Am. J. Hum. Genet. 72:961–974, 2003

Analysis of *GNAS1* and Overlapping Transcripts Identifies the Parental Origin of Mutations in Patients with Sporadic Albright Hereditary Osteodystrophy and Reveals a Model System in Which to Observe the Effects of Splicing Mutations on Translated and Untranslated Messenger RNA

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Albright hereditary osteodystrophy (AHO) is caused by heterozygous deactivating *GNAS1* mutations. There is a parent-of-origin effect. Maternally derived mutations are usually associated with resistance to parathyroid hormone termed "pseudohypoparathyroidism type Ia." Paternally derived mutations are associated with AHO but usually normal hormone responsiveness, known as "pseudo-pseudohypoparathyroidism." These observations can be explained by tissue-specific *GNAS1* imprinting. Regulation of the genomic region that encompasses *GNAS1* is complex. At least three upstream exons that splice to exon 2 of *GNAS1* and that are imprinted have been reported. NESP55 is exclusively maternally expressed, whereas exon 1A and XLαs are exclusively paternally expressed. We set out to identify the parental origin of *GNAS1* mutations in patients with AHO by searching for their mutation in the overlapping transcripts. This information would be of value in patients with sporadic disease, for predicting their endocrine phenotype and planning follow-up. In doing so, we identified mutations that resulted in nonsense-mediated decay of the mutant Gsα transcript but that were detectable in NESP55 messenger RNA (mRNA), probably because they lie within its 3′ untranslated region. Analysis of the NESP55 transcripts revealed the creation of a novel splice site in one patient and an unusual intronic mutation that caused retention of the intron in a further patient, neither of which could be detected by analysis of the Gsα complementary DNA. This cluster of overlapping transcripts represents a useful model system in which to analyze the effects that mutant sequence has on mRNA—in particular, splicing—and the mechanisms of nonsense-mediated mRNA decay.

Introduction

Albright hereditary osteodystrophy (AHO [MIM 103580]) is characterized by short adult stature, obesity, brachydactyly, and ectopic ossifications in ~60% of patients (for review, see Wilson and Trembath 1994). It is frequently associated with mild-to-moderate mental handicap. A proportion of patients with AHO have associated end-organ resistance to parathyroid hormone (PTH), known as "pseudohypoparathyroidism type I" (PHP Ia). Those who have AHO and normal endocrine responsiveness are said to have "pseudo-pseudohypoparathyroidism" (PPHP).

Patients with PHP Ia usually manifest hypocalcemia together with hyperphosphatemia and raised PTH (in the presence of normal renal function and 25-hydroxycholecalciferol) at some point in childhood. In addition, they often have clinically significant thyroid-stimulating hormone (TSH) resistance resulting in hypothyroidism (Levine et al. 1983). For reasons that are not clear, these patients frequently have intermittent prolonged periods of normocalcemia and normal thyroid function. As a result, it can be difficult to determine whether a normocalcemic, euthyroid patient with AHO has the PHP Ia form or the PPHP form, although PTH levels are usually elevated in patients with PHP Ia. In the past, the PHP Ia and PPHP forms could be distinguished by PTH-stimulation testing, which was not possible here since the supply of synthetic PTH for human injection was discontinued.

Both the PHP Ia and PPHP forms of AHO are caused by heterozygous deactivating mutations within the *GNAS1* gene, located on chromosome 20q13. *GNAS1* consists of 13 coding exons, from which four common isoforms are generated through alternative splicing of exon 3 and use of alternative splice-acceptor sites one codon apart in exon 4 (Kozasa et al. 1988). A variety of different frameshift, nonsense, and mis-

Received November 5, 2002; accepted for publication January 16, 2003; electronically published March 6, 2003.

