessary but not sufficient for the expression of hormone resistance. Parental origin of the mutation was suggested to play a role in determining the phenotypic expression of AHO (Davies & Hughes, 1993), based on a review of published familial cases of AHO; maternal transmission always resulting in PHP1a (60/60) and paternal transmission always in PPHP (6/6). To find molecular support for this hypothesis, the parental origin of the characterised GNAS1 mutations was established when possible.

For clarity, individuals in this mutation detection study are indicated by AHO/family number/ DNA number.

3.2 Establishing conditions for DGGE analysis

The size of PCR fragments amenable to analysis by DGGE is 50-500 bp (Fodde & Looskoot, 1994; Myers et al., 1987). Random mutagenesis studies suggest that the ability to detect a sequence variation is not effected by its position within the PCR fragment (Beck et al., 1993). To screen the genomic DNA encoding for GNAS1, exons 2 to 13 and their splice site consensus sequences were examined individually.
3.2.1 Primer design

Primer sequences for PCR amplification of exons 2, 3, 4, 5, 6, 7, 8, 9 and 12 and their flanking regions were kindly provided by Dr. L. Weinstein. Primer pairs for exons 10, 11 and 13 were designed using the PRIMER program. Each pair was tested for the melting pattern of the resulting PCR fragment with a 40-bp GC-clamp on either primer using the MacMelt™ program. Depending on the melt map, either the forward or reverse primer was synthesised with a 40-bp GC-clamp on its 5' end. A summary of all primers is given in table 2.2; intronic primers used for mutation detection are labelled Gsax with x indicating the exon to which flanking sequence the primer anneals.

3.2.2 Optimisation of mutation analysis by PCR and DGGE

For each exon that was analysed PCR reactions were set up using a Mg^{2+} concentration of 1.5 mM, raising the annealing temperature until the quantity of PCR product obtained dropped. PCR reactions were carried out at this optimum annealing temperature with Mg^{2+} concentrations varying between 0.5 and 2.5 mM and the resulting PCR fragments were subjected to electrophoresis on a denaturing gradient gel after heteroduplex formation. This was done because the focusing of the bands on this type of gel appeared to be dependent on the Mg^{2+} concentrations in the PCR reaction mixture. The gradient on which to run PCR fragments encompassing a particular exon initially was determined using the meltmap for that exon. From the meltmap, the melting temperature (Tm) of the fragment can be determined (fig. 3.1b). Temperature can be converted into a percentage of denaturant using the following formula: Tm = Tb + 0.3 x % denaturant, whereby Tb is the temperature at which the electrophoresis takes place (60°C). The gradient was chosen 15% above and 15% below the predicted percentage denaturant which would lead to the homozygous wild-type band being retarded halfway down the gel. When homozygous wild-type bands were far above or below the midpoint of the gel, the gradient was changed to achieve retardation of this band in the middle of the gel to enable visualisation of bandshifts.

Examination of exon 12 was complicated by the presence of three melting domains (fig. 3.1). Analysis of the PCR product generated using a GC-clamped reverse primer, was predicted to reveal any sequence variations in domain (a) (fig. 3.1b) of the fragment which would melt first upon travelling through the denaturing gradient gel, leaving domain (b) of the exon double stranded and hence inaccessible for mutation detection. PCR product was therefore digested with AluI to separate the low melting domain, and the digestion products were subjected to electrophoresis on the denaturing gradient gel. The optimised conditions for DGGE analysis of exons 2-13 are summarised in table 3.1.
Figure 3.1 Meltmap analysis of exon 12

a) Meltmap of the unclamped PCR fragment encompassing exon 12. The position of exon 12 and the flanking introns are indicated below. NC means non-complementary sequence attached to the 5' end of the forward primer.

b) Meltmap of the PCR fragment encompassing exon 12 generated using a reverse primer with a 40 bp GC-clamp attached to it. Three melting domains can be identified; (a), (b) and (c). The estimated melting temperature (Tm) of domain (a) was 66°C. Melting at this temperature would leave domain (b) double stranded hence inaccessible for mutation detection. The position of an AluI restriction site is indicated by the arrow.

c) Meltmap of the PCR fragment encompassing exon 12 following digestion with AluI. This digestion will detach the lower melting domain (a) allowing domain (b) to be denatured.
a) Intron 11 exon 12 'intron 12

b) AluI

(c) exon 12 intron 12 GC clamp
Table 3.1 DGGE conditions for mutation detection in GNAS1

<table>
<thead>
<tr>
<th>exon</th>
<th>primers</th>
<th>gel gradient (%)</th>
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<td>30-55</td>
<td>150</td>
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<td>Gsα8F/8RGC</td>
<td>35-65</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
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<td>55-80*</td>
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</table>

The primers used for amplification of each individual exon are given in the table as well as the conditions under which to screen for sequence variants in each exon. Exon 3 was analysed on 2 different gradients, either of them picking up a sequence variant and therefore acceptable for screening. Exon 12 was analysed for mutations in the low melting domain on a 25-50% gradient gel and in the high melting domain after Alul digestion of the PCR product on a 55-80% gradient gel.
3.3 Polymorphisms in GNAS1

Polymorphisms in the coding sequence were used to aid characterisation and determine the parental origin of GNAS1 mutations. Three polymorphisms are described prior to the description of the identified mutations as they were used in the characterisation of those mutations.

A neutral polymorphism in exon 5 was described (Gejman et al., 1991); a T to C transition changing the third base of the codon encoding Ile131. The polymorphism is localised within a recognition site of the restriction endonuclease *FokI*. The allele carrying a C will be digested by *FokI* and is called the (+) allele, whilst the allele carrying the T will not be digested and hence is designated the (-) allele. DGGE analysis of exon 5 and flanking regions revealed the polymorphism and comparison with *FokI* restriction digests allowed assignment of the homoduplex bands (fig. 3.8a). Homoduplex 1 represents the (+) allele and homoduplex band 2 represents the (-) allele. Bands 3 and 4 represent heteroduplexes of both alleles. From a total of 100 alleles from normal unrelated individuals, 52 were *FokI* (-) alleles and 48 were (+) alleles. The polymorphism was informative in 9 out of 25 individuals in the cohort with AHO whose parents were unaffected.

Screening of exon 7 and flanking sequence revealed an identical abnormal pattern in AHO/004/10285 and his unaffected father, in AHO/012/11061 and in AHO/060/416 and his unaffected and affected sibling. Direct sequencing revealed a heterozygous neutral C to T transition at the third base of Ile185. An *MseI* recognition site was created by this base substitution. The sequence variant was not observed in 55 other AHO affected individuals and was present on 2/102 alleles from normal unrelated individuals.

Identical atypical DGGE migration patterns have been observed upon analysis of exon 13 in individuals AHO/004/10285 and AHO/012/11061. *FokI* restriction digests of PCR products encompassing exon 13 were carried out to verify that the observed sequence variants were identical to a rare C to T transition of the third base of the codon encoding Asn371 which destroys a *FokI* restriction endonuclease recognition site in this exon (Waltman et al., 1994). The T variant was observed in 1/102 alleles from normal unrelated individuals.

A measure for the informativeness of a genetic marker is its polymorphism information content (PIC). The PIC can range from 0 (always uninformative) to 1 (always informative). The PIC value for a marker is calculated by deducting the chances of an individual being uninformative (by being homozygous or by being heterozygous whilst both parents are heterozygous) from 1 (always informative). For an autosomal marker with \( n \) alleles,

\[
PIC = 1 - \left( \sum_{i=1}^{n} p_i^2 \right) \cdot \left( \sum_{j=i+1}^{n} \sum_{k=j}^{n} 2p_i^2p_j^2 \right)
\]
where $p_i$ is the frequency of the $i$th allele (Strachan & Read, 1996). A PIC value is not identical to heterozygosity as heterozygosity does not take parental status into account (which is represented by the last term in the equation for PIC). The maximum possible PIC for a marker with two alleles is 0.375 whilst the maximal heterozygosity is 0.50. The PIC values for the exon 5, 7 and 13 markers are 0.3746, 0.0377, 0.0192 respectively.

3.4 Mutations in GNAS1 in Albright hereditary osteodystrophy

Using PCR and DGGE analysis 20 sequence variations were identified in exons 2-13. Once a sequence variant was observed, the experiment was repeated for the proband and additional family members, followed by characterisation of the variant by direct sequencing. Sequencing was performed using an independently generated PCR product. Where the mutation created or destroyed a restriction endonuclease recognition site, a further PCR fragment was subjected to restriction digestion to verify the mutation and screen for the presence of the mutation in other individuals.

A total of 19 patients with classical features of AHO in whom DGGE analysis failed to identify a potential mutation, were further investigated by direct DNA sequencing of exon 1 using the strategy outlined in figure 3.4a. Gaα bioactivity was reduced in nine patients and was unknown in the remaining ten. An additional seven mutations were identified.

To establish causality of single base pair substitutions predicted to result in amino acid substitutions, the presence of these substitutions was excluded in 38-40 normal chromosomes, their conservation amongst different Ga subunits (Pennington, 1995) and amongst Gaα subunits in different species (Itoh et al., 1986; Kaziro et al., 1991; Lambright et al., 1996) was investigated as was their position in relation to structurally and functionally important domains (Neer et al., 1988; Berlot & Bourne, 1992; Conklin & Bourne, 1993; Lambright et al., 1996).

Mutations were designated according to a nomenclature suggested by Beaudet and Tsui (Beaudet & Tsui, 1993). Amino acids are numbered according to Kozasa and colleagues (Kozasa et al., 1988) starting with the initiation codon as 1, nucleotides are numbered according to Bray and colleagues (Bray et al., 1986), the first base of the initiation codon being -33. Missense mutations are designated by the number of the changed amino acid with the wild-type amino acid prior to the number and the mutant following the number. Nonsense mutations are designated similar to missense mutations except that X is used to indicate a termination codon. Insertions, deletions and mutations affecting splice sites are designated by nucleotide number. For insertions, the designation is "ins" preceeded by the nucleotide number preceding the insertion and followed by the number of bases inserted, except when two or less bases are inserted in which case "ins" is followed by the exact nucleotides. Similar, for deletions, the designation is "del" preceeded
by the nucleotide number of the first deleted nucleotide and followed by the number of deleted bases. Splicing mutations are designated by the nucleotide position relative to the nearest exon, a nucleotide number followed by + and a number indicates the number of bases following the exon ending with that nucleotide position whilst a nucleotide number followed by - and a number indicates the number of bases preceding the exon starting with that nucleotide position. No suggestions were made for mutations in the initiation codon. Therefore I designated those as iM for the initiation codon methionine followed by + and a number indicating the nucleotide position in the codon (1, 2 or 3).

A summary of all 27 identified mutations is presented in table 3.2 whilst seven mutations are described in more detail.
Table 3.2 Mutations in GNAS1

**Exon 1**

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<th>designation</th>
<th>restriction</th>
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<th>Gα</th>
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<td>iM+1 A→G</td>
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† indicates % of normal control; n.a. means no samples were available for the Gsα bioactivity assay; c/d means restriction site created or destroyed; chr* indicates the number of normal chromosomes checked; q means Gqα, t Gtα, o Goα, i Giα; at means Arabidopsis thaliana, sc Saccharomyces cerevisiae, dd Dictyostelium discoideum, r rat, b bovine; β means mutation in β-sheet, sr specificity of interaction with receptor; aa means amino acid.
**Exon 4**

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† indicates % of normal control; n.a. means no samples were available for the Gα bioactivity assay; chr* indicates the number of normal chromosomes checked; t means Gtα, i Gicα; α means mutation in α-helix, βγ means interaction with βγ subunits; at means Arabidopsis thaliana, r rat, b bovine; aa means amino acid.
### Exon 5

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† indicates % of normal control; n.a. means no samples were available for the Gα bioactivity assay; d means restriction site destroyed; chr* indicates the number of normal chromosomes checked; α means mutation in α-helix; r means rat, b bovine; aa means amino acid.
### Exon 6

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† indicates % of normal control; n.a. means no samples were available for the Gα bioactivity assay.
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† indicates % of normal control; n.a. means no samples were available for the Gsα bioactivity assay; c/d means restriction site created or destroyed; chr* indicates the number of normal chromosomes checked; q means Gqα, t Gtα, o Goα, i Gtα; at means Arabidopsis thaliana, sc Saccharomyces cerevisiae, sp Schizosaccharomyces pombe, dd Dictyostelium discoideum, r rat, b bovine; α means mutation in α-helix, βγ means interaction with βγ subunits; aa means amino acid.
### Exon 10

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† indicates % of normal control; n.a. means no samples were available for the Gα bioactivity assay; c/d means restriction site created or destroyed; chr* indicates the number of normal chromosomes checked; q means Gqα, t Gtα, o Gοα, i Gία; at means *Arabidopsis thaliana*, sc *Saccharomyces cerevisiae*, sp *Schizosaccharomyces pombe*, dd *Dictyostelium discoideum*, r rat, b bovine; ac means interaction with adenyl cyclase; aa means amino acid.
**Exon 12**

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† indicates % of normal control; n.a. means no samples were available for the Gsα bioactivity assay.;c/d means restriction site created or destroyed; chr* indicates the number of normal chromosomes checked.
Figure 3.2 Restriction digestion analysis of exon 1

Mutations in exon 1 were identified in six individuals affected with AHO (table 3.2). The presence or absence of the mutation in available family members was established by restriction digestion analysis. Results for five families are shown whilst a further family is illustrated in figure 3.3a. Filled symbols indicate affected individuals whilst the shaded symbol indicates an unknown affection status; n.d. means not digested.
Figure 3.3 Mutation analysis in family AHO/002

a) Restriction digestion analysis confirming the presence of the mutation in all three affected family members. Individual 11029 has PPHP whilst individuals 11027 and 11026 have PHPIf.

b) Results of the DGGE analysis of exon 5 in family AHO/002. Bands 1 and 2 represent the homoduplexes of the FokI (+) and (-) alleles respectively and bands 3 and 4 the heteroduplexes of the (+) and (-) alleles. FokI genotypes are shown below the figure.

c) Sequencing analysis of the cDNA representing the maternally derived allele showing the presence of the mutation.
a) A family tree with individuals labeled N.A. and a legend indicating genotypes.

b) Another family tree with individuals labeled N.A.

A gel electrophoresis image showing bands at 151, 140, 118, 100, 82, and 66 base pairs.

b) A gel electrophoresis image showing bands at 151, 140, 118, 100, 82, and 66 base pairs.

C) A comparison of wild-type and maternally derived alleles.

Wild-type allele:

- G
- A
- T
- C

Maternally derived allele:

- G
- A
- T
- C

The maternally derived allele has a substitution of C to G.
Figure 3.4 Mutation analysis in family AHO/034

a) Schematic representation of the regions flanking exon 1, indicating the localisation of the primers for amplification of this exon. For sequence analysis, two overlapping PCR fragments were generated using primers DV-157 and MAL-2 for the 5' end of exon 1 and MET1F and MET1R for the 3' end of the exon. Both fragments were sequenced from within exon 1. Hatched areas represent highly GC-rich regions.

b) Results of PCR amplification using primers DV-157 and MET1R, suggesting the presence of a deletion in all affected members of AHO/034. Sizes are shown in bp.

c) Diagram showing that the 38 bp deletion comprises 21 bp of exon 1 and 17 bp of the following intron.
Fig. 3.5 Sequence analysis in AHO/004/10285

Sequencing analysis of the abnormal sized PCR fragment indicating the extent of the deletion (line). The deletion was 43 bp in size but the position could not be confirmed due to the presence of a 4 nucleotide repeat GTGC (****) at the breakpoints of the deletion.
Figure 3.6 An outline of the strategy to examine the effect of mutations on the expression of GNAS1

a) Schematic representation of the mRNA structure of GNAS1, indicating the position of PCR primers and FokI restriction sites, including the polymorphic sites in exons 5 and 13 (+/−). Exon 3 is shaded to indicate alternative splicing of this exon. The hatched boxes represent the probe EX12.

b) Illustration of the fragments of various PCR products encompassing the exon 5 polymorphism obtained following FokI digestion. Sizes are shown in bp. PCR amplification using primers GNAS-4 and GNAS-5 generates products from all four cDNA species generated by alternative splicing (see 1.3.2, fig. 1.5), long forms Gsα-1 and Gsα-2 and short forms Gsα-3 and Gsα-4. PCR amplification with primers GNAS-3 and GNAS-5 or GNAS-6 will generate products from the long forms only, Gsα-3 (Gsα-4). Primer sequences are shown in table 2.2.

c) Illustration of the FokI restriction fragment sizes of various cDNA species generated by alternative splicing (see 1.3.2, fig. 1.5), long forms Gsα-1 (Gsα-2) and short forms, Gsα-3 (Gsα-4) following amplification with primers GNAS-1 and GNAS-13. The predicted sizes of restriction fragments after size separation, Southern blotting and hybridisation with probe EX12 are indicated.
Figure 3.7 RNA analysis of GNAS1 mutations in AHO/004/10285 and AHO/006/11151

a) PCR amplification using primers GNAS-3 and GNAS-6 is shown in the panel on the left, confirming the presence of a normal sized fragment in all three individuals but an additional smaller fragment in AHO/006/11151. The middle panel shows the PCR products generated using primers GNAS-3 and GNAS-5 and the fragments obtained following FokI digestion (F) demonstrating the presence of the wild-type allele (+) only. The panel on the right shows the fragments obtained following FokI digestion of PCR products generated using primers GNAS-4 and GNAS-5 demonstrating the presence of both alleles in the heterozygous control and AHO/006/11151. Primer sequences are shown in table 2.2.

b) Autoradiograph of the cDNAs generated using primers GNAS-1 and GNAS-13 and hybridised with EX12 (fig. 6.3c). The control is an individual homozygous for the FokI(+) allele. Normal, p indicates the wild-type allele in AHO/004/10285 which is paternally derived whilst mutant, m indicates the mutant allele which is maternally derived.

c) Sequence analysis of the abnormal sized fragment in AHO/006/11151 (see left panel in a) showing the absence of exon 5.
b) IS < normal, p — mutant, m —

310
281/271
234
198
118

310
281/271
234
198
118

EXON

GATC

normal, p —

mutant, m —
a) The upper photograph shows the DGGE analysis of exon 5, identifying the *FokI* genotype and the sequence variation in AHO/006/11151. The lower photograph shows the *FokI* restriction fragments from PCR products from the same individuals, size separated on an agarose gel. The individuals genotypes are shown between the photograph. Comparison of the two figures showed that the homoduplex band 1 arises from the *FokI* (+) allele and the homoduplex band 2 from the *FokI* (-) allele. Bands 3 and 4 represent heteroduplexes of the (+) and (-) alleles. The DGGE analysis also shows that the sequence variation is present on the paternally derived allele.

b) Sequence analysis of exon 5 and flanking regions in AHO/006/11151 identifying a G→A substitution at position +1 of intron 5 (left) which was confirmed, by sequence analysis of the individuals *FokI* (-) allele, to have arisen on the paternally derived allele (right).
b) Intron

wild-type

AHO/006/11151

paternally derived allele
Figure 3.9 DGGE analysis of exons 5 and 6

DGGE analysis of exon 5 showing the presence of the FokI polymorphism. Band 1 indicates the homoduplex of FokI (+) alleles and band 2 from the homoduplex of FokI (-) alleles. Bands 3 and 4 represent heteroduplexes of the (+) and (-) alleles. In addition, sequence variations were observed in AHO/006/11151, AHO/043/361, AHO/18/260, AHO/052/615 and AHO/055/624 (*).

DGGE analysis of exon 6 showing the presence of sequence variations in AHO/024/12004 and AHO/057/668.
a) An MseI polymorphism was identified in exon 7 (AHO/004/10285) as well as a 4 bp deletion (AHO/005/11091). Both sequence variations were used to verify that the 4 bp deletion in the 2 affected offspring in AHO/060 was carried on the maternally derived MseI (-) allele.

b) Partial coding sequence of exon 7 of GNAS1. The 4 bp deletion is overlined. A CT repeat (**) is present at the breakpoints of the deletion. Below is shown the consensus sequence derived from comparison of deletion hotspots in other genes (Krawczak and Cooper, 1991).
a) $MseI^+ -WT$

- $MseI - \Delta 4bp$

$MseI$ genotype

- $- \Delta$
- $- \Delta$
- $+\Delta$
- $+\Delta$
- $+\Delta$
- $- \Delta$
- $- \Delta$

b) GATCAAGCAGGCTGACTATGTGCCGAGCGA

** AAGA

TG GGTC
Figure 3.11 DGGE analysis of exon 8

DGGE analysis of exon 8 showing the presence of a sequence variation in AHO/013/165.
Figure 3.12 DGGE analysis of exons 9 and 10

DGGE analysis of exon 9 reveals sequence variations in AHO/021/284 and AHO/051/613. Duplicate samples of the two affected individuals are also illustrated.

DGGE analysis of exon 10 demonstrates sequence variations in the two affected individuals from family AHO/016 and in simplex cases AHO/032/12425 and AHO/023/11249.
DGGE analysis of the higher melting domain (b) in exon 12 (see fig. 3.1c), after AluI digestion of the PCR product encompassing the entire exon 12, shows the presence of a sequence variation in the two affected offspring of family AHO/001 and their mother. The shaded symbol indicates that her affection status is unknown. The sequence variation is not detected upon DGGE analysis of the entire the PCR fragment (fig. 3.1b) which is expected to detect mutations in the lower melting domain (a), suggesting that the chosen approach to screen exon 12 for mutations is legitimate.

DGGE analysis of exon 13 showing the presence of sequence variations in AHO/045/375 and AHO/012/11061.
Exon 1, AHO/002, H41D

A C→G substitution in codon 41 (H41D) was identified in individual 11027 who has PHP1a. The sequence variation created a BsaI recognition site and restriction digestion analysis showed that the base pair substitution was present in the patient's mother who has PPHP, and in his halfsister who has PHP1a (fig. 3.3a). The substitution was not present in the patient's father or maternal grandmother. The single nucleotide change will substitute the basic amino acid histidine (His) with the acidic amino acid aspartic acid (Asp) at position 41. His41 is a residue conserved in Gsa subunits in mammals and is part of a β-sheet. Therefore it is very likely that the amino acid substitution has caused the AHO phenotype in this family.

The exon 5 FokI polymorphism was used to establish the parental origin of the disease allele prior to mutation detection (fig. 3.3b). Genotyping of affected individuals 11029, 11027 and 11026 revealed cosegregation of the FokI (-) allele with the reduced Gsa bioactivity and the AHO phenotype indicating that this allele carried the Gsa mutation. The grandmother 11030 is homozygous for the FokI (+) allele hence the mutant allele must be grandpaternally derived. Thus within one family a paternal Gsa mutation has resulted in the PPHP phenotype while subsequent maternal transmission has resulted in offspring with PHP1a. These results are in keeping with the hypothesis that paternal transmission leads to PPHP and maternal transmission to PHP1a. The determination of the parental origin in this family has been reported elsewhere (Wilson et al., 1994).

Sequencing of the full length transcript (exons 1-13) identified the presence of the mutation on the FokI (-) allele. These data confirm the previous observation of cosegregation of the FokI (-) allele with the AHO phenotype and reduced Gsa bioactivity in this family.

Exon 1, AHO/034, 86del38

Upon PCR amplification of exon 1 and flanking regions, the normal sized 535 bp fragment was observed in all family members and in addition, a smaller fragment of approximately 500 bp was present in all 8 affected individuals in this family (fig 3.4). In affected individuals PCR amplification of the 5' end of exon 1 (primers DV-157 and MAL-2) generated a fragment of normal size whilst amplification of the 3' end of exon 1 (primers MET1F and MET1R) generated a product of the predicted size and a smaller sized fragment (data not shown). Hence, a deletion was predicted to be located downstream of primer MAL-2 (fig. 3.4a). Sequencing of the smaller PCR fragment revealed a 38 bp deletion comprising 21 nucleotides of the 3' end of exon 1 and 17 nucleotides of the following intron thus eliminating the donor splice site of exon 1. A premature termination codon would be created within intron 1 leading to the incorporation of at least 116 alternative amino acids. Biochemical data on this family have been reported by Fischer and colleagues (Fischer et al., 1983).
A single PCR product corresponding to the expected 159 bp fragment was identified in 12 affected individuals with either PHP1a or PPHP whilst in individual 10285, who has PHP1a, an additional smaller fragment was detected that was not present in either of the unaffected parents (data not shown). Direct sequencing of the smaller fragment revealed a 43 bp deletion comprising at least 35 nucleotides of the 3' end of exon 4 and extending into the following intron (fig. 3.5) thus omitting the donor splice site of exon 4. The 159 bp fragment was of wild-type sequence identical to that seen in both parents (data not shown). This de novo mutation is the largest deletion in GNAS1 described so far and has been reported elsewhere (Oude Luttikhuis et al., 1994).

To investigate the expression of the mutant allele, cDNA was synthesised from RNA isolated from the lymphoblastoid cell line CB0017. The outline of the investigation is depicted in figure 3.6. PCR amplification across the region containing the deletion using primers GNAS-3 and GNAS-6 or GNAS-4 and GNAS-5 revealed normal sized fragments (fig. 3.7a). These data did not reveal the expression of any mutant mRNA. The family were informative for the exon 13 FokI polymorphism; the proband had inherited the (-) allele paternally and the (+ •) allele maternally (data not shown). Therefore this polymorphism was used to investigate the expression of the mutant allele further, as outlined in figure 3.6c. Autoradiography revealed a single band in the control lane identifying the FokI (+) alleles and confirming complete digestion (fig. 3.7b). In the 10285 lane, two fragments were observed. A very weak signal from the maternally derived (+) allele was detected and a strong signal from the paternally derived (-) allele indicating that low quantities of the (+) allele were expressed in lymphocytes and that therefore this allele is carrying the mutation. Thus, these data demonstrated that the mutation is expressed and is present on the maternally derived allele. This is in keeping with the hypothesis that maternal transmission leads to PHP1a.

DGGE analysis of exon 5 detected the FokI polymorphism present in this exon and genotypes were confirmed by restriction digestion analysis (fig. 3.8a). The unaffected father and mother of the proband 11151, who has PPHP, are homozygous (-) and (+ •) for the FokI polymorphism respectively. Individual 11151 was confirmed by FokI restriction digestion analysis to be heterozygous. However, on DGGE she showed an altered banding pattern compared with other heterozygotes indicating the presence of a sequence variation within the analysed fragment. Furthermore, the absence of a paternally derived homoduplex demonstrated that the sequence alteration had arisen on the paternally derived allele. Direct sequencing of the amplified genomic DNA from individual 11151 revealed a heterozygous G→A substitution at the donor splice site of exon 5 (fig. 3.8b), which was not present in either parent (data not shown). To confirm the parental origin of the GNAS1 mutation, the FokI (-) allele (representing the paternally derived allele) was sequenced to
show the presence of the mutation (fig. 3.8b). This result is in keeping with the hypothesis that paternal origin of GNAS1 mutations leads to PPHP and is a valuable addition to the small number of reported paternal transmissions of AHO leading to PPHP. This mutation has been reported elsewhere (Wilson et al., 1994).

The strategy to study the expression of this splice site mutation is outlined in figure 3.6b. Synthesis of cDNA from RNA isolated from the lymphoblastoid cell line CBO011 was performed. Upon amplification of the long mRNA forms only, using primers GNAS-3 and GNAS-6, the normal sized and a smaller fragment were observed in individual 11151 (fig. 3.7a) suggesting a deletion within the amplified region. PCR amplification of exons 3-5 and subsequent FokI digestion showed that only the (wild-type) (+) allele was present, suggesting that exon 5 was at least deleted in part. Sequencing of the smaller exons 3-6 fragment revealed exon 5 in its entirety was deleted on the mutant allele (3.7c) as a consequence of exon skipping. Splicing of exon 5 does not lead to a frameshift in the mRNA and therefore it is likely that a truncated, non-functional protein is created which lacks 40 amino acids. It is of interest that upon amplification of exons 4-5 using primers GNAS-4 and GNAS-5 (amplifying both long and short cDNA species) both alleles appeared to be present. As PCR amplification of the long forms only showed absent expression of the mutant (-) allele, the observed (-) allele must be of the short form(s) of the mRNA. These data imply that different splice mechanisms exist for the alternative splice products and that the mutation affects only certain products, not others.

**Exon 7, AHO/005, 535del4**

Identical deletions of 4 base pairs in this exon had been described previously in three unrelated kindreds (Weinstein et al., 1992; Weinstein et al., 1994) suggesting a possible deletion hotspot. As a rapid means of screening our cohort of patients, radio-labelled PCR products encompassing exon 7 were size separated on polyacrylamide gels. Autoradiography revealed a smaller than expected fragment in individual 11091, who has PHP1a (data not shown). A normal sized fragment was present in the patient’s healthy mother and all other individuals screened. The deceased father had no recorded history of AHO. Parental origin of this deletion could not be determined due to the absence of informative polymorphisms. Sequencing analysis revealed that the deletion in this patient was identical to those described by Weinstein and colleagues. This deletion has been reported elsewhere (Yu et al., 1995 (family 4)).

**Exon 7, AHO/060, 535del4**

Upon DGGE analysis of exon 7, two homoduplexes and two heteroduplexes were observed in individual 416, who has PHP1a. Comparison with individuals heterozygous (AHO/004/10285) and homozygous (-) (AHO/002/11027) for the exon 7 MseI polymorphism and an individual with a 4 bp deletion in this exon (AHO/005/11091, see above) suggested that an identical deletion was present on the MseI (-) allele in individual
416 (fig. 3.10a). Sequence analysis confirmed that the deletion was identical to the one present in AHO/005/11091 and those described by Weinstein and colleagues (Weinstein et al., 1992; Weinstein et al., 1994) and also the heterozygous status of 416 for the Msel polymorphism. DGGE analysis showed that the affected mother and brother of 416 carried the same mutation and verified that the mutation in 416 and 426 was maternally derived.

3.5 Predicted effect of GNAS1 mutations on the G\(\alpha\) protein

An illustration of the predicted mutant G\(\alpha\) proteins resulting from GNAS1 mutations is presented in figure 3.14.

3.5.1 Mutations in the initiation codon

A mutation in the initiation codon identical to one identified in this study (iM+1A\(\rightarrow\)G) was reported previously (Patten et al., 1990). Immunochemical studies suggested that the mutant G\(\alpha\) transcripts are translated into abnormal G\(\alpha\) proteins, lacking the N-terminus. Initiation of translation may occur at the first AUG downstream of the mutated initiation codon resulting in a protein lacking the first 59 amino acids of a normal G\(\alpha\) molecule, although this codon is not present within the sequence optimal for recognition of AUG as an initiation codon (Kozak, 1989). The second initiation codon mutation (iM+2T\(\rightarrow\)A) is likely to result in an identical mutated G\(\alpha\) protein.

3.5.2 Missense mutations

All eight single base pair substitutions associated with the AHO phenotype are predicted to result in G\(\alpha\) proteins incorporating an incorrect amino acid. The substituted amino acids were always replaced by amino acids with different chemical characteristics which are likely to disrupt correct protein folding and hence protein function.

3.5.3 Nonsense mutations

The two nonsense mutations are likely to result in truncated G\(\alpha\) proteins lacking the amino acids following the premature termination codon.

3.5.4 Insertions/deletions

All identified insertions and deletions may lead to frameshifts thereby generating alternative termination codons. In all but one (1149insG) the newly created termination codon is upstream of the wild-type termination codon predicted to lead to premature termination of translation and shortened proteins. As a consequence of the frameshift, the amino acids incorporated in the mutant G\(\alpha\) protein, following the insertion or deletion differ from those in the normal G\(\alpha\) proteins.
Figure 3.14 The predicted effects of GNAS1 mutations on the Gsα protein

Schematic representation of the predicted effects of GNAS1 mutations on the Gsα protein. Illustrated is the protein generated upon translation of the long form mRNA Gsα-1 (see 1.3.2, fig. 1.5). The mutations are predicted to have the same effect on the protein generated upon translation of the other mRNA species except where exon skipping is concerned. In that case exon skipping occurs in the long mRNA species whilst it is unknown what effect the mutation has on the short mRNA species (AHO/006/11151).
EXON 1 2 3 4 5 6 7 8 9 10 11 12 13

- iM+1A->G
- iM+2T->A
- E27X
- 45insC
- H41D
- L46P
- 86del38
- 244del43
- 270delAG
- I103T
- A108P
- 309insT
- 402+1G->A
- 402+2T->C
- 430delTG
- 500+5del4
- 535del4
- 535del4
- 556-1G->A
- 630-1G->C
- R231C
- T242I
- F246S
- E259V
- R342X
- 1149insG

- incorrectly incorporated amino acids
- possibly incorrectly incorporated amino acids
- spliced out exon
- amino acid substitution
3.5.5 Splice site mutations

The six identified mutations in consensus splice sequences comprise four donor splice sites and two acceptor splice sites. The donor splice site mutations are likely to lead to skipping of the exon preceding the mutation (fig. 3.15). Skipping of exon 5 will not cause a frameshift and therefore a shortened protein lacking the 40 amino acids encoded by this exon will be produced. In contrast, skipping of exon 6 will lead to a frameshift which will result in the incorporation of alternative amino acids until the prematurely generated termination codon is reached. The acceptor splice site mutations are likely to cause the same effect on the protein either by generating a new acceptor splice site (556-1G→A) or by using an alternative splice site (630-1G→C) (fig. 3.16).

3.6 GNAS1 genotype and Albright hereditary osteodystrophy phenotype

A review of 31 published reports of AHO involving two or more generations indicated that the phenotypic expression in the offspring depends on the sex of the transmitting parent (Davies & Hughes, 1993). Maternal transmission lead to PHPIa in 60/60 offspring and paternal transmission to PPHP in 6/6 offspring. Molecular data to confirm which parent was affected were available in three kindreds (Patten et al., 1990; Weinstein et al., 1990).

To find additional molecular support for these observations, parental origin of GNAS1 mutations was established in 17 parent-to-child transmissions (table 3.3). In familial AHO the affected parent was identified by the presence of the mutation whilst in sporadic AHO exonic polymorphisms were used to establish the origin of the mutated allele (see AHO/006 in 3.4). Maternal transmission/origin of the mutation resulted in offspring with PHPIa whilst paternal origin of the mutation lead to PPHP in offspring. In one family, a mutation on the paternally derived allele caused PPHP whilst subsequent maternal transmission resulted in PHPIa. These data provide molecular support for the hypothesis that maternal transmission leads to PHPIa and paternal transmission to PPHP.

Inspection of the obtained data did not reveal a consistent pattern between the type of mutation and the somatic features of AHO and severity of mental retardation implying that individuals with mutations predicted to lead to truncated Gsα proteins were not more severely affected than those with mutations leading to amino acid substitutions. However, subcutaneous calcifications were absent in a higher proportion of individuals with missense mutations (90%) than in individuals with any other type of mutations (16%). No positional effect was observed. The phenotypic characteristics of unrelated individuals with mutations that are predicted to have an identical effect on the Gsα protein were compared and no common phenotype was observed. For example, subcutaneous calcifications were present in individuals AHO/006/11151 and AHO/018/260 who had splice site mutations 402+1G→A and 402+2T→C respectively, and absent in...
Figure 3.15 Effects of donor splice site mutations on mRNA processing and resulting protein

Schematic representation of the donor splice site mutations and their effect on the processing of the long form mRNA Gsα-1 and the Gsα protein.

a) The donor splice site consensus sequence in primates according to Cooper and Krawczak (1993). The nucleotide frequencies in certain positions are indicated.

b) Schematic drawing indicating the wild-type and the mutated sequence (bold) leading to exon skipping. Skipping of exon 5 will not lead to a frameshift and therefore the mutant protein will resemble the wild-type protein lacking the amino acids encoded by exon 5.

c) Schematic drawing indicating the wild-type and the mutated sequence leading to exon skipping. An overlapping repeat sequence is present (overlined) which may have resulted in the 4 bp deletion (**). Skipping of exon 6 will create a frameshift and lead to the incorporation of 11 incorrect amino acids.
a) donor splice site consensus sequence

<table>
<thead>
<tr>
<th>exon</th>
<th>intron</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>-1</td>
</tr>
<tr>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>+3</td>
<td>+4</td>
</tr>
<tr>
<td>+5</td>
<td>+6</td>
</tr>
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<td>G78</td>
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<tr>
<td>G100</td>
<td>T100</td>
</tr>
<tr>
<td>A57</td>
<td>A71</td>
</tr>
<tr>
<td>G84</td>
<td>T47</td>
</tr>
</tbody>
</table>

b) wild-type

- exon 4: GAAgtac
- exon 5: ACC
- exon 6: CCCgtaagct

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Glu</td>
</tr>
<tr>
<td>5</td>
<td>Thr</td>
</tr>
<tr>
<td>6</td>
<td>Pro</td>
</tr>
</tbody>
</table>

402+1G->A

- exon 4: GAAgtac
- exon 5: ACC
- exon 6: CCCgtaagct

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Glu</td>
</tr>
<tr>
<td>5</td>
<td>Thr</td>
</tr>
<tr>
<td>6</td>
<td>Pro</td>
</tr>
</tbody>
</table>

402+2T->C

- exon 4: GAAgtac
- exon 5: ACC
- exon 6: CCCgtaagct

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Glu</td>
</tr>
<tr>
<td>5</td>
<td>Thr</td>
</tr>
<tr>
<td>6</td>
<td>Pro</td>
</tr>
</tbody>
</table>

mutant

- exon 4: ATT GAA
- exon 6: GAA TTC

Ile Glu Glu Phe

c) wild-type

- exon 5: CCCgtaagct
- exon 6: GAA
- exon 7: CAG TAgtaagtaacgc

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Pro</td>
</tr>
<tr>
<td>6</td>
<td>Glu</td>
</tr>
<tr>
<td>7</td>
<td>Gln Ty</td>
</tr>
</tbody>
</table>

500+4del4

- exon 5: CCCgtaagct
- exon 6: GAA
- exon 7: CTT CTC

<table>
<thead>
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<th>Exon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
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<td>6</td>
<td>Glu</td>
</tr>
<tr>
<td>7</td>
<td>Leu Pro</td>
</tr>
</tbody>
</table>

mutant

- exon 5: CCT CCC
- exon 7: CTT CCT GGA CAA GAT CGA CCG TAG TAA CCG

Pro Pro Leu Pro Gly Gln Asp Arg Arg Asp Gln Ala Gly Stop
Figure 3.16 Effects of acceptor splice site mutations on mRNA processing and resulting protein

Schematic representation of the acceptor splice site mutations and their effect on the processing of the long form mRNA Gσα-1 and the Gσα protein.

a) The acceptor splice site consensus sequence in primates according to Cooper and Krawczak (1993). The nucleotide frequencies in certain positions are indicated.

b) Schematic drawing indicating the wild-type and the mutated sequence. The G→A substitution will create a new acceptor splice site downstream which will lead to the deletion of 1 bp (hatched). As a consequence the reading frame will be changed leading to the incorporation of 7 incorrect amino acids.