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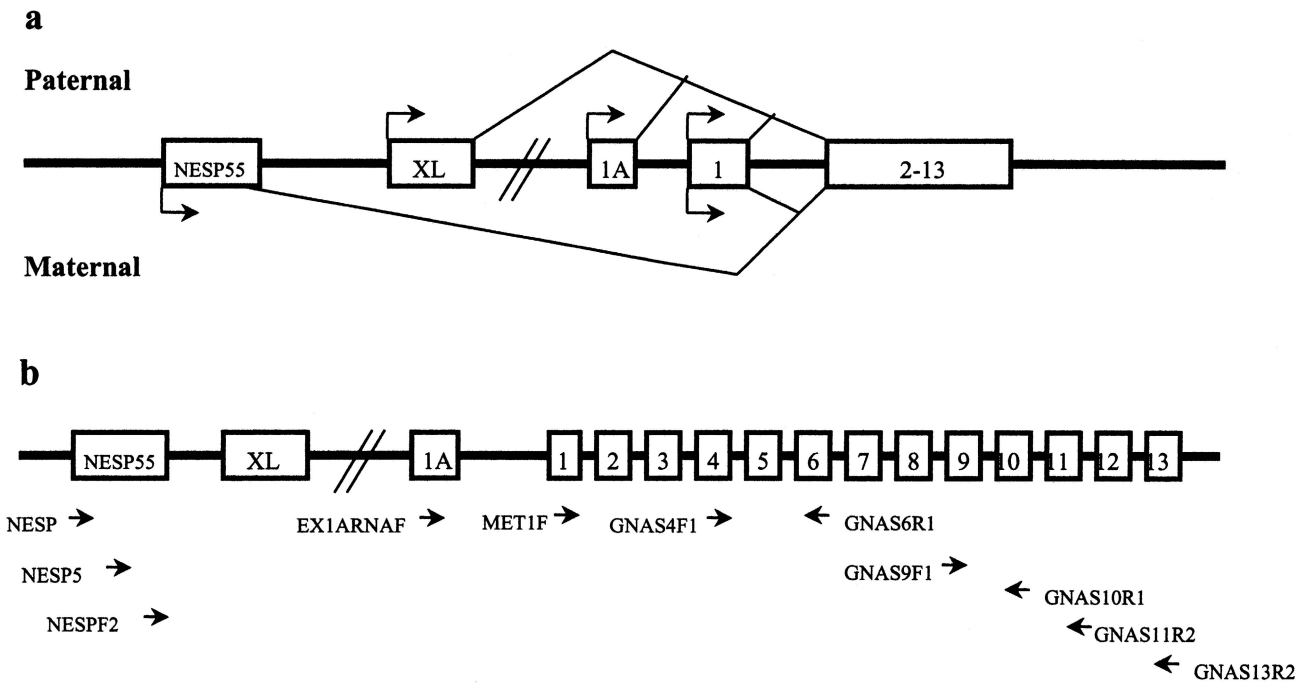

Figure 1 Schematic representation of the *GNAS1* cluster, including exons 1–13 of *GNAS1* and upstream imprinted exons NESP55, XLαs, and exon 1A. *a,* Summary of imprinting. Arrows above and below depict paternally and maternally expressed transcripts, respectively. The transcripts for Gsα and XLαs have ORFs starting in their unique 5′ exons and ending in exon 13. Exons 2–13 of *GNAS1* lie within the 3′ UTR of NESP55. Exon 1A is probably not translated. *b,* Location of primers used to amplify the Gsα, NESP55, and exon 1A transcripts (not to scale).

sense mutations throughout the gene have been reported (for review, see Aldred and Trembath 2000). *GNAS1* is widely expressed and encodes the α subunit of the heterotrimeric G protein, Gs, which transduces signals between certain cell-surface receptors (including those for PTH, TSH, and thyrotropin-releasing hormone) and intracellular adenylyl cyclase (for review, see Bourne and Stryer 1992). Patients with either form of AHO have an ~50% reduction in bioactivity of the Gsα subunit in all tissues measured, including erythrocytes, lymphocytes, platelets, and fibroblasts (Farfel and Bourne 1980; Levine et al. 1980; Bourne et al. 1981; Farfel et al. 1982). In families in which AHO is segregating, the strongest predictor of the endocrine phenotype is the parent of origin (Davies and Hughes 1993). Maternal transmission is usually associated with the PHP Ia form, and paternal transmission is usually associated with the PPHP form, although there has been an exception (Schuster et al. 1994). These observations are best explained by a tissue-specific imprinting model in which *GNAS1* is biallelically expressed in most tissues but maternally expressed in certain cells, such as those of the proximal renal tubule. The AHO phenotype results from a general 50% Gsα-bioactivity reduction caused by deactivating mutations of either allele, whereas associated PHP Ia occurs only if the maternal allele is mutated, since that is the only allele expressed in the imprinted tissues. Support for this model has come from *Gnas* knockout mice, from mice with uniparental disomy for the homologous region encompassing *Gnas,* and from studies of human pituitary and thyroid (Yu et al. 1998; Peters et al. 1999; Hayward et al. 2001; Germain-Lee et al. 2002; Mantovani et al. 2002).