c) Schematic drawing indicating the wild-type and the mutated sequence. A 6 bp sequence identical to 6 bp in the acceptor splice site is present 19 bp upstream (overlined). The mutation in the wild-type acceptor splice site may lead to the use of the upstream repeat sequence as an alternative acceptor splice site. This will change the reading frame leading to the incorporation of 8 incorrect amino acids.
a) acceptor splice site consensus sequence

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<tr>
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</thead>
<tbody>
<tr>
<td>-6 -5 -4 -3 -2 -1</td>
<td>+1 +2</td>
</tr>
<tr>
<td>T47 T46 C29 C74 A100 G100</td>
<td>G49 T37</td>
</tr>
</tbody>
</table>

b) wild-type

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<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT  CAGgtgtgcaa</td>
<td>caatttgtttcagGAC  CTG</td>
</tr>
<tr>
<td>Asp  Gln</td>
<td>Asp  Leu</td>
</tr>
</tbody>
</table>

mutant

<table>
<thead>
<tr>
<th>Exon 7</th>
<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT  CAG ACC  TGC  TTC  GCT  GCC  GTG  TCC  TGA</td>
<td>Asp  Gln  Thr  Cys  Phe  Ala  Ala  Val  Ser  Stop</td>
</tr>
</tbody>
</table>

c) wild-type

<table>
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<th>Exon 9</th>
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</thead>
<tbody>
<tr>
<td>TTC  CAgtaagcc</td>
<td>ggaataaccagctgtcctcctccccaccagC  ATG</td>
</tr>
<tr>
<td>Phe  His</td>
<td>s  Met</td>
</tr>
</tbody>
</table>

mutant

<table>
<thead>
<tr>
<th>Exon 8</th>
<th>Exon 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTC  CAgtaagcc</td>
<td>ggaataaccagCTGTCCTCCTCCCCACCAGCATGT'TGA</td>
</tr>
<tr>
<td>Phe  His</td>
<td>sCysProProProHisGlnHisValStop</td>
</tr>
</tbody>
</table>
Table 3.3 Parental origin of GNAS1 mutations

<table>
<thead>
<tr>
<th>parental transmission/origin†</th>
<th>kindreds</th>
<th>phenotype in offspring</th>
<th>number of affected offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>maternal transmission</td>
<td>6*</td>
<td>PHPIa</td>
<td>13</td>
</tr>
<tr>
<td>maternal origin</td>
<td>1</td>
<td>PHPIa</td>
<td>1</td>
</tr>
<tr>
<td>paternal transmission</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>paternal origin</td>
<td>3*</td>
<td>PPHP</td>
<td>3</td>
</tr>
</tbody>
</table>

† Maternal transmission indicates transmission of the mutation by the mother and maternal origin indicates that the mutation has arisen on the maternally derived allele.
* In one family, a mutation on the paternally derived allele caused PPHP whilst subsequent maternal transmission resulted in PHPIa.
AHO/052/615 who had mutation 402+1G→A. Furthermore, mental retardation in these three patients was either moderate, mild or absent. Variable phenotypes were also observed amongst affected sibs; both individuals AHO/001/11011 and AHO/001/11012 have brachydactyly but the digits involved and the severity of their metacarpal shortening differs. These data strongly suggest that the nature of the mutation is not solely responsible for the development of the physical features of AHO and mental retardation but that other factors (epigenetic and/or environmental) influence which phenotypic characteristics are displayed. The same is true for the development of hormone resistance associated with AHO as identical mutations can cause PPHP as well as PHP1a, even within one family (AHO/002).

3.7 Discussion

A search of all 13 exons of GNAS1 which included exon-intron boundaries revealed 27 heterozygous mutations of which 25 were unique. The entire cohort of 58 probands was screened for mutations in exons 2-13 regardless of the presence of a disease causing mutation in any of those exons and never revealed more than one mutation in any individual. Individuals with mutations in exons 2-13 were not screened for the presence of mutations in exon 1 as only one causal mutation was presumed to be present in each affected individual. The identified mutations were variable in nature, comprising 2 point mutations in the initiation codon, 8 missense and 2 nonsense mutations, 3 insertions, 6 deletions and 6 splice site mutations. All eight missense mutations were believed to be causal as they were absent in at least 38 alleles from normal unrelated controls whilst six missense mutations occurred in residues conserved amongst α subunits of different species (Itoh et al., 1986; Kaziro et al., 1991; Lambright et al., 1996) and all eight in residues conserved between different kinds of G proteins (Pennington, 1995). The predicted effect of missense mutations on the Gsa protein is the substitution of an amino acid whilst all remaining mutations are predicted to lead to the generation of truncated proteins (table 3.2 and fig. 3.14). Six missense mutations were located in structurally important domains such as α-helices and β-sheets (Lambright et al., 1996), two in regions involved in interaction with βγ subunits (Conklin & Bourne, 1993) and three in regions involved in interaction with adenylyl cyclase (Berlot & Bourne, 1992). Amino acid substitutions in any of these domains are likely to disrupt the structure and consequently function of the protein.

AHO-causing mutations were distributed throughout the gene (fig. 3.17). However 7/27 (26%) of the mutations appeared clustered within the first exon, all unique except for one (IM+1A→G) identical to a previously published mutation (Patten et al., 1990). Previous reports of mutations in GNAS1 always excluded exon 1 because the highly GC-rich sequence surrounding this exon makes it difficult to generate PCR products for mutation analysis. The N-terminus of Gsa is one of the sites of interaction with βγ
Figure 3.17 Overview of GNAS1 mutations

Diagram showing the distribution of the identified GNAS1 mutations and their position in functionally important domains. G-1 to G-5 are regions conserved amongst members of the GTPase superfamily.
(Conklin & Bourne, 1993); the first 25 amino acids of the α subunit, all encoded by exon 1, are essential for βγ binding (Denker et al., 1992). The initiation codon mutations will generate truncated Gsa proteins unable to interact with βγ and as a consequence block signal transduction. In addition, the N-terminal region of Gsa is a contact site for the receptor, as is the C-terminal region. The last few amino acids of the C-terminus contribute to the specificity of the receptor-G protein interaction (Conklin & Bourne, 1993). The single nucleotide insertion 3 base pairs before the termination codon is likely to result in a truncated protein that does not interact with the Gsa-coupled receptor. Interaction with effectors (adenylyl cyclase) occurs in a region between residues 236 and 356. Scanning mutagenesis experiments have shown that this stretch of amino acids is required for activation of adenylyl cyclase (Berlot & Bourne, 1992). The mutations in exons 10 and 12 are located within this region and are therefore likely to lead to proteins incapable of generating a second messenger through adenylyl cyclase activation.

The exact position of all deletions but one (86del38) and all insertions could not be established because of repeated sequence at the boundaries of the deletions (2-5 bp) and insertions (1-2 bp). Repeat sequences at deletion boundaries are frequently observed and Efstratiadis and colleagues (Efstratiadis et al., 1980) suggested that short repeats (2-8 bp) may be involved in the generation of these deletions by slipped mispairing. The repeat sequences present at the deletion boundaries of 244del43 (GTGC) and 535del4 (CT) are shown in figures 3.5 and 3.10b respectively. The deletion 535del4 has so far been observed in seven kindreds (Nakamoto et al., 1992; Weinstein et al., 1992; Yu et al., 1995, this study) and therefore represents a deletion hotspot. Slipped mispairing alone however is unlikely to be sufficient to cause this deletion as 2 bp direct repeats frequently occur within the gene and no other deletion hotspots have been identified. An alternative mechanism involves the arrest of DNA polymerase α. A 6 bp consensus sequence (TG(A/G)(A/C)(T/G)(A/C) found in GNAS1 and deletion hotspots in other human genes (Krawczak & Cooper, 1991) is consistent with an arrest site for this enzyme. The breakpoints of the GNAS1 deletion correspond with the observations of breakpoints 3′ or 3′ to the second residue (G) and/or last residue (A/C) of the consensus sequence. Hotspots in seven human genes always involved non-identical deletions within a short stretch of DNA (≤16 bp) (Krawczak & Cooper, 1991). Slipped mispairing can account for insertions just as for deletions (Cooper & Krawczak, 1993); a 2 bp direct repeat (TG) is present at the insertion boundary in 309insTG.

All identified deletions and insertions are predicted to lead to the generation of a premature termination codon. Studies of triosephosphate isomerase and dihydrofolate reductase have shown that the presence of premature termination codons can lead to diminished mRNA expression (Urlaub et al., 1989; Cheng et al., 1990). Translation into the penultimate exon may be necessary for normal RNA processing and transport to the cytoplasm. The 4 bp deletion 535del4, which is expected to lead to the generation of a premature termination codon before the penultimate exon, was found to be minimally
expressed or not expressed at all (Weinstein et al., 1992). The deletion 244del43 however showed low levels of expression of the mutant allele (fig. 3.7b). The detected fragment encoded exons 12 and 13 only (fig. 3.6c) and was of expected size. Restoration of the reading frame by additional exon skipping was suggested as an explanation for the observed size reduction of dystrophin present at greatly reduced levels in individuals with Duchenne Muscular Dystrophy (MIM 310200) who had frameshift mutations (Nicholson et al., 1992).

Measurement of Gsα bioactivity was performed in 41/58 probands. Reduced bioactivity (< 80% compared to an unrelated control sample) was observed in 24 of those 41 individuals whilst normal bioactivity (≥ 95%) was observed in 11 individuals. The remaining individuals had values between 80 and 95% making it difficult to identify those individuals as having reduced or normal bioactivity. The range of bioactivity values, as measured by the cys complementation assay, is wide and should be used as a guideline rather than a criteria for diagnosis of PHP1a/PPHP. Therefore all 58 probands were included in the mutation screening.

DGGE is a sensitive mutation detection technique (~99%) (Fodde & Loosekoot, 1994) and when applied to GNAS1 revealed sequence 24 variants in exons 3 (data not shown) (4.2%), 4 (12.5%), 5 (25%), 6 (8.3%), 7 (12.5%), 8 (4.2%), 9 (8.3%), 10 (12.5%), 12 (4.2%) and 13 (8.3%). Any sequence variants present within these exons were therefore expected to be observed. Sequence variants in the remaining exons (2 and 11) were either not present or not detected under the conditions used. Of the 24 individuals with reduced Gsα bioactivity, mutations were found in 20 implying that mutations in the remaining 4, who had typical features of AHO, are likely to be located in the regulatory sequences (such as GC-boxes in the promoter region) or in introns. It is unlikely that the insensitivity of DGGE can account for the absence of GNAS1 mutations in 31/58 individuals (53%) from our cohort. The lack of GNAS1 mutations in those individuals may be explained by locus heterogeneity. Microdeletions of chromosome 2q37 have been identified in one individual with AHO and normal Gsα bioactivity (Wilson et al., 1995) and in two related individuals with AHO whose Gsα bioactivity values were unknown (5.4).

DGGE analysis has been successfully used for diagnostic applications and proven useful for the analysis of conditions displaying a heterogeneous mutation spectrum (Beck et al., 1993; Fodde & Loosekoot, 1994). Based on these facts and the spectrum of GNAS1 mutations identified in this study, DGGE appears a good technique for the analysis of GNAS1 mutations in exons 2-13. However, the technique could be improved for diagnostic purposes by devising gel electrophoresis conditions enabling the screening of all exons at once. Mutations in exon 1 will remain to be identified by direct DNA sequencing as this exon is not amenable for DGGE analysis.

Within the same family, identical mutations gave rise to AHO with and without hormone resistance suggesting that the nature of the mutation is not the determinant for development of hormone resistance. Parental origin of the mutation was suggested to play
a role (possibly by genomic imprinting) in determining the phenotypic expression of AHO (Davies & Hughes, 1993) based on a review of published familial cases of AHO. Our data correspond with the observation that maternal transmission leads to PHPIa and paternal transmission to PPHP (Davies & Hughes, 1993) and hence provide molecular support for a parental effect.

As the characteristic features of AHO, round face, obesity, subcutaneous calcifications, brachydactyly and mental retardation are not always present in all patients we investigated whether molecular heterogeneity of GNAS1 mutations may account for (at least in part) variation in the observed phenotypic characteristics. A higher proportion of individuals with mutations predicted to lead to truncated Gsa proteins had subcutaneous calcifications than individuals with mutations predicted to lead to amino acid substitutions. No other features appeared to be related to GNAS1 genotype. In contrast, mutations with identical predicted effects on the Gsa protein showed no common phenotype indicating that other factors (epigenetic and/or environmental) besides the nature of the mutation may be necessary to cause the physical features and mental retardation characteristic of AHO. Alternative pathways may be involved that compensate for Gsa defects.
Chapter 4: GNAS1 expression in hormone-responsive tissues

4.1 Preface

Deactivating mutations in GNAS1 can cause AHO with and without hormone resistance in affected members of the same family indicating that the nature of the mutation alone is not sufficient to explain the difference in hormone responsiveness (Patten et al., 1990; Nakamoto et al., 1992; Weinstein et al., 1990; Miric et al., 1993; Yu et al., 1995; chapter 3, this study). A review of published familial cases of AHO suggested that PHPIa was inherited maternally and PPHP paternally (Davies & Hughes, 1993). One hypothesis proposed to explain the observed parental effect is the genetic mechanism termed imprinting. In this instance, imprinting of GNAS1 would occur in cells or tissues responsive to PTH and other hormones stimulating cAMP synthesis through adenylyl cyclase.

Imprinting would result in monoallelic expression and to test this hypothesis (illustrated in 1.4.2, fig. 1.6), the expression pattern of GNAS1 was examined in hormone-responsive tissues.

4.2 DNA studies of human leucocytes, renal cortex and proximal tubules and thyroid

Mono- or biallelic expression of a gene may be established in samples heterozygous for an exonic polymorphism. Detection of a single expressed allele of GNAS1 in tissue samples of heterozygous individuals would implicate imprinting of GNAS1.

A variety of tissue samples were screened for heterozygosity for a FokI polymorphism located in exon 5 of GNAS1 (Gejman et al., 1991). These included hormone responsive tissues, renal proximal tubules and thyroid, and hormone unresponsive tissues, namely leucocytes and renal cortex. The results are presented in table 4.1. Cultured cells consisting of >95% renal proximal tubules were obtained from two individuals (#6 and #7). Heterozygosity for the FokI polymorphism was confirmed using genomic DNA from these two proximal tubules samples.
Table 4.1 Identification of tissue samples informative for a *FokI* polymorphism in exon 5 of GNAS1§

<table>
<thead>
<tr>
<th>tissue sample</th>
<th><em>FokI</em> genotype*</th>
<th>informative</th>
</tr>
</thead>
<tbody>
<tr>
<td>leucocytes # 1</td>
<td>+/-</td>
<td>yes</td>
</tr>
<tr>
<td>leucocytes # 2</td>
<td>+/-</td>
<td>yes</td>
</tr>
<tr>
<td>leucocytes # 3</td>
<td>+/-</td>
<td>no</td>
</tr>
<tr>
<td>leucocytes # 4</td>
<td>+/-</td>
<td>no</td>
</tr>
<tr>
<td>leucocytes # 5</td>
<td>+/-</td>
<td>no</td>
</tr>
<tr>
<td>renal cortex # 6</td>
<td>+/-</td>
<td>yes</td>
</tr>
<tr>
<td>renal cortex # 7†</td>
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<td>yes</td>
</tr>
<tr>
<td>renal cortex # 8</td>
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<tr>
<td>renal proximal tubules # 6</td>
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</tr>
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<td>thyroid # 9</td>
<td>+/-</td>
<td>yes</td>
</tr>
<tr>
<td>thyroid # 10</td>
<td>+/-</td>
<td>yes</td>
</tr>
</tbody>
</table>

§ Gejman *et al.* (1991), frequencies for the *FokI* (-) and (+) alleles are 0.53 and 0.47 respectively.
* *FokI* genotypes were determined using genomic DNA template for PCR amplification with primers Gsa4F and Gsa5R (table 2.2).
† *FokI* genotype was determined using combined genomic and cDNA template for PCR amplification with primers GNAS-4 and GNAS-5 (table 2.2).
4.3 RNA studies of human leucocytes, renal cortex and proximal tubules and thyroid

The strategy to examine the expression pattern of GNAS1 is illustrated in figure 4.1. Using this approach, biallelic expression would be indicated by the presence of all four fragments following hybridisation with EX4. Complete digestion of the nested PCR products was determined by the presence of a single fragment of 276 bp, detected by hybridisation with EX9 using parallel Southern blots or reprobing of filters stripped of EX4. Figure 4.2 is representative of the data obtained using this experimental approach. Complete digestion of the PCR fragments was verified (fig. 4.2a). The presence of all cDNA products was identified in all the examined tissues with no qualitative difference noted between hormone responsive and unresponsive samples respectively (fig. 4.2b). These experiments do no exclude quantitative differences in the expression of the different GNAS1 transcripts in the various samples analysed.

4.4 RNA studies of human osteoblasts

The GNAS1 expression pattern was examined in a further hormone responsive cell type using a simplified strategy outlined in figure 4.3a. The (+) and (-) alleles were identified in two of the five samples (#13 and #15; fig. 4.3b) indicating biallelic expression of GNAS1 in cultured osteoblasts.

4.5 Discussion

GNAS1 expression was studied in human proximal tubules, thyroid and osteoblasts to test the hypothesis that tissue specific imprinting is the mechanism responsible for the difference in hormone responsiveness seen in the phenotypic variants PHP1a and PPHP. These tissues and cells were selected as they are responsive to PTH and other hormones acting via stimulation of adenylyl cyclase. Leucocytes and human renal cortex were also studied as control cells and tissue, unresponsive to hormone stimulation.

The results demonstrated biallelic expression in all the tissues studied. However, quantitative differences in the expression of GNAS1 alleles were suggested by the appearance of varying intensity of the restriction fragments in several samples (e.g. #7, #9 and #10). Densitometry was not performed as the nested PCR was not developed as a quantitative assay. Similar observations have not been reported for other known imprinted genes.

The observed biallelic expression does not disprove the hypothesis that genomic imprinting of GNAS1 occurs in hormone-responsive tissues. The tissues studied consisted
Figure 4.1 An outline of the strategy to examine the expression pattern of GNAS1

a) Schematic representation of the mRNA structure of GNAS1, indicating the position of PCR primers and *FokI* restriction sites (F), including the polymorphic site in exon 5 (+/-). Exon 3 is shaded to indicate alternative splicing of this exon. The hatched boxes represent the probes EX4 and EX9.

b) Indication of the various cDNA species generated by alternative splicing (see 1.3.2, fig. 1.5), long forms Gsα-1 (Gsα-2) and short forms, Gsα-3 (Gsα-4) following amplification with primers GNAS-1 and GNAS-13 (first round PCR). Sizes are shown in bp. Below are shown the products of a second round (nested) PCR using primers GNAS-2 and GNAS-11. Primer sequences are shown in table 2.2.

c) Illustration of the fragment sizes of the nested PCR products obtained following *FokI* digestion.

d) Demonstration of the predicted sizes of fragments identified following size separation, Southern blotting and hybridisation with the probes EX4 and EX9.
Figure 4.2 The GNAS1 expression pattern in human leucocytes, renal cortex and proximal tubules and thyroid

a) and b) Autoradiographs of cDNAs generated using nested PCR (fig. 4.1d) and hybridised with (a) EX9 and (b) EX4. The reproducibility of the assay was determined by the generation of nested PCR products using three independently generated first round PCR products (fig. 4.1b).
CO%
a%
o%
I
s
I
i
I
0 >
I
o
&

a)

b)

276 bp long and short

long (-)
short (-)
long (+)
short (+)
Figure 4.3 The GNAS1 expression pattern in human osteoblasts

a) Schematic representation of the mRNA structure in the vicinity of the exon 5 FokI polymorphism, indicating the location of the PCR primers GNAS-4 and GNAS-5 and the polymorphic restriction site. PCR using these primers will amplify all four cDNA species (Gsα-1, Gsα-2, Gsα-3 and Gsα-4). Underneath, the sizes of the PCR fragments obtained following FokI digestion are shown in bp.

b) Agarose gel showing the fragments obtained following FokI digestion of PCR products using all four cDNA species as template. Sizes of the fragments from the ΦX/HaeIII marker are shown in bp.
of a heterogeneous cell population and imprinting may be confined to one cell type. Studying individual cells would prove technically difficult as clonal cell lines would have to be established and may not reflect true in vivo expression because it is not known whether the imprinting process is affected by cell culture. The renal proximal tubules and osteoblasts studied may have lost their imprinting during cell culture. Furthermore, the four different Gsα transcripts may behave differently, some may have both alleles expressed, others only one. The observed fragments of the long and short forms (fig. 4.2b) constitute each at least two fragments differing by 3 base pairs. Therefore, the intensity of the bands is the result of hybridisation of the probe to fragments of two Gsα mRNA species and may mask the expression pattern of individual transcripts. However, these experiments provide no support for the hypothesis of monoallelic (imprinted) regulation of GNAS1 expression. Further explanations for the parental effect in the hormone responsive phenotype include posttranscriptional or posttranslational modifications of GNAS1 in hormone responsive tissues or hormone-related regulation of the β and/or γ subunits of Gs.

During the course of the experiments described in this chapter, two independent studies found no support for imprinting of GNAS1. A study of GNAS1 expression in ten different tissues from human fetuses at gestational ages from 6 to 13 weeks, by FokI restriction digestion analysis and quantification of the resulting fragments, found no evidence for tissue specific imprinting at least in early embryogenesis (Campbell et al., 1994). Williamson and colleagues studied expression of Gnas, the mouse homologue of GNAS1, in tissues from mice with maternal duplication/paternal deficiency and its reciprocal for the distal region of chromosome 2 (Williamson et al., 1994). Gnas, is localised to region 2E1-2H3 on chromosome 2 (Ashley et al., 1987), a region known to show imprinting effects (Cattanach & Kirk, 1985). Biallelic expression was found suggesting that Gnas was not imprinted.

A parental effect has also been observed in myotonic dystrophy (MIM 160900); a congenital form is exclusively inherited from affected mothers. Imprinting of the DM-kinase gene was suggested and excluded by demonstration of biallelic expression in various human and mouse tissues (Jansen et al., 1993). The molecular mechanism accounting for this effect remains unclear.

In conclusion, genomic imprinting of GNAS1 has not been shown in the hormone-responsive tissues renal proximal tubules, thyroid and osteoblasts. Should GNAS1 expression be regulated by genomic imprinting, the imprint is unlikely to be present in the whole target tissue but confined to the hormone-responsive cells within that target tissue.
Chapter 5: Deletion mapping in Albright hereditary osteodystrophy-like syndrome

5.1 Preface

Phelan and colleagues reported two unrelated individuals with a phenotype resembling AHO (AHO-like) who had cytogenetically detectable deletions of the terminal region of chromosome 2 (Phelan et al., 1993). This suggested the existence of a clinical syndrome distinct from AHO as caused by mutations of GNAS1 located on chromosome 20. To search for further evidence of an AHO-like syndrome mapping to chromosome 2q37, three groups of individuals were studied. Group 1 consisted of twelve individuals with AHO, normal karyotype and normal Gsα bioactivity, group 2 was composed of seven individuals with a brachydactyly phenotype and mild to moderate mental retardation, normal karyotype and unknown Gsα bioactivity and group 3 consisted of six individuals with cytogenetically visible deletions of chromosome 2q37 who had mental retardation and brachydactyly. All twelve group 1 individuals and one group 2 individual were originally ascertained for the GNAS1 mutation screening study because of their apparent AHO phenotype. Individuals from groups 1 and 2 were screened for the presence of submicroscopic deletions and group 3 individuals were screened in order to characterise the molecular extent of their deletions. Group 3 individuals are referred to as del2/family number/DNA number.

5.2 Microsatellite analysis

Short tandem repeat polymorphisms, or microsatellites, consisting of short repeats of (CA)n, were used to establish whether an individual had both a paternally and a maternally derived allele. These microsatellite markers were used because they can be readily analysed by PCR and because their relative order has been established based on linkage analysis and may be represented on a single genetic linkage map. New linkage maps are continuously produced as more microsatellite markers are developed. The most recently available version of the Généthon linkage map was used in this study (Dib et al., 1996).

Genomic DNA of the individuals and their available parents was amplified by PCR using the primers described in table 2.2. The radioactively labelled PCR products were size separated on denaturing polyacrylamide gels and subjected to autoradiography. By comparing the alleles present in parents and their affected offspring, the presence or absence of a deletion can be demonstrated. Alternatively, the parental mating may be
uninformative when the offspring is homozygous for a single allele common to both parents (fig. 5.1b).

A total of 11 microsatellites was analysed and the results are summarised in figure 5.2 which shows the current ordering of the microsatellites (Dib et al., 1996). Data obtained from individuals AHO/011/10780 and del2/001/198 have been published elsewhere (Wilson et al., 1995) and were referred to as KW and RA respectively. Individual AHO/054/419 in figure 5.2 is a relative of individual AHO/054/622 and will be discussed later (5.4). In groups 1 and 2, informative data on 15/19 individuals produced no evidence of a deletion in the terminal region of chromosome 2q. Two AHO individuals (AHO/012/11061 and AHO/050/609) whose fathers were unavailable for study (5.5) showed apparent homozygosity for all eight tested microsatellite markers in this region. Two further AHO individuals (AHO/011/10780 with normal Gsa levels and AHO/054/622 with unknown Gsa levels) showed evidence of a microdeletion in the terminal region of chromosome 2q. Deletions were confirmed in all six individuals in group 3. All eight individuals had overlapping deletions. The region between markers D2S2285/D2S2253 and D2S125, at least 3 cM in size, was deleted in all eight individuals. The proximal boundary of the deletion lies within an 8 cM region between D2S338/D2S345 and D2S2285/D2S2253 and the terminal boundary lies between D2S125 and the 2q telomere.

Three deletions were maternally derived and five were paternally derived suggesting that the parental origin of the deletion plays no role in determining the disease phenotype.

5.3 Minisatellite analysis

The minisatellite D2S90 was analysed using two complementary methods. First, genomic DNA from the individuals and their available parents was digested with the restriction endonuclease MspI and genotyped by Southern analysis. As the mutation rate at this locus is high (13% in the male germline and 0.4% in the female germline (Buaid & Vergnaud, 1994)), the absence of a small, mutated parental allele beyond the resolution of detection by Southern analysis required demonstration of such an allele by PCR amplification across the repeat. The combination of Southern analysis to detect large fragments and PCR analysis to detect small fragments allows confirmation of a deletion at this locus.

Seven of the individuals with deletions at microsatellite loci showed a deletion at the D2S90 locus whilst the eighth individual was uninformative for this locus. The family of individual AHO/054/622 was analysed by PCR only as the DNA samples were shown to be of insufficient quality to perform Southern analysis. The results of the PCR analysis of D2S90 are shown in figure 5.1d. The affected individual showed the presence of a
Figure 5.1 Deletion mapping in family AHO/054

a) Analysis of the microsatellite marker D2S331 demonstrating that the affected individual has no deletion; m means maternally derived allele and p means paternally derived allele in offspring.

b) Analysis of the microsatellite marker D2S206 demonstrating an uninformative situation.

c) Analysis of the microsatellite marker D2S125 demonstrating the presence of a paternally derived deletion in the affected individual.

d) Analysis of the minisatellite marker by PCR demonstrating the presence of a paternally derived deletion in the affected individual. The unaffected sister has inherited a mutated paternally derived allele (p*).
a) D2S331
no deletion

b) D2S206
uninformative

c) D2S125
paternally derived deletion

d) D2S90
paternally derived deletion
Figure 5.2 Summary of identified deletions on chromosome 2q37

Diagram showing the extent of the deletions present in an individual with AHO and normal Gsα levels, two related individuals with AHO and unknown Gsα levels and six individuals with cytogenetically visible deletions of 2q37. The ordering of the markers and the distance between them is according to Dib et al. (1996) distances marked with * are according to Gyapay et al. (1994). The location of D2S90 is unclear hence indicated with a thin line left of the microsatellites. The solid line right of the microsatellites indicates the region deleted in all patients whilst the dashed lines indicate the regions in which the proximal and terminal endpoint of the deletions are localised.

NI means not informative, ND not determined, no Δ no deletion, Δm/Δp deletion on the maternally/paternally derived allele.
single allele the same size as one of the maternal alleles suggesting a paternally derived deletion. This individual's sister had two alleles, a normal sized maternally derived allele and a mutated paternally derived allele.

Individual AHO/050/609 with normal karyotype and normal Gsα levels was identified with an apparent paternally derived deletion of D2S206 and all other distal microsatellites (fig. 5.2) suggesting the presence of a large deletion, despite his normal karyotype. All six individuals with cytogenetically visible deletions of 2q37 had smaller molecular deletions than individual AHO/050/609 appeared to have. In addition, this individual was not deleted for D2S90 whereas other (micro)deletion individuals were, which made this individual crucial in determining whether D2S90 was localised within the commonly deleted region.

5.4 Cryptic translocation in a large Dutch family

Individual AHO/054/622 (with normal karyotype and unknown Gsα levels) who has a paternally derived microdeletion on 2q37, is a member of a large five generation Dutch family with 14 affected members (individual V11, fig. 5.3a). The presence of numerous affected individuals, all with apparently unaffected parents suggests the segregation of a balanced translocation within this family.

A balanced translocation is a transfer of material between chromosomes. While no genetic material is lost or gained with the exchange, the individual is at risk of producing chromosomally unbalanced offspring with a deletion of one region of the genome and triploidy of another region upon segregation of the chromosomes at meiosis (fig. 5.3b). The translocation is said to be cryptic when it is undetectable by normal karyotyping.

Genomic DNA from individual AHO/054/419 (V3, fig. 5.3a) and her parents was analysed for eight microsatellites. The mother was an assumed carrier of the unbalanced translocation and a deletion on the maternally derived chromosome was demonstrated (fig. 5.2). Genotype data of the parents of AHO/054/622 and AHO/054/419 produced no evidence for molecular rearrangements giving further support for the presence of a translocation in this family.

Independent cytogenetic investigation using fluorescence in situ hybridisation with a 2q probe confirmed the presence of a balanced translocation between the long arms of chromosomes 2 and 8 in the father of individual AHO/054/622 (IV10, fig. 5.3a) (Dr. R. Hennekam (Institute of Human Genetics, Department of Clinical Genetics, University of Amsterdam, Amsterdam, The Netherlands), personal communication). The translocation is designated t(2:8)(q37.3:qter).
Figure 5.3 Translocation in a large Dutch kindred

a) Pedigree of a large Dutch family with multiple members affected with AHO. Individuals V3 and V11 (AHO/054/419 and AHO/054/622 respectively) are shown to have a microdeletion in the 2q37 region consequence of an unbalanced translocation. Individual IV10 (AHO/054/405) is the carrier of a balanced translocation between the long arms of chromosomes 2 and 8, designated t(2:8)(q37.3:qter).

b) Schematic representation of the quadrivalent formed at meiosis. Segregation of the chromosomes can lead to a balanced or unbalanced translocation in the gametes.
a) 

I
II
III
IV
V

AHO/054/419
AHO/054/622

meiotic quadrivalent

b) 

2

der(8)

der(2)

balanced translocation in gamete segregating chromosomes 2+8 and der(2)+der(8)

unbalanced translocation in gamete segregating chromosomes 2+der(2) and 8+der(8) or 2+der(8) and 8+der(2)
5.5 Paternity testing

Individuals AHO/054/622, del2/001/198 and del2/003/389 (fig. 5.2) had paternally derived deletions which were crucial in determining the proximal endpoint of the deletion. Paternity was tested in the last two individuals whilst paternity in individual AHO/054/622 was known to be correct based on the presence of a balanced translocation in the father. Two further individuals with paternally derived deletions (individuals del2/002/394 and del2/006/404) did not need testing for correct paternity as they were irrelevant in determining the proximal endpoint of the deletion. Genotypes were determined using microsatellite markers D6S273, D11S903 and D20S93 (table 2.2) with observed heterozygosities of 77%, 75% and 100% respectively. All three markers were used for individual del2/001/198 and the last two for individual del2/003/389. No evidence of incorrect paternity was found and therefore the identified deletions are likely to be the result of a de novo deletion on the paternally derived allele.

Individual AHO/050/609 with an apparent paternally derived deletion, was not deleted for D2S90 and therefore was important to establish if D2S90 was localised within the commonly deleted region. Paternity was tested using microsatellites D6S273, D11S903, D20S93 and a microsatellite with a heterozygosity of 63% present in the elastin gene (ELN). Informative data were obtained for D6S273, D11S903 and D20S93 whereby the individual had inherited one allele maternally whilst the other allele did not correspond with any of the two alleles of the father indicating incorrect paternity. As a result, the data regarding the extent of the deletion obtained for this individual were not considered further.

5.6 Probability of homozygosity against deletion in two Albright hereditary osteodystrophy individuals

Two individuals (AHO/012/11061 and AHO/050/609) were identified who were apparently homozygous for all eight of the microsatellites tested (D2S206, D2S331, D2S336, D2S338, D2S345, D2S125, D2S395 and D2S140). Incorrect paternity was shown in individual AHO/050/609 (5.5) whilst the father of individual AHO/012/11061 was unavailable for study.

A deletion in or uniparental disomy of this region of the genome could explain the observation of a single allele. Uniparental disomy was excluded in both individuals because they were heterozygous at the D2S211 locus, localised to chromosome 2q34-37 which is proximal to the analysed 2q37 microsatellites. The presence of a deletion cannot be confirmed by molecular analysis. Cytogenetic investigation will be required to determine true homozygosity from hemizygosity.
The probability that either individual was homozygous for all eight markers by chance alone was calculated. The first step was determining the size of the allele present at each of the eight loci. Subsequently, the population frequency of the allele with that size \((f_a)\) was obtained from the Genome Database (GDB). The probability of being homozygous for the allele present at each locus was determined by its frequency square \((f_a^2)\) according to the Hardy-Weinberg principle. The probability of being homozygous for all eight loci (assuming independent segregation of these loci) was calculated by multiplying \((f_a^2)\) for locus 1 with \((f_a^2)\) for locus 2 with \((f_a^2)\) for locus 3 and so on. The probability that individual AHO/012/11061 was homozygous for all eight markers by chance was \(3.88 \times 10^{-11}\) as calculated using the above method whilst the probability was \(1.2 \times 10^{-11}\) for individual AHO/050/609 and therefore it is very likely these individuals have a deletion in this region.

5.7 Discussion

The possibility of a second locus for AHO present on distal chromosome 2, through identification of two unrelated individuals with an AHO-like phenotype who had cytogenetically visible deletions of 2q37 (Phelan et al., 1993), was further investigated. A proportion of the individuals in our cohort had normal Gsa bioactivity and were considered candidates for a 2q defect as the Gsa gene is localised on chromosome 20. These individuals were screened for submicroscopic deletions as were individuals with a brachydactyly, mental retardation phenotype and normal karyotype. Deletions were identified in two individuals, one with AHO and normal Gsa bioactivity (AHO/010/10780) and one with AHO and unknown Gsa bioactivity (AHO/054/622) confirming a locus for AHO-like syndrome on chromosome 2q37. Two other AHO individuals with normal Gsa bioactivity (AHO/012/11061, AHO/050/609) are likely to have a deletion. However, no evidence for deletions was found in the 15 remaining individuals with normal karyotypes implying deletions are not a common mutation event leading to AHO-like syndrome. Furthermore, the molecular extent of the deletion was determined in individuals with cytogenetically detectable deletions of 2q37. Parental origin of the chromosomes with the deletions was established and showed paternal origin in 5 cases and maternal origin in 3 cases. No evidence for incorrect paternity was found in individuals whose deletions were critical in determining the smallest region commonly deleted.

The individuals with 2q37 deletions reported in this study have short stature, stocky build, round face, brachydactyly, and mental retardation in common. Thus far, 23 individuals with deletions involving 2q37 have been described, 20 of those in the last five years (Young et al., 1983; Sanchez & Pantano, 1984; Gorski et al., 1989; Coldwell et al., 1992; Lin et al., 1992; Stein et al., 1992; Haag et al., 1993; Waters et al., 1993; Fisher et
Two individuals had normal Gsα levels determined by immunochemistry (Phelan et al., 1995). Cytogenetic studies on individual del2/006/404 in this study have been described previously (Phelan et al., 1995). Frequently observed features include round face, short stature, stocky build, depressed nasal bridge, hypotonia and brachydactyly. Nearly half of the individuals reported are too young to confirm the presence of brachydactyly. Mental retardation was observed in all individuals. Other features observed are syndactyly, a condition characterised by soft tissue fusion between the digits of the hands and/or feet, in three individuals (Young et al., 1983; Sanchez & Pantano, 1984; Wang et al., 1994) and eczema in five individuals (Gorski et al., 1989; Fisher et al., 1994; Wilson et al., 1995). Syndactyly was generally associated with deletions extending proximal to 2q37 whilst eczema occurred in individuals with deletions of 2q37.

The microsatellites used for the study are ordered according to the current version of the Généthon genetic map (Dib et al., 1996) (fig. 5.2), constructed using the algorithm MultiMap (Matise et al., 1994). As genetic maps are based on statistical data, the order of markers on this type of map should be considered as the highest probable order. Locus order determined by linkage analysis can be confirmed by independent physical mapping. Low-resolution genetic maps (which contain relatively few markers) generally agree with physical maps but high-resolution genetic maps may show conflicting order because genotyping errors lead to a higher percentage of incorrect marker orders on high-resolution maps (NIH/CEPH Collaborative Mapping Group, 1992). Microsatellite markers in the 2q37 region are close together reflecting a high-resolution linkage map. Odds of 1000:1 are considered to give good support for a particular order of loci. However, the markers terminal of D2S336 are placed on the map with odds lower than 1000:1 which means that the order of the markers may be unreliable. No recombinations were observed in informative genotyped families for markers D2S338 and D2S345 and therefore their relative order could not be established. The same is true for markers D2S2285 and D2S2253. A Généthon physical map was recently generated covering about 75% of the human genome (Chumakov et al., 1995). Unfortunately the terminal region of 2q was not covered so the correct order of the distal markers could not be confirmed. No other physical maps of this region are available which enable ordering of the microsatellite markers. Comparison of the characterised deletions showed that the smallest region deleted in all individuals lies between microsatellites D2S2285/D2S2253 and D2S125 which are a genetic distance of 3 cM apart (fig. 5.2). The proximal endpoint of the critical region lies between markers D2S338/D2S345 and D2S2285/D2S2253, an 8 cM region. The terminal endpoint of the critical region could not be determined with any confidence because the most terminal marker, D2S2338 was found to be not deleted in AHO/054/419. Possible interpretations of these data include that the individual has not got a terminal deletion of...
2q but has an interstitial deletion, or the marker D2S2338 has been placed wrongly on the genetic map and should be placed proximal of D2S2285/D2S2253. Confirmation of either possibility awaits the generation of a physical map of this region of the genome or the identification of deletions in additional individuals with AHO-like features. Therefore as the position of the most distal marker on the map is unreliable, the terminal endpoint of the critical region has to lie between D2S125 and the telomere. No marker terminal of D2S125 apart from the D2S2338 marker, has been found which is not deleted in any of the individuals which makes it impossible to determine whether the AHO-like critical region is associated with an interstitial deletion or healing of a terminal deletion. Telomeres are essential structures present at the ends of chromosomes and broken chromosome ends need repairing to prevent fusion to other chromosomes and loss of terminal sequences. Repair of chromosome ends can be achieved by telomere repair or telomere capture. Telomere repair involving the de novo addition of telomere repeat sequences to the breakpoint of a terminal deletion on chromosome 16 associated with α thalassaemia has been reported (Wilkie et al., 1990; Lamb et al., 1993; Flint et al., 1994). This mutation was stably inherited demonstrating that telomeric sequences can stabilise chromosome ends. The repair mechanism is unclear but may be mediated by telomerase. Stabilisation of chromosome ends by acquiring telomeric sequences from another chromosome is called telomere capture (Meltzer et al., 1993; Flint et al., 1996). In this model, the donor chromosome would become unstable or participate in a secondary recombination event.