Regulation of the genomic region that encompasses *GNAS1* is complex (see fig. 1*a*). Three overlapping transcripts, each of which has an alternative upstream exon spliced to exons 2–13 of *GNAS1,* have been reported. Exon 1A lies ~2.5 kb upstream of *GNAS1* exon 1. Exon 1A–containing transcripts are exclusively paternally expressed in a wide range of tissues and are probably not translated. Exon 1A appears to be intimately involved in the tissue-specific imprinting of *GNAS1,* since individuals with abnormal imprinting of exon 1A—specifically, an unmethylated maternal allele—have isolated PTH resistance known as "PHP Ib" (Liu et al. 2000). Another transcript, known as "extra-large Gsα" (XLαs), comprises a novel exon, 35 kb upstream of *GNAS1* exon 1, that forms a continuous ORF with exons 2–13 of *GNAS1;* this transcript is also exclusively paternally expressed. The third transcript, NESP55 (a neuroendocrine secretory protein), has a novel exon ~14 kb upstream of XLαs and is spliced to *GNAS1* exons 2–13, which appear to lie

within its 3′ UTR. The function of NESP55 is not known, but it appears to be exclusively maternally expressed (Hayward et al. 1998). NESP55 is spanned by a paternally expressed antisense transcript that originates upstream of XLαs and that is likely to be involved in suppressing the paternal NESP55 allele (Hayward and Bonthron 2000). Further complexity is suggested by the presence of an alternative 3′ exon, contained within intron 3 of *GNAS1,* that includes an alternative stop codon and polyadenylation site (Crawford et al. 1993).

We set out to test whether these overlapping imprinted transcripts could be used to determine the parental origin of *GNAS1* mutations. That would be of potential clinical value for predicting the endocrine phenotype in patients with sporadic AHO and for quantifying the extent of the parental-origin effect, given that it may not be absolute. Using a common polymorphism (c.393T/C) in exon 5 of *GNAS1,* we have confirmed that imprinting of the NESP55 and exon 1A transcripts is conserved in control lymphoblastoid cell lines. We have tested a cohort of nine patients with AHO who have mutations in exons 2–13 of *GNAS1* from whom lymphoblastoid cell lines were available. In one patient known to have inherited the condition from her mother and in a further five patients with de novo mutations who have PHP Ia, we have demonstrated that the mutation is present in the NESP55 transcript but not in the exon 1A–containing transcript, confirming that it originated on the maternal *GNAS1* allele. In one patient with PPHP in whom paternal origin has been demonstrated previously (Wilson et al. 1994) and in two further patients with probable PPHP, paternal origin was confirmed.

Four of the patients in the cohort that we studied had mutations predicted to cause frameshifts and premature termination codons. These mutations were not detectable in the Gsα cDNA, presumably owing to nonsense-mediated decay (NMD) of mRNA. However, the mutations, which were all maternally derived, were detected in the NESP55 transcripts, where they lie within the 3′ UTR. In one patient, a single-nucleotide substitution at position −11 from the intron 4 splice-acceptor site appears to have resulted in retention of intron 4 in the NESP55 transcript. In another patient, a single-nucleotide change at position −1 of the intron 7 splice-acceptor site resulted in the use of a novel splice-acceptor site 1 nt downstream in the NESP55 transcript.

Subjects and Methods

Patients

Patients with AHO were recruited through U.K. clinical geneticists. In some instances, affected parents were ascertained after the diagnosis of AHO in their child. Most patients were seen at home for examination and for collection of samples for baseline biochemistry, including urea, electrolytes, calcium, phosphate, albumin, creatinine, and thyroid function, as well as lymphocyte DNA and lymphoblastoid cell lines. Samples for PTH were not collected, because of the requirement for rapid separation of the plasma. When available, additional information was obtained from the local clinicians and hospital notes. For patients who were treated with vitamin D preparations and/or thyroxine, details of their biochemical results at presentation were obtained when possible from notes or the referring clinician. Ethical committee approval was obtained for the study.

DNA Preparation

DNA and RNA were extracted from Epstein-Barr virus–transformed lymphoblastoid cell lines, which were grown in RPMI media (Gibco BRL) with 10% fetal bovine serum (Sigma), 1% L-Glutamate (Gibco BRL), and 0.1% penicillin/streptomycin (Sigma). DNA was extracted using standard methods.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from lymphoblast cell pellets and fetal tissue by using Trizol, following the manufacturer's instructions (Gibco BRL). Each sample was treated with DNase (Gibco BRL), and cDNA synthesis was performed on ~2 μg of total RNA by using Superscript II (Gibco BRL), following the manufacturer's instructions. The reverse-transcriptase reaction contained both an oligo dT and a random nonomer primer. The resulting cDNA products were amplified using standard hypoxanthine-guanine phosphoribosyltransferase–gene primers and were visualized on a 2% agarose gel to detect genomic contamination.