The common features of round face, short stature, depressed nasal bridge, stocky build, brachydactyly and mental retardation suggest that a gene (or genes) involved in skeletal morphogenesis or neurodevelopment lies within the region commonly deleted in individuals with AHO-like syndrome. The observations in the three groups of individuals screened for deletions on chromosome 2q37 suggest that AHO-like syndrome is caused by chromosomal deletions as no evidence was obtained suggesting that mutations in a single gene cause AHO-like syndrome. Deletions leave an individual with a single copy of a chromosomal region and certain genes localised in this deleted region may show haploinsufficiency. This term describes the inactivation of one of a pair of alleles leading to reduced gene dosage, expression or protein activity. By means of positional cloning, a number of genes have been identified which are haploinsufficient in chromosome deletion syndromes. These genes can be divided into two groups (Wilkie, 1994); genes encoding proteins that are subunits of multimere complexes in which stoichiometry could be important for function (including transcription factors and proteins involved in signal transduction), and genes that need a certain threshold level for appropriate action. Developmental pathways are particularly sensitive to levels of certain critical proteins as shown by experiments in Drosophila embryos. Appropriate levels are especially important for proteins involved in rate limiting steps. Not necessarily all genes in the deleted region show haploinsufficiency. Genes causing a recessive phenotype are unlikely to contribute to the phenotype associated with the deletion syndrome. If AHO-like syndrome was caused
by deletion of a number of contiguous genes, a gene responsible for brachydactyly type E would be expected amongst these genes.

Possible candidate genes can be divided into two groups; those encoding proteins acting via the Gsa signal transducing pathway and those acting through alternative pathways. Defects in genes encoding proteins of the Gsa pathway may give rise to a similar phenotype as defects in Gsa. This is plausible because the hormone responsive form of AHO (PPHP) is very difficult to distinguish from AHO-like syndrome. However, subcutaneous calcifications have never been observed in AHO-like syndrome whilst they may occur in PPHP, although not always. The difference in Gsa bioactivity is really the only clear distinction that can be made. Therefore, genes encoding proteins such as adenylyl cyclase, cAMP phosphodiesterase or G protein coupled receptors should be considered candidates for AHO-like syndrome. Genes for seven types of adenylyl cyclase have been identified and mapped. Each is localised on a different chromosome however none of the genes has been localised to 2q (Stengel et al., 1992; Gaudin et al., 1994).

Genes for two G protein coupled receptors are localised to distal 2q. The vasointestinal peptide receptor, encoded by RDC1 was mapped to 2q37 by chromosomal in situ hybridisation with a cDNA clone isolated from a human cDNA library (Libert et al., 1991). The 5-hydroxytryptamine receptor 2B, encoded by HTR2B, was mapped to 2q36.3-q37.1 also by chromosomal in situ hybridisation (Le Coniat et al., 1996) and mediates many physiological functions of serotonin, a neurotransmitter.

Unrelated pathways involving developmental genes could also explain the observed phenotypic abnormalities in AHO-like syndrome. The homeobox containing gene GBX2 gene was mapped to chromosome 2q37 by fluorescence in situ hybridisation (Matsui et al., 1993). Homeobox containing genes encode sequence specific DNA-binding transcription factors. Proteins like these are important in development. Mutations in the homeobox containing PAX3 gene can cause Waardenburg syndrome type I (MIM 193500) (Tassabehji et al., 1995) and the skeletal malformation campomelic dysplasia (MIM 211970) can be cause by mutations in the SOX9 gene which belongs to a family of DNA-binding proteins (Kwok et al., 1995). A lot of information relating to embryonic development comes from studies on the fly Drosophila Melanogaster. It is of interest that recently by comparison of Drosophila mutant genes with human cDNAs, a cDNA localised to chromosome 2q37 was identified which was homologous to the twist mutant (Banfi et al., 1996). The sequence of this cDNA corresponds to the twist-related protein which is a basic helix-loop-helix transcription factor essential for head mesenchyme formation and acting as an inhibitor of muscle differentiation (Fuchtbauer, 1995). Human cDNAs homologous to Drosophila mutants have revealed a gene which is localised in the smallest region deleted in individuals with DiGeorge syndrome (MIM 188400) and expressed in the affected tissues emphasising the usefulness of comparative genetics (Pizzuti et al., 1996).
Recently, the gene encoding the protein called SHIP (for SH2-containing inositol phosphatase) (Damen et al., 1996; Lioubin et al., 1996) was localised to chromosome 2q36-37 (G.Krystal (Terry Fox Laboratory, British Columbia Cancer Agency, University of British Columbia, Vancouver, Canada), personal communication). SHIP is a signal transduction protein involved in a new growth factor-receptor signalling pathway in hematopoietic cells. Genes related to SHIP, each involved in the metabolism of phosphatidylinositol polyphosphates, can cause human disease including Lowe oculocerebrorenal syndrome (MIM 309000) (Zhang et al., 1995) and ataxia telangiectasia (MIM 208900) (Savitsky et al., 1995). Further genes mapped to chromosome 2q37 include COL6A3 (Weil et al., 1988), HDLBP (high-density lipoprotein-binding protein) (Xia et al., 1993), SAG (S-antigen) (Valverde et al., 1994), PDCD1 (programmed cell death 1) (Shinohara et al., 1994), glucagon (pancreatic hormone) (Schroeder et al., 1984), VGL (vigilin) (Plenz et al., 1994), ALPI (intestinal alkaline phosphatase) (Wu et al., 1993) and ALPP (placental alkaline phosphatase) (Raimondi et al., 1988).

Chromosome 2q37 deletions have not been detected as a common mutation event responsible for AHO-like syndrome. This may be due to smaller mutations not identified by deletion mapping or alternatively by mutations in other regions of the genome. One region of interest is on the long arm of chromosome 15. A mother and her daughter, both affected with AHO were found to have a cytogenetic deletion of chromosome 15q11-13 (Hedeland et al., 1992). Deletions in this region are also associated with Prader-Willi and Angelman syndrome. A further region associated with AHO is on the short arm of chromosome 5p. An individual with PHP1 who also had features of cri du chat syndrome (MIM 123450), localised to chromosome 5p, was found to have a deletion of this region (Kee et al., 1976). These two chromosomal localisations may be involved in those AHO individuals who have no defect on chromosome 2q37.

The deleted region common to all AHO-like individuals is larger than 3 cM. The number of genes that may be present in this region is likely to be large and limit the investigation of their role in AHO-like syndrome individually. Therefore it is important to redefine the size of the commonly deleted region. A region smaller than 3 cM may be identified by deletion mapping in additionally ascertained individuals with the AHO features brachydactyly and mental retardation or by screening the currently available individuals with newly characterised markers on 2q37. It is essential that a physical map of chromosome 2q37 should be generated which will be helpful in characterisation of additional polymorphic markers in this region. Furthermore, the presence or absence of expressed sequence tags (ESTs) in the commonly deleted region can be established using a physical map. ESTs expressed in the tissues affected in AHO-like syndrome (brain and bone) can then quickly be screened. When present in the commonly deleted region, the genes associated with these ESTs become candidate genes involved in AHO-like syndrome.
Chapter 6: Exclusion of linkage between isolated autosomal dominant brachydactyly type E and candidate regions 2q37 and 20q13

6.1 Preface

The term brachydactyly refers to shortening of the digits due to anomalous development of any of the phalanges or metacarpals. Bell (Bell, 1951) reviewed 124 pedigrees containing 1336 individuals with this malformation and defined seven distinct groups; A1, A2, A3, B, C, D and E. Brachydactyly type E (BE) (MIM 113300) is characterised specifically by one or more short metacarpals and/or metatarsals. A lack of symmetry and lack of shortening of corresponding bones of the hands and feet are common together with variable expression within and between families. BE has been observed as an isolated malformation (Brailsford, 1945; Newcombe & Keats, 1969; Cartwright et al., 1980) as well as part of distinct syndromes including Turner syndrome (Poznanski et al., 1977), Albright hereditary osteodystrophy (AHO) (Albright et al., 1942; Albright et al., 1952), Biemond syndrome (Biemond, 1934) and Ruvalcaba syndrome (Ruvalcaba et al., 1971). Previous studies (Wilson et al., 1995; chapter 5, this study) revealed chromosome 2q37 as a candidate region for BE based on the presence of (micro)deletions in five patients with short metacarpals and metatarsals, mental retardation and other dysmorphic features similar to those seen in AHO. The aim of this study was to evaluate these chromosomal loci as possible candidate regions for familial BE, and in particular to assess whether (micro)deletions on chromosome 2q37 might represent a contiguous deletion syndrome involving deletion of a number of genes, including the gene responsible for BE.

6.2 Ascertainment of a family with isolated autosomal dominant brachydactyly type E

A large pedigree with isolated autosomal dominant BE was described by Brailsford (Brailsford, 1945). Ten affected members in three generations of this family were studied by radiography. Dr. D. Williams (Department of Clinical Genetics, Leicester Royal Infirmary, Leicester) recontacted surviving members of this family (fig. 6.1), examined seven individuals of whom five were affected and took blood samples for DNA isolation from eight individuals. Shortening of metacarpals and metatarsals was observed in each of the examined individuals. Other congenital malformations, short stature and mental retardation were not observed.
Figure 6.1 Pedigree of a family with autosomal dominant brachydactyly type E

Individuals IV:1, IV:4 and all individuals of generation V were added to the pedigree originally described by Brailsford (Brailsford, 1945). Below each examined individual, the number of the affected digits is stated (fig. 1.1). H3,4;3 means short 3rd and 4th metacarpal of the left hand and short 3rd metacarpal of the right hand. F3,4,5;4,5 means short 3rd, 4th and 5th metatarsal of the left foot and short 4th and 5th metatarsal of the right foot.
6.3 Genotype analysis of polymorphic markers on chromosomes 2q and 20q in a family with isolated autosomal dominant brachydactyly type E

Molecular analysis was performed in eight members of the above mentioned family with BE. For chromosome 2q analysis, six microsatellite markers (D2S125, D2S140, D2S395, D2S338, D2S345 and D2S336) and a single variable number of tandem repeat (VNTR) marker (D2S90), were used. Primer sequences are described in table 2.2. Genotypes were determined empirically as follows (fig. 6.2); the total number of alleles in the family was determined and each allele was given a number, beginning with 1 for the smallest allele. Genotypes for chromosome 20 markers were determined using an endonuclease restriction site (FokI) polymorphism in exon 5 of the Gsα gene (Gejman et al., 1991), a dinucleotide repeat in intron 3 of the same gene (Granqvist et al., 1992) and a tetranucleotide repeat (D20S93) which maps to a maximum distance of 4 cM distal to the Gsα gene (Melis et al., 1993). Genotypes for the last two markers were determined as described above and genotypes for the FokI polymorphism were determined by designating the allele carrying the restriction site as allele 1 and the allele without the restriction site as allele 2.

Once genotypes were determined, haplotypes were inferred. The results of the 2q study are shown in figure 6.3a. Direct inspection showed that no common haplotypes were shared by all affected individuals, excluding linkage of autosomal dominant BE in this family to this region of the genome. The results of the 20q study are shown in figure 6.3b. The family was uninformative for both polymorphisms in Gsα and fully informative for the flanking marker D20S93. Direct inspection revealed that no common haplotypes were shared by all affected individuals and the data therefore exclude the Gsα gene in the development of the BE phenotype in this family.

6.4 Discussion

Two candidate loci were studied because of the known association of BE with AHO, caused by mutations in the Gsα gene on chromosome 20q13 and an AHO-like syndrome defined by (micro)deletions at chromosome 2q37. The results showed no evidence of linkage between BE in the investigated family and the two regions of the genome. Other syndromes featuring BE include Biemond syndrome (Biemond, 1934) and Ruvalcaba syndrome (Ruvalcaba et al., 1971) and their localisation provide further candidate loci to be considered for BE.

AHO-like syndrome could be a contiguous deletion syndrome involving the gene responsible for BE. Criteria for the identification of a contiguous deletion syndrome are: a correlation between the extent of the deletion and the phenotype, mapping of more than one disease gene in the deleted region of the chromosome and mapping and cloning of
Figure 6.2 Genotype analysis of the microsatellite marker D2S336

Autoradiograph showing the different sized alleles at the D2S336 locus present in the examined individuals of the family with autosomal dominant brachydactyly type E. Alleles are numbered according to size starting with 1 for the smallest allele.
Figure 6.3 Haplotypes for the 2q and 20q markers

a) Haplotypes for the 2q markers in seven examined individuals. The numbers given for individual IV:3 indicate DNA results and not haplotypes.

b) Haplotypes for the 20q markers in the same seven individuals. The numbers given for individual IV:3 indicate DNA results and not haplotypes.
a) Markers Alleles
D2S336 1,2,3,4,5
D2S345 1,2
D2S338 1,2,3
D2S395 1,2,3,4
D2S14 0,1,2,3
D2S125 1,2,3,4,5
D2S90 1,2,3,4

b) Markers Alleles
D20S93 1,2,3,4,5,6,7
GNASin3 1,2
GNASex5 1,2
each gene responsible for part of the observed phenotype (Ballabio, 1991). Although previous data (Wilson et al., 1995; chapter 5, this study) suggest a correlation between the extent of the deletion and the phenotype (brachydactyly E and mental retardation are present in all patients with a (micro)deletion), the data presented here give no evidence of support for AHO-like syndrome being a contiguous deletion syndrome.

Homeobox containing genes (HOX genes) are implicated in the control of distal limb development (Redline et al., 1992) and should be considered candidate genes for BE. Recently, mutations in the HOXD13 were shown to cause altered growth and branching patterns in individuals affected with synpolydactyly which is characterised by webbing between fingers and duplication of fingers (Muragaki et al., 1996). Another group of candidates include growth factor genes expressed during limb development and which may have stimulatory or inhibitory effects on limb growth and patterning (Redline et al., 1992; Niswander & Martin, 1993). The genes HOXD, MSX1 and MSX2 (previously called HOX7 and HOX8) and fibroblast growth factor (FGF)-1 and -2 have been excluded in two families with brachydactyly type A1 (MIM 112500), characterised by shortening of the middle phalanx of all digits in hands and feet (Mastrobattista et al., 1995). These genes should be considered in other types of brachydactyly. Further candidate genes include Gdf5, BMP-2 and FGF-8. Growth/differentiation factors (GDFs) are related to known bone- and cartilage-inducing molecules, the bone morphogenetic proteins (BMPs) (reviewed in Rosen & Thies, 1992). Mutations in Gdf5 are known to cause the mouse brachypodium (bp) phenotype (Storm et al., 1994). Skeletons of mice homozygous for a Gdf5 mutation showed that the long bones of the limb and the feet were shorter than controls. Additional defects included reduced length of metacarpals and metatarsals. BMP-2 can interfere with limb growth by counteracting the growth promoting effect of FGF-4 (Niswander & Martin, 1993) and FGF-8 is expressed in developing limbs in regions that direct outgrowth (Crossley & Martin, 1995). Elucidation of the genes involved in the various types of brachydactyly may help to understand the molecular mechanisms involved in limb development.
Chapter 7: Concluding remarks

At the commencement of this study, data were beginning to emerge that contributed to our understanding of the molecular basis of AHO. Reduced levels of the α subunit of the stimulatory G protein (Gsa) were observed in individuals with AHO with and without hormone resistance (PHP1a and PHP1b respectively). Deactivating mutations in the gene encoding Gsa (GNAS1) were shown to cause PHP1a and PHP1b and the gene was localised to the chromosomal region 20q13. However, it became clear that within a single kindred, identical mutations could cause both PHP1a and PHP1b. It was therefore necessary to consider possible explanations for this phenomena. A survey of published familial cases of AHO revealed that maternal transmission exclusively lead to PHP1a and paternal transmission to PHP1b. Genomic imprinting of GNAS1 was suggested as a possible mechanism to explain these observations.

Individuals affected with AHO were screened for mutations in exons 2-13 of GNAS1 using PCR and DGGE and an assay was devised to screen exon 1 using PCR and direct sequencing. In addition to the six mutations characterised before this study began, a total of 27 heterozygous mutations were identified of which 25 were unique. The mutations were distributed throughout the gene although 7/27 (26%) were present in exon 1. Clustering of mutations in this part of GNAS1 was unknown because this exon was not screened for mutations previously by any other group that reported GNAS1 mutations. A relatively common 4 bp deletion (present in seven kindreds, two identified in this study) represents a hotspot in exon 7 of this gene. The identified mutations comprised eight single base pair substitutions predicted to lead to amino acid substitutions and nineteen mutations predicted to lead to truncated Gsa proteins. The use of exonic polymorphisms in the recognition sites of restriction endonucleases enabled the identification of the parental source of the mutated allele in ten affected individuals. All cases supported the hypothesis that maternally derived GNAS1 mutations lead to PHP1a and paternally derived GNAS1 mutations lead to PHP1b.

Analysis of the expression of GNAS1 mRNA from hormone responsive tissues (renal proximal tubules, thyroid and osteoblasts) demonstrated that tissue-specific imprinting of GNAS1 does not occur and therefore cannot explain the phenomena of a parental effect in the aetiology of AHO.

In addition to the analysis of the GNAS1 locus on chromosome 20q13, this study has identified a second locus for AHO (AHO-like syndrome) on chromosome 2q37 by deletion mapping. AHO-like syndrome could represent a contiguous deletion syndrome although no support has been found for the presence of a locus for brachydactyly type E (one of the characteristic features of AHO) in this region of the genome.
There remain many unanswered questions concerning the genetics of AHO. A large number of GNAS1 mutations have now been identified in individuals with AHO, although not in all individuals with Gsα deficiency who were studied. It was beyond the scope of this study to develop techniques for the analysis of the promotor region of GNAS1, the structure of which precludes all standard mutation detection techniques. The nature of the control mechanism of GNAS1 expression remains unknown and it may be that defects in such are responsible for reductions in the quantity of Gsα protein.

Further investigation into the expression of hormone responsiveness is required to explain the observed parental effect. The possibility of genomic imprinting at the cellular level should be considered, although practical difficulties may be encountered that will require the development of highly sensitive assays.

The second locus for AHO on chromosome 2q37 requires further investigation. The nature of the defect, single gene defect or contiguous deletion syndrome, will need to be established and the gene(s) responsible identified and characterised.

Furthermore, defects in other regions of the genome should be considered in individuals with AHO who have no abnormalities in GNAS1 or on chromosomes 2q37.
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Matsui, T., Hirai, M., Hirano, M. & Kurosawa, Y. (1993) The HOX complex neighbored by the EVX gene, as well as two other homeobox-containing genes, the GBX-class and the EN-class, are located on the same chromosomes 2 and 7 in humans. FEBS Letters, 336, 107-110.


Appendices: publications including work presented in this thesis

A  Genomics (1994) 21, 455-457
B  Journal of Medical Genetics (1994) 31, 835-839
E  Journal of Medical Genetics (1996) 33, 873-876
Appendix A


reprint
Characterization of a de Novo 43-bp Deletion of the Gsα Gene (GNAS1) in Albright Hereditary Osteodystrophy

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Received November 9, 1993; revised February 20, 1994

Albright hereditary osteodystrophy (AHO) is an autosomal dominant disorder characterized by short stature, obesity, mental retardation, subcutaneous calcification, and brachymetaphalangia. Two distinct forms of AHO exist: pseudohypoparathyroidism type I (PHPI) and pseudopseudohypoparathyroidism (PPHP). The classification is dependent upon the presence or absence, respectively, of resistance to parathyroid and other hormones that bind to Gs-protein-coupled membrane receptors stimulating adenylyl cyclase. Gs is a heterotrimeric protein comprising α, β, and γ subunits encoded by separate genes. Reduced activity of the α subunit of Gs has been found in patients with AHO; single basepair substitutions (8, 9, 11) and deletions up to 4 bp (8, 11, 13). However, large deletions or gene rearrangements within or including the Gsα locus have not previously been identified (2, 7, 8).

Genomic DNA was isolated from peripheral leukocytes from 13 unrelated AHO patients. Exon 4 and flanking intronic sequence of GNAS1 were PCR amplified under the following conditions. A reaction mixture containing genomic DNA, deoxynucleotides (200 μM each), forward and reverse oligonucleotide primer (1 μM each), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl2, and 1 U Taq DNA polymerase (Applied Biotechnologies) was heated for 5 min at 93°C, followed by 35 cycles of 93°C for 1 min and 58°C for 30 s and a final primer extension of 2 min at 70°C. The PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized using UV light. Sequences for the oligonucleotide primers are given in the legend to Fig. 1. Gel-purified fragments were dissolved in 10 μl of H2O and sequenced using a modified cycle sequencing method. Asymmetric PCR was performed using 1 μl of DNA template, 75 μM deoxynucleotides, and 1 μM forward or reverse primer for 15 cycles as above. The product was precipitated, redissolved in 5 μl of H2O, and cycle sequenced using primers endlabeled with [γ-32P]dATP (Amersham) and DNA Tag polymerase. The products were mixed with an equal volume of formamide loading dye (95% formamide and 20 mM EDTA), heat-denatured, and run on an 8% denaturing poly

FIG. 1. PCR-amplified fragments encompassing exon 4 from 10285 and both unaffected parents (10286 and 10287). The sequences of the oligonucleotide primers used for amplification of exon 4 are 5’-GTACGTACCCAGTACTCCTAATCTGACATTG 3’ (forward primer) and 5’-GCTACAAGACAGACAGACAGCAGCAAG 3’ (reverse primer). The first lane is size marker φX174/HaeIII. The normal size fragment of 159 bp was present in all three individuals, while 10285 has an additional 116-bp band.

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Mutations in the Gsa subunit genes have been reported in several human genetic disorders and malignancies. Patients with McCune-Albright syndrome, some somatotrophinomas and thyroid hyperplasia, express constitutively activated Gsa subunits due to substitution of the amino acid residue Arg<sup>201</sup> or Gln<sup>207</sup> (6, 12). In contrast, in AHO heterogeneous missense mutations of Gsa that lead to a reduction in Gsa bioactivity have been reported. We predict that the 43-bp deletion would lead to a premature stop codon, 62 codons downstream of the deletion.

The de novo mutation reported here is the largest deletion in the Gsa gene described so far for AHO patients. It is of interest that at the breakpoints of the deletion, there is a 4-nucleotide homology GTGC (Fig. 2); hence, the exact position of the deletion cannot be defined. Repeated sequence at deletion boundaries has previously been observed (10) and may predispose to deletion events through unequal crossover. However, this repeat is unlikely to represent a deletion hot spot within the Gsa gene, as it has not been previously reported or observed in our cohort of affected individuals. Despite the large deletion, this patient has the typical PHP1a phenotype. The molecular mechanisms underlying the form of AHO, either PHP1a or PHP2, remain unclear. Recently, tissue-specific imprinting of GNAS1 has been proposed, based upon the sex of the transmitting parent in families with AHO (3). However, we have been unable to assign parental origin for this mutation, as the family is uninformative for all expressed polymorphisms tested (4).

ACKNOWLEDGMENTS

We thank Dr. L. Weinstein for providing sequence data for oligonucleotides. Cyc S49 mouse lymphoma cell membranes were kindly donated by Dr. C. Van Dop. L.C.W. is an MRC (UK) Training Fellow, and support from the Research Trust for Metabolic Diseases of Childhood is gratefully acknowledged.

REFERENCES


Appendix B

Parental origin of Gsα gene mutations in Albright's hereditary osteodystrophy (1994)
*Journal of Medical Genetics*, 31, 835-839.

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Parental origin of $G_s\alpha$ gene mutations in Albright's hereditary osteodystrophy

Louise C Wilson, Monique E M Oude Luttikhuis, Peter T Clayton, William D Fraser, Richard C Trembath

Abstract

Heterozygous mutations of the $G_s\alpha$ gene leading to reduced $G_s\alpha$ activity have been identified in patients with Albright's hereditary osteodystrophy (AHO). However, AHO may be associated with hormone resistance (pseudohypoparathyroidism type Ia, PHPIa) or a normal response (pseudopseudohypoparathyroidism, PPHP). As both disorders may occur within the same family, the relationship between $G_s\alpha$ genotype and phenotype remains unresolved.

The AHO phenotype may be dependent upon the sex of the parent transmitting the $G_s\alpha$ mutation, perhaps through a gene imprinting mechanism. We have used an intragenic $G_s\alpha$ FokI polymorphism to determine the parental origin of $G_s\alpha$ gene mutations in sporadic and familial AHO. We now show that a de novo G→A substitution at the exon 5 donor splice junction in a child with PPHP was paternally derived. Furthermore, in a female with PPHP, the $G_s\alpha$ abnormality was shown to be of paternal origin, while subsequent maternal processing and transmission resulted in PHPIa in two offspring. As transmission of PPHP has rarely been reported, determining parental origin of the disease allele in sporadic cases may provide insight into the mechanism of hormone resistance in AHO.

(J Med Genet 1994;31:835-839)

Albright's hereditary osteodystrophy (AHO) is an autosomal dominantly inherited syndrome of short stature, obesity, round face, brachymetaphalangism, subcutaneous ossification, and variable mental retardation. Many subjects with AHO have associated pseudohypoparathyroidism type I (PHPI), failing to respond normally to exogenous PTH and phosphate excretion after exogenous PTH. Resistance to other adenylyl cyclase stimulating hormones including TSH, LH, and FSH is frequently also observed. The remaining subjects, who respond normally to exogenous PTH, have pseudopseudohypoparathyroidism (PHPH)." Upon extracellular binding of many hormones to their specific receptors, intracellular heterotrimERIC G protein is activated. G proteins comprising $\alpha$, $\beta$, and $\gamma$ subunits can either stimulate (Gs) or inhibit (Gi) the formation of cAMP through adenylyl cyclase. The majority of patients with PHPH have a 50% reduction in $G_s\alpha$ levels in membranes from various tissues including erythrocytes and renal cortex and these patients are classified as PHPIa. Interestingly, levels of $G_s\alpha$ activity are similarly reduced in patients with PPHP.

The human $G_s\alpha$ gene has been mapped to chromosome 20q13, is 20 kb in length, and has 13 exons. Heterozygous $G_s\alpha$ gene mutations, either single base pair substitutions or small deletions of up to 43 bp, have been identified in patients with AHO. Within five separate kindreds, related persons with PHPIa and PPHP have been observed to cosegregate with the same $G_s\alpha$ mutation. Hence, molecular heterogeneity of $G_s\alpha$ mutations is not sufficient to explain the phenotypic difference in hormone responsiveness seen in PHPIa and PPHP.

A recent review of published familial cases of AHO suggests that parental origin of the mutated allele may influence phenotype since 66/66 offspring with PHPH resulted from maternal transmission and 6/6 cases with PPHP resulted from paternal transmission. Such effects may be the result of genomic imprinting. Extensive studies in the mouse have led to identification of chromosome regions involved in imprinting. The mouse homologue of $G_s\alpha$ (GNAS) has been localised to distal chromosome 2 (2E1-2H3), a region known to show imprinting effects. In a further series of four families, we have noted phenotype concordance in all nine sibs, as predicted by an imprinting hypothesis (L Wilson, unpublished observations). A single instance of paternal transmission to an offspring with PHPH has recently been described, but the $G_s\alpha$ mutation in this family has not been characterised.

In view of the paucity of reported male transmissions of AHO, we sought to test these observations in two persons with sporadic PPHP using a coding sequence polymorphism in order to determine the parental origin of the $G_s\alpha$ disease alleles.

Subjects and methods

PATIENTS

AHO 6 (II-1) (fig 1), is a 14 year old girl with short stocky build (height < 3rd centile, weight 50th centile), a round face, subcutaneous calcification, marked brachymetaphalangism, developmental delay, and grand mal fits. Her only medication is Carbamazepine. She was born after an uneventful pregnancy at 37 weeks' gestation by emergency caesarian section for fetal distress and footling breech presentation and weighed 2100g. The neonatal course was complicated by bilateral pneumothoraces. At 2 weeks she was noted to have
abnormal renal function (urea 6.7 mmol/l, NR 2.8–8.6; creatinine 122 μmol/l, NR 42–74; glomerular filtration rate 16 ml/min/1.73 m²).

Renal imaging by ultrasound, 99mTc diethylene triamine penta-acetic acid (DTPA) scan, intravenous urogram, and cystogram was normal.

At 14 years of age, all serum calcium levels had been normal but her renal function remained impaired complicating assessment of her endocrine status. Serum calcium (7.7 mmol/l, NR 2.2–2.58), Mg 0.52 mmol/l (NR 0.78–1.65), basal intact PTH 53 ng/l (NR <102), and ovariectomy Gsa bioactivity was reduced to 76% of the normal control compared with 126% and 127% respectively in her clinically normal father and mother. These findings are compatible with a diagnosis of PPHP.

AHO 2 II-2 (fig 2) is a 22 year old female with short stature (<3rd centile), marked shortening of the fourth and fifth metacarpals and distal first phalanges, but no ectopic calcification. She is normocalcaemic (Ca 2.45 mmol/l, NR 2.22–2.58), Pi 0.88 mmol/l (0.9–1.38), basal intact PTH 10 ng/l (NR <5–45) with normal urinary cAMP and phosphaturic responses to PTH (table). Currently, she is treated with Carbimazole for hyperthyroidism (free T3 16.5 pmol/l (NR <6–8), positive antithyroid microsomal antibody 1:100 000). Her erythrocyte Gsa activity was 14.2% compared with 27% in her clinically normal siblings. These findings are compatible with a diagnosis of PPHP.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Basal urinary cAMP/Cr</th>
<th>Peak urinary cAMP/Cr</th>
<th>Basal urinary Pi/Cr</th>
<th>Peak urinary Pi/Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHO 6, II-1</td>
<td>0.4</td>
<td>0.8</td>
<td>0.90</td>
<td>1.82</td>
</tr>
<tr>
<td>AHO 2, II-2</td>
<td>0.3</td>
<td>0.39</td>
<td>0.65</td>
<td>2.9</td>
</tr>
<tr>
<td>AHO 2, III-1</td>
<td>0.67</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

PTH infusion testing was performed using a bolus of synthetic PTH-(1-34) (Rorer pharmaceuticals) at 3 U/kg in 10 ml of diluent (Rorer pharmaceuticals). Basal levels were the mean urinary cAMP (μmol/l) to urinary creatinine (mmol/l) ratios and urinary phosphate (mmol/l) to creatinine ratios for two 30 minute urine samples taken in the hour before administration of PTH. Following PTH infusion urine samples were collected at 30 minute intervals for three hours and the maximum cAMP and phosphate to creatinine ratios achieved are recorded. Normal responses are a greater than six fold rise in cAMP/Cr and three fold rise in Pi/Cr.

Figure 1 The upper photograph shows the DGGE analysis of exon 5, identifying the FokI genotype and the sequence variation in subject II-1 from AHO 6. The lower photograph shows the FokI digests on PCR products of the same persons on a 2% agarose gel. The genotypes of the subjects are shown between the photographs. The sequences of the oligonucleotide primers used for amplification of exon 5 are: 5' CGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCCGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCAGCCAGTGCTGTTCTTGACC GG (forward primer) and 5' CCGTTATGGACATGCTTGCAGCG (reverse primer). Comparison of the two figures showed that homoduplex band 2 arises from the FokI (+) allele and homoduplex band 1 from the FokI (−) allele. The DGGE analysis also showed that the variant pattern in II-1 is present on the paternally derived allele.

Figure 2 Results of DGGE analysis of exon 5 in subject II-1. Bands 1 and 2 represent the homoduplexes from the (+) and (−) alleles. The symbol denotes the PHPHa phenotype and the symbol denotes the PHPHa phenotype.
reduced to 54%. These data support a diagnosis of PPHP. Her mother, I-2, is clinically normal with Gsa activity of 110%. Her dead father was of normal stature with no known endocrine disorder.

The proband's daughter, III-2, was induced at term. The neonatal period was complicated by the development of necrotising enterocolitis on the second day. Bradycardia was noted soon after birth and proved secondary to hypothyroidism (T4 47 mmol/l (NR 90-195), TSH > 30 mU/l (NR 0-5-6)) Thyroid microsomal antibodies were weakly positive. Transient hypocalcaemia (Ca 1-83 mmol/l (NR 2-2-2-6)) was documented. Now aged 4 years she has a round face, obesity, developmental delay, and brachymetaphalangism but no ectopic calcification. She has subsequently remained normocalcaemic but had a markedly blunted urinary cAMP and phosphate response to intravenous PTH (table). Erythrocyte Gsa levels are reduced to 60% of a normal control. These clinical and metabolic features are compatible with a diagnosis of PHP1a. The proband's son, III-1, also presented with hypothyroidism in the neonatal period. At 1 year he has a round face, stocky build, and developmental delay. He has remained normocalcaemic and has not been assessed by PTH infusion testing. His Gsa levels are reduced to 64% of a control.

MEASUREMENT OF GSÅ BIOACTIVITY
Two-five ml samples of venous blood in citrate anticoagulant were frozen on dry ice and stored at −70°C. Measurement of erythrocyte Gsa bioactivity was by cyc-reconstitution assay as described by Bourne et al and membranes were kindly provided by Dr C Van Dop. Puriﬁcation of [32P]cAMP was as described by Salomon et al using Dowex Alumina chromatography and [3H]cAMP to monitor recovery. Sample eluates were counted in 8ml of scintillation fluid (Universol ES) in a dual channel scintillation counter. Values are the mean of duplicates corrected for cAMP recovery and expressed as a percentage of the [32P]cAMP production in a concurrent sample from an unrelated healthy control.

PCR AMPLIFICATION OF GENOMIC DNA
Genomic DNA isolated from peripheral leucocytes was used for amplification in a 50μl reaction mixture containing deoxynucleotides (200μmol/l each), forward and reverse oligonucleotide primer (1μmol/l each), 50mmol/1 KCl, 10mmol/l Tris-HCl (pH 9-0), 0-1% Triton X-100, 1-5mmol/l MgCl2 and 1U Tag DNA polymerase (Applied Biotechnologies). Amplification consisted of denaturation at 93°C followed by 35 cycles of 93°C for one minute and 60°C for 30 seconds and a final primer extension of two minutes at 70°C. The PCR products were analysed on 2% agarose gels, stained with ethidium bromide, and visualised by UV light. Sequences for the oligonucleotide primers were obtained from L S Weinstein and are mentioned in the legend to fig 1.

DGGE ANALYSIS OF PCR AMPLIFIED DNA FRAGMENTS
PCR amplified DNA fragments were analysed by DGGE as described previously. The samples were subjected to electrophoresis for 5-5 hours at 150V and 60°C in an 8% polyacrylamide gel containing a gradient linearly increasing from 40 to 70% (100% denaturant is 7 mol/l urea and 40% (v/v) formamide). After electrophoresis, the DNA fragments were visualised by ethidium bromide staining.

DNA SEQUENCING
PCR amplified DNA fragments were sequenced using a PCR based assay. Gel puriﬁed fragments were dissolved in 10μl H2O. Asymmetrical PCR was performed using 1μl of the puriﬁed fragment, 75μmol/l deoxynucleotides, and 1μmol/l forward or reverse primer for 15 cycles at 94°C for 1 minute and 60°C for 1 minute. Sequences for the oligonucleotide primers were obtained from L S Weinstein and are mentioned in the legend to fig 1.
cycles as described, followed by another purification, after which the DNA was dissolved in 5 µl H2O. The generated template was subsequently used in cycle sequencing, using primers end labelled with [γ-32P]dATP (Amersham) and DNA Taq polymerase. The products were mixed with an equal volume of formamide loading dye (95% formamide and 20 mmol/l EDTA), heat denatured, and run on an 8% denaturing polyacrylamide gel at 50 W for two to four hours, followed by x-ray autoradiography.

Results
PARENTAL ORIGIN OF A G→A SUBSTITUTION IN AHO 6
Analysis of PCR amplified products encompassing exon 5 by DGGE detected sequence variation within this exon and genotypes were confirmed by FokI restriction analysis. Comparison of DGGE and restriction fragment analyses allowed assignment of homoduplex bands (fig 1). The father (I-1) and mother (II-2) of the proband (II-1) are homozygous negative and positive respectively for the diallelic FokI polymorphism. The proband was confirmed by FokI restriction fragment analysis to be heterozygous. However, on DGGE analysis she was found to have an altered banding pattern compared with all other heterozygotes indicating the presence of a DNA sequence alteration within this amplified fragment. Furthermore, the absence of a paternally derived homoduplex confirmed that the mutation had arisen on the paternal allele. Direct sequencing of amplified genomic DNA encompassing exon 5 from the patient II-1 showed a heterozygous G→A substitution (fig 3B) which was not present in either parent.

To confirm the parental origin of this Gsa mutation, the PCR amplified exon 5 fragment in II-1 was digested with FokI. The minus (−) allele was purified from an agarose gel and sequenced to show the presence of the mutation (fig 3C). The de novo origin of the mutation was confirmed through multilocus DNA fingerprinting for paternity (data not shown). The G→A substitution was not observed in 78 normal chromosomes.

PARENTAL ORIGIN OF THE MUTANT GSα IN AHO 2
PCR products encompassing exon 5 were subjected to DGGE. The analysis (fig 2) showed the family to be informative for the FokI polymorphism. Genotyping of affected subjects II-2, III-1, and III-2 showed cosegregation of the (−) allele with the reduced Gsa activity and the AHO phenotypes indicating that this allele carries the Gsa mutation. The grandmother I-2 is homozygous for the (+) allele hence the mutant allele is grandpaternally derived. Thus within one family a paternal Gsa mutation has resulted in the PPHP phenotype while subsequent maternal transmission has resulted in offspring with PHP1a.

Discussion
This is the first report showing the parental origin of a Gsa gene mutation in sporadic AHO. This is of particular interest in the light of the proposal that genomic imprinting may explain the alternative phenotypes, PHP1a or PPHP that result from identical Gsa mutations. Only six cases of paternal transmission have previously been reported. This may be because of reduced male fertility or alternatively a certain bias should these offspring have normal endocrine function. Under these circumstances, identifying the parental origin of Gsa mutations in sporadic PPHP cases is a valuable approach to testing this apparent parental effect on phenotype.

The proband in family AHO 6 has a classical AHO phenotype. The diagnosis ofPHP1a is based on her normal response to PTH infusion testing, normocalcaemia, normal thyroid function and reduced Gsa levels. While the presence of renal impairment in this subject would have caused difficulty in interpreting a blunted cAMP and phosphaturic response to exogenous PTH, it does not alter the significance of a normal response. We have identified a heterozygous G→A substitution within the consensus donor splice sequence at position +1 of intron 5 which is likely to result in loss of the donor splice site. In addition, we have shown that this mutation is paternally derived and has arisen de novo since paternity has been confirmed by DNA fingerprinting (data not shown). The FokI polymorphism results exclude the possibility of uniparental disomy.

In family AHO 2, subject II-2 has unequivocal PPHP based on her AHO phenotype, normal response to exogenous PTH, and reduction in erythrocyte Gsa levels. While the de novo nature of the AHO cannot be confirmed since her father is dead, an imprinted hypothesis predicts equivalent outcomes from a paternally derived de novo mutation and a paternal transmission. Hyperthyroidism is not a typical feature of AHO and strongly positive antithyroid microsomal antibody titres in II-2 supports a coincidental autoimmune aetiology. Interestingly both her offspring have presented with neonatal hypothyroidism, a feature which has only been described in PHP1a.55 The diagnosis has been confirmed in III-2 by a blunted response to exogenous PTH and the reduced erythrocyte Gsa levels. Our data show the "switch" in phenotype is dependent on the sex of the transmitting parent across two generations.

There is accumulating evidence that genomic imprinting plays a role in human disease. Genomic imprinting of the Gsa gene would not explain the clinical observations in AHO as equivalent reductions in Gsa bicarbonate activity are found in PHP1a and PPHP in all tissues tested to date.35-38 However, imprinting at a tissue or even cellular specific level could account for these findings. A simple model would require that cells such as renal tubular cells express only the maternal Gsa allele and other cells such as erythrocytes express paternal alleles. A mutation in the maternal allele would result in renal resistance to PTH. Elsewhere mutation in either parental allele would result in a 50% reduction in measured Gsa activity giving rise to the AHO phenotype. A patient...
Gsa mutation would not be expressed in the imprinted cells resulting in no observed PTH resistance and hence PHPH. Of note, the finding of Downs et al. that Gsa levels in the total renal cortical membranes of a patient with PHPH were 30 to 40% of controls is compatible with a cellular specific imprinting model. Tissue specific imprinting has been reported in the rat, where a gene paternal tissue is expressed in all embryonic tissues except the chord and leptomeninges where both parental alleles are expressed.

PTH responsive cells suitable for direct testing of a cell specific imprinting hypothesis are not readily accessible and no animal model of AHO has been identified. At present, evidence for imprinting is derived solely from clinical observation of parental effects and transmission has resulted in PHPH. Cla­

ifiersation of the nature of any parental effect on phenotype will be of particular significance for accurate genetic counselling in AHO as well as understanding the pathological mechanisms responsible for development of the disorder.

We are grateful to Mr Neil Periam for technical assistance and Dr Peter for help with DNA fingerprinting. LCW is an MRC (UK) clinical training fellow. This work was supported by a grant from the Research Trust for Metabolic Diseases of Childhood (RCT). Finally, we thank the families for their cooperation.

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Appendix C


reprint
Brachydactyly and Mental Retardation: An Albright Hereditary Osteodystrophy-like Syndrome Localized to 2q37


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Summary
We report five patients with a combination of brachymetaphalangia and mental retardation, similar to that observed in Albright hereditary osteodystrophy (AHO). Four patients had cytogenetically visible de novo deletions of chromosome 2q37. The fifth patient was cytogenetically normal and had normal bioactivity of the α subunit of Gs (Gsα), the protein that is defective in AHO. In this patient, we have used a combination of highly polymorphic molecular markers and FISH to demonstrate a microdeletion at 2q37. The common region of deletion overlap involves the most telomeric 2q marker, D2S125, and extends proximally for a maximum distance of 17.6 cM. We suggest this represents a consistent phenotype associated with some deletions at 2q37 and that genes important for skeletal and neurodevelopment lie within this region. Screening for deletions at this locus should be considered in individuals with brachymetaphalangia and mental retardation. Furthermore, 2q37 represents a candidate region for type E brachydactyly.

Introduction
The delineation of dysmorphic mental retardation syndromes on the basis of a constellation of physical and biochemical features has proved valuable in identifying individuals whose disorder has a common underlying etiology. In a number of these dysmorphic syndromes, the finding of patients with de novo cytogenetic abnormality has contributed to the localization and subsequent characterization of the underlying molecular genetic defect (Ewart et al. 1993; Reiner et al. 1993).
Patients and Methods

Patient 1 (KW)

KW is a 12-year-old female, the first child of healthy unrelated parents, born by breech delivery at 36 wk gestation, weighing 2.34 kg, with Apgar scores of 5 at 1 min and 8 at 5 min. All developmental milestones were delayed, and she now attends a special school, with severe learning difficulties. She has had intermittent grand mal seizures from the age of 5 years, despite normocalcemia throughout. Electroencephalography was consistent with primary generalized epilepsy. A computed-tomography head scan showed narrowing of the front of the skull, with a degree of craniosynostosis. From the age of 7 years, she rapidly gained weight, while remaining short in stature. Her bone age at 10 years was delayed, assessed by carpal index as 8 years. She has a left amblyopia secondary to strabismus and astigmatism.

Presently, her height is 126 cm (<3rd percentile), weight 36.4 kg (25th percentile), and occipitofrontal circumference (OFC) 50 cm (<3rd percentile). She has a round face, with low, flattened nasal bridge and dental crowding but no cutaneous or intracranial calcification (fig. 1, top). She has shortened fourth and fifth metacarpals with overlying knuckle dimples and shortened terminal phalanges (fig. 1, middle). Her feet are small and broad, with marked shortening of the third and fourth metatarsals (fig. 1, bottom). She has a moderately severe lumbar lordosis.

Investigations revealed that plasma calcium, phosphate, urea, creatinine, magnesium, vitamin D, and intact PTH were all within normal limits, as were thyroid function, thyrotropin-releasing hormone, and luteinizing-hormone-releasing-hormone (LHRH) tests. Response to exogenous PTH was normal, with a 90-fold rise in urinary cAMP/creatine. Gsa levels, the average from two separate assays, were 90%, 125%, and 99% of a healthy unrelated control, in the patient, father, and mother, respectively. Chromosomes were 46,XX normal female, with no visible deletion on 2q on prometaphase spreads.

Patient 2 (LC)

LC is a 12-year-old boy, born to healthy unrelated parents after a normal pregnancy and delivery, weighing 3.5 kg. All developmental milestones were delayed, and he now attends a special school, with moderate learning difficulties. He had surgery for strabismus at age 12 years. Flexural eczema and sparse white hair were present from birth, with subsequent intermittent hair loss and total scalp alopecia from 5 years of age. There is a paternal history of atopy.

Presently, he is prepubertal, with a height of 149.5 cm (50th percentile), weight 56.8 kg (97th percentile), and OFC 53 cm (25th–50th percentile). He has no scalp hair and sparse eyebrows and eyelashes, but his nails, teeth, and sweating are normal. He has a round face, prominent ears with large fleshy lobes, prominent columella, bilaterally

Figure 1  Top, KW at 12 years of age, showing round face, short neck, and small nose with low flattened nasal bridge. Middle, Plain radiograph of hand from KW at 12 years of age, illustrating both shortening of the distal phalanx of thumb and of fourth and fifth metacarpals and cone-shaped third metacarpal epiphysis. Bottom, Feet of KW, showing marked third- and fourth-metatarsal shortening.
Figure 2  Top. Hands of LC, demonstrating shortening of the fourth and fifth metacarpals and proximally placed thumbs. Middle, Fist of JD, illustrating knuckle dimples overlying the shortened third, fourth, and fifth metacarpals and the short distal phalanx of the thumb. Bottom, Feet of RA, showing marked third-, fourth-, and fifth-metatarsal shortening.

shortened fourth and fifth metacarpals (fig. 2, top), proximally placed thumbs, and small feet with short fourth and fifth toes.

Plasma calcium, albumin, urea, and creatinine were within normal limits, while phosphate was slightly raised, at 1.68 mM (normal range 0.8–1.44 mM). PTH was not tested. Thyroid function was normal. Karyotype analysis on peripheral lymphocytes was 46,XY,del (2)(q37), and parental chromosomes were normal.

Patient 3 (JD)

JD is a 14-year-old girl, the only child of healthy unrelated parents, born at term by emergency cesarean section for fetal distress, weighing 3.4 kg. She responded rapidly to resuscitation for profound bradycardia and apnea and had no further neonatal problems. All developmental milestones were delayed. She now has moderate mental retardation and major behavioral problems with aggression and self-mutilation. She has had eczema from infancy and has slow-growing fine scalp hair. Pubertal development has been normal.

Presently, her height is 150 cm (10th percentile), weight 50 kg (25th–50th percentile), and OFC 54 cm (50th percentile). She has a mild left ptosis, upslanting palpebral fissures, frontal bossing, prominent columella, and prominent large ears with fleshy earlobes. She has marked shortening of the third, fourth, and fifth metacarpals (fig. 2, middle) and third and fourth metatarsals bilaterally. Skeletal survey showed a thoracic kyphoscoliosis and bilateral coxa valga.

Investigation revealed normal renal function, corrected plasma calcium, phosphate, and intact PTH. Routine chromosomal analysis showed an abnormal 46,XX,del (2)(q37) karyotype. Parental chromosomes were normal.

Patient 4 (CH)

CH is a 20-year-old female, the youngest of three children, born uneventfully to healthy unrelated parents and weighing 4.09 kg. Severe eczema was present from the neonatal period. Developmental milestones were delayed, and poor height and weight gain were noted. Skeletal survey at this time showed a slightly advanced bone age, some epiphyseal irregularity, and flattened vertebrae T12 and L1 with anterior beaking.

Between 12 and 17 years of age, she received growth hormone for short stature, with an initial improvement in growth velocity. Detailed endocrine assessment at 20 years of age confirmed growth hormone deficiency, with maximal levels of 2.3 mU/L during insulin-induced hypoglycemia but a normal cortisol response. Thyroid function has remained normal. Menarche occurred at 14 years of age but with amenorrhea for the last 4 years. Gonadotrophin response to LHRH was normal but with undetectable estradiol and progesterone. Pelvic ultrasound demonstrated a small uterus, and the left ovary was visualized. Plasma calcium, phosphate, urea, creatinine, and intact PTH were all normal.

At 20 years of age, she has moderate learning difficulties. Her height is 139.7 cm (<3d percentile), weight 50 kg (10th–25th percentile), and OFC 54 cm (25th percentile).
She has very fine scalp hair, sparse eyebrows and lashes, flexural eczema, keratoconus, and minimal development of secondary sexual characteristics. Her face is round, with a beaked nose, prominent columella, and low-set prominent ears with fleshy earlobes. She has shortening of the first, fourth, and fifth metacarpals and of the first metatarsal, fourth, and fifth toes and a hypoplastic left ulna. Blood chromosome analysis showed 46,XX,del(2)(q37.1), while parental chromosomes were normal.

Patient 5 (RA) RA is a 15-year-old girl, the first child of healthy unrelated parents, born at 36 wk gestation, following a normal pregnancy and delivery, weighing 2.45 kg. Bilateral dislocating hips associated with acetabular dysplasia were noted soon after birth. Other skeletal abnormalities include dislocated radial heads, bowing of the radius and ulna, mild lumbar scoliosis, and joint laxity at the wrists, fingers, knees, and ankles. Additional findings on skeletal survey are mild dysplasia of the distal femoral epiphyses, metacarpal and metatarsal shortening, and a normal bone age. Dentition is normal, and there is no skin laxity. Myopia and an alternating divergent squint were noted at 2 years of age, but fundal examinations have been normal. Menarche was at 14 years of age. Developmental milestones were mildly delayed, and she currently attends a special school, with moderate learning difficulties. Her mother has myopia and short stature but no skeletal abnormalities.

Presently, her height is 135.6 cm (<3d percentile), weight 35.2 kg (<3d percentile), and OFC 53.2 cm (10th percentile). She has frontotemporal atrophy, deep set eyes, a flat midface, mild micrognathia, and prominent upper lip. Genetic marker studies, including serum calcium, were normal. Karyotyping of cultured lymphocytes showed 46,XX,del(2) (q37.2 or q37.3). Parental chromosomes were normal. The clinical features of these patients are summarized in table 1.

Measurement of Gsa Bioactivity

Between 2 and 5 ml of venous blood in citrate anticoagulant were frozen on dry ice and stored at −70°C. Measurement of syncthyocyte Gsa bioactivity was by cys-reconstitution assay, as described by Bourne et al. (1983), and membranes were provided by Dr. C. Van Dop. Purification of [32P]cAMP was as described by Gyapay et al. (1994), and annealing temperatures were as follows: AFM259yc9 (D2S206), AFM-182ya5 (D2S140) and AFM356te5 (D2S395), AEM275yf5 (D2S336), AFM269yd9 (D2S331), and AEM112yd4 (D2S125) were amplified by PCR, using standard methods. Primer sequences were as described by Gyapay et al. (1994), and annealing temperatures were as follows: AFM259yc9, 53°C; AFM182ya5, 56°C; AFM356te5, 62°C; AFM275yf5 and AFM269yd9, 60°C; and AEM-112yd4, 55°C. Samples were mixed with an equal volume of formamide denaturing buffer (95% formamide, 20 mM Tris-borate EDTA and 13 mM sodium sulfate, pH 8.0); 1 mM each of dATP, dCTP, dGTP, dTTP; 113 pg polymerase. Primer sequences were as follows: forward, 5' GGT GTA GAG CTC TGG TGA GTC AGG 3' (Buard and Vergnaud 1994); and reverse, 5' GGC CTT CTC CCT GT A ACC AGT TAC 3' (J. Buard, personal communication), and annealing temperature was 68°C.

Dinucleotide repeats AFM259yc9 (D2S206), and AFM182ya5 (D2S140) in KW

Using D2S90/CEBII in KW

Genetic Marker Studies

Genomic DNA from patients and their parents was prepared from peripheral blood lymphocytes or lymphoblastoid cell lines by standard methods. Markers CEBI1, D2S3, and D2S90 (CEB1) were typed by Southern analysis of unamplified DNA digested with MspI or HinfI (D2S90 and CEBI1) or with PstI (D2S3), using standard methods. D2S90 was additionally typed by Southern analysis of PCR product made by 23 cycles of amplification in a reaction volume of 22 μl containing 200 ng of genomic DNA, 1 μM each of forward and reverse primers, and 45 mM Tris HCl (pH 8.8); 11 mM ammonium sulphate; 4.5 mM MgCl2; 6.7 mM 2-mercaptoethanol; 4.4 mM EDTA (pH 8.0); 1 mM each of dATP, dCTP, dGTP, dTTP; 113 μg of BSA/ml; and 1 U of Taq polymerase. Primer sequences were as follows: forward, 5' GGT GTA GAG CTC TGC TGA GTC AGG 3' (Buard and Vergnaud 1994); and reverse, 5' GGC CTT CTC CCT GT A ACC AGT TAC 3' (J. Buard, personal communication), and annealing temperature was 68°C.

Metaphases were prepared from a lymphoblastoid cell line, by standard methods. The cosmid CEBI (containing the VNTR at D2S90) was labeled with biotin-11-dUTP by nick-translation using a concentration of DNAase I sufficient to produce labeled fragments 300–600 bp in length (Buckler and Rack 1993). One hundred nanograms of conjugated probe and 2.5 μg of competitor DNA were denatured in hybridization mix for 5 min at 55°C and preannealed for 10 min at 37°C. Slides were denatured at 70°C in 70% formamide and 2 × SSC, were washed in 2 × SSC, and were dehydrated through an alcohol series. Hybridization was carried out at 42°C overnight, and the
Table I

Summary of Clinical Features and Comparison among Subjects with AHO

<table>
<thead>
<tr>
<th>AHO Characteristic</th>
<th>KW (12)</th>
<th>LC (12)</th>
<th>JD (14)</th>
<th>CH (20)</th>
<th>RA (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short stature</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stocky build</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mild/moderate MR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Soft-tissue ossification</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brachymetaphalangia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Seizures</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endocrine:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHP: resistance to PTH and other hormones</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPHP: normal endocrine responses</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Strabismus</td>
<td>Eczema, fine hair, self-mutilation, thoracic kyphoscoliosis, coxa valga</td>
<td>Strabismus, eczema, sparse hair, alopecia, proximally placed thumbs</td>
<td>Deficient growth hormone, estradiol, and progesterone Eczema, sparse hair, hypoplastic ulna, keratoconus</td>
<td>Congenital dislocating hips, dislocated radial heads, bowed radius and ulna, joint laxity, myopia, strabismus</td>
</tr>
</tbody>
</table>

Note.—Subject ages (in years) are given in parentheses. A plus sign (+) indicates that a subject is positive for a characteristic, and a minus sign (−) indicates that a subject is negative for a characteristic.

slides were then washed in 2 × SSC for 10 min and in 0.1 × SSC for 40 min. Probes were detected by alternate layers of fluorescein isothiocyanate-conjugated avidin (5 μg/ml; Vector Laboratories) and biotinylated anti-avidin (5 μg/ml; Vector Laboratories). After a final wash in PBS, the slides were counterstained in antifade (Vector Laboratories) containing 0.5 μg of propidium iodide/ml. A confocal laser microscope (Biorad MRC 600) was used for image collection and analysis.

Results

Analysis of Microsatellite Markers

A total of 13 polymorphic markers mapping within 2q37 were analyzed, and the results are summarized in figure 3,
which summarizes the data and shows current ordering of the markers analyzed (Gyapay et al. 1994). All five patients have overlapping deletions within 2q37. Four have maternal deletions, and paternity therefore has not been tested. In patient RA, paternity has been confirmed by analysis at nine independent microsatellite loci. D2S338 marks the centromeric boundary of the common region of deletion. The proximal extent of the deletion cannot presently be defined for KW, within the 22-cM region between D2S125 and D2S336 (fig. 4). Similarly, no informative marker telomeric to D2S90 and D2S125 has yet been found.

**Analysis of D2S90 in KW**

Both parents were heterozygous for alleles at D2S90; however, only a single paternally derived allele was detected in KW. Owing to the high mutation rate at this locus (1.5% in sperm, 0.4% in ova [Vergnaud et al. 1991]), the presence of a small allele undetected by Southern analysis was excluded by PCR amplification across the repeat (fig. 5). This confirmed the presence of a single paternally derived allele. To exclude both uniparental isodisomy and mutation of the maternal allele to a size indistinguishable from the paternal allele, FISH using D2S90 was performed (fig. 6), which confirmed the deletion of this locus in 25/25 cells examined.

**Discussion**

We report five unrelated patients with overlapping deletions, involving chromosome 2q37, in whom the salient
consistent clinical features are brachymetaphalangia and mental retardation. In four, cytogenetic deletions had been detected. In KW, despite the absence of cytogenetic abnormality, we were prompted to search for microdeletions at 2q37, by her AHO phenotype with normal erythrocyte Gsα levels, rendering the Gsα locus on 2q13 unlikely to be of etiological importance. The deletion in KW is the only 2q37 microdeletion described to date. It is not as yet possible to determine whether this represents an interstitial deletion or a healing of a terminal deletion through telomere repair or capture. The minimum region of deletion overlap in our five patients involves D2S125, the most telomeric 2q marker described, and extends proximally for a maximum distance of 17.6 cM.

Our patients and those in the preliminary report by Phelan et al. (1993) have, in common, metacarpal and metatarsal shortening, moderate mental retardation, short stature, round faces, short necks, and shortened noses with flat nasal bridges. Neither our patients nor, apparently, those of Phelan et al. manifested any endocrine abnormality compatible with PHP. Taken together, these data suggest that a gene(s) important for skeletal morphogenesis and neurodevelopment lies within the region identified in this study. The possibility exists that protein(s) encoded by genes at 2q37 act through a Gsα-transduced pathway. This may result in the phenotypic similarity to Gsα-deficient AHO, but without hormone resistance, since other Gsα-transduced pathways would remain intact. It is noteworthy therefore that the gene for one G-protein-coupled receptor, human RDC1 (GPRN1), has been mapped to 2q37 (Libert et al. 1991). Alternatively, the phenotypic abnormalities may result from haploinsufficiency for developmental genes acting through an unrelated pathway—for example, the homeobox gene GBX2, which has recently been mapped to 2q37 (Matsui et al. 1993).

Eight children with isolated deletions involving 2q37 have been reported elsewhere (Young et al. 1983; Sanchez and Pantano 1984; Gorski et al. 1989; Coldwell et al. 1992; Stein et al. 1992; Waters et al. 1993; Wang et al. 1994). The oldest, at 8 years, had certain features in common with our patients—namely, seizures, small nose with depressed nasal bridge, and developmental delay—but no brachymetaphalangia was noted (Stein et al. 1992). Of the remainder, the oldest was 4 years 10 mo (Lin et al. 1992). This child was described as having small hands and feet. Another child, at 9 mo of age, was said to have hypoplastic second phalanges of the fingers and a hypoplastic first phalanx of the big toe (Sanchez and Pantano 1984). Metacarpal and metatarsal shortening was not described; however, in AHO, these clinical features have been shown to evolve with age, because of both reduced longitudinal growth and premature epiphyseal fusion, and thus such features may not be apparent in young children. Three of the reported cases had cutaneous syndactyly (Young et al. 1983; Sanchez and Pantano, 1984; Wang et al. 1994), as has been described in several children with more centromeric distal 2q deletions (reviewed by Ramer et al. 1989). Syndactyly was not present in any of the patients we describe; however, an additional feature noted in all four of our cytogenetic deletion cases was that of cutaneous eczema, also recorded in the report by Gorski et al. (1989) of a 21-mo-old boy with 46,XY, del(2q37).

Other conditions in which brachymetaphalangia is a major feature include brachydactyly type E and acrodysostosis, both of which may be difficult to distinguish from PPHP and have been suggested to belong to the same disease spectrum (Ablow et al. 1977; Poznanski et al. 1977). In type E brachydactyly, short stature may also be present. In acrodysostosis, cutaneous ossification is absent, metacarpal shortening is usually more generalized, and nasal hypoplasia is more severe. We are not aware that Gsα levels have been measured in such patients. Clearly, 2q37 is an alternative candidate region for these disease loci.

We now suggest there are three groups of patients in whom cytogenetic and molecular investigation of 2q37 may be valuable: those with brachymetaphalangia and mental retardation; those with the AHO phenotype but normal Gsα levels; and those with type E brachydactyly or acrodysostosis. Further studies of these three groups are now indicated and may help to define the critical region of 2q37, as well as the range of clinical manifestations of this new chromosomal deletion syndrome.

Acknowledgments

We thank Dr. Giles Vernaud and Jerome Buard for making available primer sequence and cosmids for D2S90(CEB1) and CEB1. We are grateful to Dr. Nicola Royle and Dr. John Armour for helpful discussions and to Shaojie Di Wang and Neil Periam for technical support. L.C.W. is a Medical Research Council, UK
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Appendix D


reprint
A deletion hot-spot in exon 7 of the Gsα gene (GNAS1) in patients with Albright hereditary osteodystrophy


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Received June 9, 1995; Revised and Accepted June 30, 1995

Albright hereditary osteodystrophy (AHO) is a familial disorder characterized by short stature, obesity, rounded facies and skeletal defects including brachydactyly and subcutaneous ossifications. Some affected patients have only the somatic features which are characteristic of the AHO phenotype [pseudopseudohypoparathyroidism (PPHP)] while others have these features in association with resistance to multiple hormones that are coupled to stimulation of adenylyl cyclase [pseudohypoparathyroidism (PHP)]. Molecular studies have identified heterozygous loss-of-function mutations in the gene which encodes the Gsα subunit of Gs (GNAS1) in many AHO patients (both with PHP and PPHP). Gs is the guanine nucleotide regulatory protein (G protein) which stimulates adenylyl cyclase. These mutations are consistent with the observations that most AHO patients have reduced steady-state levels of Gsα mRNA (1,2) and Gs protein as measured by functional assays (2–4). GNAS1 is located on the distal long arm of chromosome 20 (5) and contains 13 exons (6). The mutations identified to date include splice-junction and frameshift mutations as well as missense mutations which alter protein function (7–13). All of the GNAS1 mutations identified thus far have been found only in single independent AHO kindreds.

We previously have identified a 4 bp deletion mutation in exon 7 of GNAS1 in lymphoblasts which were derived from an AHO patient and showed that the mutation disrupted mRNA expression (Fig. 1) (9). We now report that the identical mutation is present in four further unrelated AHO kindreds. Genomic DNA was isolated from blood using a previously described method (8) or the IsoQuick kit (MicroProbe Corp., Garden Grove, CA). A 200 bp genomic DNA fragment including GNAS1 exon 7 was amplified using the following primers according to a previously published protocol (8): sense, 5’ GCCGCCGCCGCTCCGGCCGCCGCCGCCGCCGCCGCCGCCGC- CGCGCCGCCGCAATTGATGTGACGGCTTG 3’ (GC-clamp is underlined); antisense, 5’ GTAGTTTGGAAAGAGGCCTCAGTATGGCGAAGGCTCAG 3’. In each case the mutation was confirmed on at least two independent blood samples and control reactions with no DNA were run in all PCR products to rule out contamination. The identical heterozygous 4 bp deletion was found to be present in four further unrelated patients as determined by abnormal migration of the heteroduplexes in nondenaturing polyacrylamide gels, as previously described (9). In each case the mutation was confirmed by direct sequencing of the PCR products. The data on the four kindreds is summarized in Table 1.

It is unlikely that any of these kindreds are related. Both kindreds 1 and 3 are from the Midwest USA. However, haplotype analysis of the probands using a FokI polymorphism present in exon 5 of GNAS1 (5) revealed that the mutant alleles in the two kindreds are unrelated (data not shown). Kindred 2 is of Puerto Rican background and recently immigrated to New York while kindred 4 is from England. It is therefore very unlikely that they are related to each other or

Figure 1. Coding sequence of GNAS1 exon 7 is shown in the top line (6) with the the 4 bp deletion mutation underlined. The deletion was previously described as CTGA (9) but is indicated here as GACT. Whether the deletion is defined as CTGA or GACT, the sequence of the mutant allele is identical. CT dinucleotide repeats in the vicinity and within the deletion are overlined. Note the lack of palindromic sequences in this region. Shown below is a consensus sequence which has been derived from comparison of deletion hot-spot sites in other genes (16).

<table>
<thead>
<tr>
<th>Kindred 1</th>
<th>AHO</th>
<th>Hormone resistance</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Father</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Female proband</td>
<td>Yes</td>
<td>Yes</td>
<td>Present</td>
</tr>
<tr>
<td>Brother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Brother</td>
<td>No</td>
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<td>Absent</td>
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<tr>
<td>Kindred 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Father</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother*</td>
<td>NA</td>
<td>No</td>
<td>Present</td>
</tr>
<tr>
<td>Father</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Female proband</td>
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</tr>
<tr>
<td>Brother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
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<tr>
<td>Sister</td>
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<td>No</td>
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</tr>
<tr>
<td>Kindred 4</td>
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<tr>
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<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>Male proband</td>
<td>Yes</td>
<td>Yes</td>
<td>Present</td>
</tr>
</tbody>
</table>

Table 1. AHO kindreds with 4 base pair deletion mutation

*To whom correspondence should be addressed at: Metabolic Diseases Branch, NIDDK/NIH, Building 10, Room 8C101, Bethesda, MD 20892–1752, USA

Subject was not available for direct clinical evaluation by the investigators.

Table 1. AHO kindreds with 4 base pair deletion mutation

<table>
<thead>
<tr>
<th>Kindred 1</th>
<th>AHO</th>
<th>Hormone resistance</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Father</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Female proband</td>
<td>Yes</td>
<td>Yes</td>
<td>Present</td>
</tr>
<tr>
<td>Brother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Brother</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Kindred 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Father</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Female proband</td>
<td>Yes</td>
<td>Yes</td>
<td>Absent</td>
</tr>
<tr>
<td>Kindred 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother*</td>
<td>NA</td>
<td>No</td>
<td>Present</td>
</tr>
<tr>
<td>Father</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Female proband</td>
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</tr>
<tr>
<td>Brother</td>
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<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Sister</td>
<td>No</td>
<td>No</td>
<td>Absent</td>
</tr>
<tr>
<td>Kindred 4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mother</td>
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<td>No</td>
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</tr>
<tr>
<td>Male proband</td>
<td>Yes</td>
<td>Yes</td>
<td>Present</td>
</tr>
</tbody>
</table>

NA, not available.

*To whom correspondence should be addressed at: Metabolic Diseases Branch, NIDDK/NIH, Building 10, Room 8C101, Bethesda, MD 20892–1752, USA
to the other two kindreds. Clinical evaluation and genetic analysis demonstrates that the mutation identified in the kindred 1 proband is a de novo mutation while that identified in kindred 2 is inherited from the mother of the proband. The fathers in kindreds 2 and 4 were unavailable for genetic analysis, therefore it was impossible to confirm whether or not the probands had de novo mutations. It has recently been reported that patients who present with PHP inherit the disease from their mother while those with PPHP inherit the disease from their father, suggesting that the GNAS1 gene may be an imprinted gene (14). If this model is accurate, one would predict that the probands in all four kindreds, who have PHP, would have inherited the mutation from their mothers. In fact the proband in kindred 3 did inherit the mutation from her mother (Table 1). The absence of mutation (as well as lack of clinical features) in the mothers of the other three probands suggests that in these kindreds the disease may be caused by de novo mutations.

Including the prior published report (5) we have identified this 4 bp deletion mutation in a total of five out of 35 independent AHO kindreds which have been examined to date. In four of these kindreds membrane Gs was not measured by functional assay and therefore the defect in these kindreds may be at another genetic locus. We are also aware of another functional assay and therefore the defect in these kindreds may be at another genetic locus. We are also aware of another AHO kindred in which the identical deletion was identified (15). All other GNAS1 mutations identified to date have been unique to each AHO kindred. This 4 bp region within exon 7 thus appears to be a deletion hot-spot.

Hot-spots for small deletions have been identified in two settings: regions with repeat sequences and regions in which the sequence confers the formation of a quasipalindrome (16,17). Inspection of exon 7 and surrounding regions does not suggest the presence of quasipalindromic sequences which could explain a hot-spot for a microdeletion (Fig. 1). The presence of CT dinucleotide repeats flanking the deletion is consistent with the slipped strand mispairing model in which there is misalignment of a CT repeat during replication and excision of the resulting 4 bp single stranded loop (16). By inspection of deletion hot-spots in other human genes, Krawczak and Cooper (16) defined a 6 bp consensus sequence which is found also in other sporadic gene deletions and translocations. This consensus sequence is consistent with previously defined arrest sites for DNA polymerase α and has been shown in vitro to be especially prone to frameshift deletion. The GNAS1 deletion described here coincides with this consensus sequence (Fig. 1). Often the deletion breakpoints are 5' or 3' to the second residue (G) and/or last residue (A) of the consensus sequence (16) which precisely correspond to the breakpoints of the GNAS1 deletion. Therefore slipped strand mispairing and arrest of DNA polymerase α are mechanisms that are likely to contribute to the relatively frequent occurrence of this 4 bp deletion.

ACKNOWLEDGEMENTS

L.C.W. is an MRC clinical training fellow. R.C.T. acknowledges grant support from the Research Trust for Metabolic Diseases in Children.

REFERENCES

Appendix E

Isolated autosomal dominant type E brachydactyly: exclusion of linkage to candidate regions 2q37 and 20q13 (1996) Journal of Medical Genetics, 33, 873-876

reprint
Isolated autosomal dominant type E brachydactyly: exclusion of linkage to candidate regions 2q37 and 20q13

M E M Oude Luttikhuis, D K Williams, R C Trembath

Abstract
Type E brachydactyly is a digital malformation which characteristically causes an asymmetrical shortening of one or more metacarpals or metatarsals or both. Although commonly seen as part of a syndrome, it can be inherited as an autosomal dominant characteristic, the gene acting with variable expressivity, but complete penetrance. As an Albright hereditary osteodystrophy (AHO)-like syndrome including brachydactyly type E and mental retardation may be caused by (micro) deletions at chromosome 2q37, this region together with the AHO locus at chromosome 20q13 were considered as candidate loci for brachydactyly type E.

In this paper we describe a family with isolated autosomal dominant type E brachydactyly in whom molecular analysis excludes linkage to these regions, providing support for further genetic heterogeneity of this trait.

Key words: brachydactyly type E; Albright hereditary osteodystrophy; Gsa gene.

The brachydactylies are a heterogeneous group of digital anomalies classified by Bell in 1951. Bell reviewed 124 pedigrees containing 1336

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Figure 1  Brailsford's original family with autosomal dominant brachydactyly type E, pedigree updated 1995.
Table 1 Summary of clinical features described

<table>
<thead>
<tr>
<th>Subject</th>
<th>Metacarpal shortening</th>
<th>Metatarsal shortening</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left hand</td>
<td>Right hand</td>
</tr>
<tr>
<td>IV.2</td>
<td>3, 4</td>
<td>3, 4</td>
</tr>
<tr>
<td>IV.3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>V.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>V.3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>V.5</td>
<td>3, 4</td>
<td>3</td>
</tr>
</tbody>
</table>

people with this malformation and on anatomical grounds defined seven clear groups. Type E brachydactyly is distinguished by shortening of one or more of the metacarpals or metatarsals or both. A striking feature is the common lack of symmetry and lack of shortening of corresponding bones of the hands and feet, together with intrafamilial variation. At that time Bell also recognised that this type of brachydactyly was heterogeneous, featuring in several genetic conditions (notably Turner syndrome and Albright hereditary osteodystrophy) in addition to being inherited as a distinct autosomal dominant characteristic. In 1995, chromosome 2q37 was suggested as a candidate region for type E brachydactyly, based on the presence of (micro)deletions in five patients with short metacarpals, mental retardation, and other dysmorphic features similar to that observed in Albright hereditary osteodystrophy, which is caused by mutations in the GNAS gene located on chromosome 20q13. The purpose of this study was to evaluate these chromosomal loci as possible candidate regions for familial brachydactyly type E, and in particular to assess whether microdeletions on chromosome 2q37 might represent a contiguous gene syndrome involving a gene for brachydactyly type E. Molecular analysis was therefore performed in eight members of a family with autosomal dominant brachydactyly type E using six microsatellite markers and a single VNTR on chromosome 2q37, together with three markers on chromosome 20q13.

Patients, materials, and methods
We recontacted surviving members of a brachydactyly type E family originally described by Brailsford (fig 1). The clinical features of those with the characteristic are summarised in table 1. Hand x rays of IV.3 showing bilateral shortening of the third metacarpals and x rays of the feet showing bilateral shortening of the first and fourth metatarsals are shown in fig 2. Other congenital malformations, short stature, and mental retardation were not observed.

DNA studies
Genomic DNA was isolated from peripheral leucocytes using standard methods. Genotypes
Markers Alleles
D20S93 1,2,3,4,5,6,7
GNASin3 1,2
GNASex5 1,2

Figure 3 Chromosome 20q13 haplotype data.

for chromosome 20 markers were determined using an endonuclease restriction site polymorphism (FokI) in exon 5 of Gsa, a dinucleotide repeat in intron 3 of the same gene, and a highly polymorphic tetranucleotide repeat (D20S93) which maps to a maximum distance of 4 cM distal to the Gsa gene. PCR amplification of D20S93 was performed in the buffer described by Wilson et al. for 23 cycles at an annealing temperature of 62°C. The dinucleotide repeat markers D2S125, D2S395, D2S345, D2S336, and D2S338 were PCR amplified in the presence of [α-32P]-dCTP at annealing temperatures of 55°C, 62°C, 60°C, 60°C, and 60°C respectively. The first four markers were amplified in the same buffer as that used in the D20S93 study while marker D2S338 was amplified in the buffer used for exon 5 amplification in the presence of 1 mmol/l MgCl₂. The PCR products of a further marker, D2S140, were radiolabelled using a reverse primer end labelled with [γ-32P]-ATP in a reaction with the D20S93 buffer at an annealing temperature of 56°C. All PCR reactions were performed for 23 cycles, subjected to electrophoresis on 6% denaturing polyacrylamide gels, and visualised by autoradiography. The primer sequences were described by Gyapay et al. The genotypes for the VNTR D2S90 were determined as previously described by Wilson et al.

Results
The family analysed in this study contained six people with classical type E brachydactyly. The results of the chromosome 20q13 study are summarised in fig 3. The family was uninformative for both polymorphisms in Gsa and fully informative for the flanking marker D20S93. Direct inspection showed that no common haplotypes are shared by all affected people and the data therefore exclude the Gsa gene in the development of the brachydactyly E phenotype in this family. The genotypes for each marker on terminal chromosome 2q were determined and haplotypes inferred (fig 4). Inspection failed to show a common haplotype shared by all affected subjects, excluding linkage of autosomal dominant brachydactyly type E in this family to this region of the genome. Two point linkage analysis of disease locus against each marker at 2q confirmed the absence of linkage for all markers using MLINK, with exclusion (lod ≤ -2) to recombination fractions (θ) of 0.15 or more (data not shown).

Discussion
We chose to study two candidate loci because of the known association of brachydactyly type E with Albright hereditary osteodystrophy, caused by mutations in the Gsa gene on chromosome 20q13 and an AHO-like syndrome recently defined by (micro)deletions at chromosome 2q37, which could be a contiguous gene defect involving a locus for brachydactyly type E. These results show no evidence of linkage to these two regions of the genome and give no evidence of support for a contiguous gene defect in the AHO-like syndrome. Other syndromes featuring brachydactyly type E include Biemond syndrome I and Ruvalcaba syndrome and their localisation will enable further candidate loci for familial isolated brachydactyly type E to be considered. In addition, homeobox containing genes and genes encoding growth factors, both classes of genes implicated in the control of distal limb development, should be considered. Indeed the genes HOXD, MSX1, MSX2, FGF-1, and FGF-2 have recently been excluded in two families with type A1 brachydactyly. These genes should clearly be considered in other subtypes of brachydactyly.

The first two authors contributed equally to this work.

Markers Alleles
D2S336 1,2,3,4,5
D2S345 1,2
D2S338 1,2,3
D2S395 1,2,3,4,5
D2S140 1,2,3
D2S125 1,2,3,4,5
D2S90 1,2,3,4

Parentheses indicate inferred haplotype
(5) (3)
(2) (1)
? ?
(2) (3)
(3) (3)
(2) (4)
(1) (2)

Figure 4 Chromosome 2q37 haplotype data.