Screening of GNAS1 *Exons 2–13 and Adjacent Introns, by Use of Denaturing High-Performance Liquid Chromatography (DHPLC)*

Previously reported mutations were confirmed in the cell lines by sequencing of the relevant exon, using BigDye terminator technology (Applied Biosystems), and were run on an ABI 377 DNA Sequencer. Screening for unknown mutations was performed using DHPLC (Transgenomic WAVE DNA Fragment Analysis System). The optimal column temperatures and acetonitrile gradients were calculated for each fragment by using the WAVEMaker program (conditions available on request). Each fragment was amplified using the primer sets and conditions listed in table 1, with Hot Star *Taq* polymerase (Qiagen), following the manufacturer's instructions. Any amplicons exhibiting altered elution peaks were sequenced as described above.

Table 1

Primers and Conditions for Amplification of Gsα, NESP55, and Exon 1A Transcripts

Primer Name	Primer Sequence	PCR Annealing Temperature/No. of Cycles
GNAS4F	ATG AAA GCA GTA CTC CTA ACT GA	
GNAS5R	GTG CCC ATG TGC AGG GCT GTC ACT CAT GTT	57°C/30[a]
GNAS7F	TGA GCC TGA CCT TGT AGA GAG ACA CA	
GNAS8R	GGT TAT TCC AGA GGG ACT GGG GTG AA	57°C/30[a]
GNAS9F	GGT TTC TTG ACA TTC ACC CCA G	
GNAS10R	CTC TAT AAA CAG TGC AGA CCA G	57°C/30[a]
GNAS4F1	TGA GAG GGC AAC CAA AGT G	
GNAS6R1	CCT TGG CAT GCT CAT AGA ATT	57°C/30[a]
MET1F	GGA ACA GTA AGA CCG AGG ACC	
GNAS11R2	CTC AGG AGT AGT GTA GCG AGC A	62°C/35[a]
NESP	CTT AAC GCC CAC CAC CGC TC	
GNAS11R2	CTC AGG AGT AGT GTA GCG AGC A	6, 34[b]
NESP5	TCG GAA TCT GAC CAC GAG CA	
GNAS10R1	CAC GAA GAT GAT GGC AGT CAC	6, 34[b]
NESP5	TCG GAA TCT GAC CAC GAG CA	
GNAS11R2	CTC AGG AGT AGT GTA GCG AGC A	6, 34[b]
NESP5	TCG GAA TCT GAC CAC GAG CA	
GNAS13R2	TTA GAG CAG CTG GTA CTG ACG	63°C/40[a]
NESPF2	GAA GGA GCC CAA GGA GGA GAA GCA GCG GC	
GNAS11R2	CTC AGG AGT AGT GTA GCG AGC A	63°C/35[a]
EX1ARNAF	CTG CGT CAG GTG GCT GGC	
GNAS10R1	CAC GAA GAT GAT GGC AGT CAC	64°C/35[a]
EX1ARNAF	CTG CGT CAG GTG GCT GGC	
GNAS11R2	CTC AGG AGT AGT GTA GCG AGC A	64°C/35[a]

[a] PCRs performed under the following conditions: 96°C for 15 min (5 min for Megamix PCR premix), then 96°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min for the number of cycles indicated.

[b] PCRs performed under the following conditions: 96°C for 15 min (96°C for 30 s, 66°C for 30 s, and 72°C for 45 s) for 1 cycle, then repeated for 5 cycles with .5°C decrement of annealing temperature, concluding with 96°C for 30 s, 63°C for 30 s, and 72°C for 45 s for 34 cycles.

Imprinting Analysis of NESP55, Exon 1A, and Gsα Transcripts in Lymphoblast and Fetal RNA

For the detection of heterozygotes for the exon 5 *Fok*I polymorphism (c.393T/C), DNA extracted from normal lymphoblast cells and cDNA from fetal tissue samples was amplified using primers GNAS4F/GNAS5R, for the DNA samples, and GNAS4F1/GNAS6R1, for the cDNA samples, and Megamix PCR premix (Microzone) (table 1 and fig. 1*b*). The products were digested with *Fok*I (NEB) and were separated on a 2% agarose gel. Seven heterozygous normal control lymphoblast cDNA samples, two heterozygous fetal cDNAs (lung and kidney), and cDNA from patient AHO-33 were amplified for the NESP55, exon 1A, and Gsα transcripts, by using NESPF2/GNAS10R1, EX1ARNAF/GNAS10R1, and MET1F/GNAS10R1, respectively. The authenticity of each amplicon was verified by sequencing as described above (see the previous subsection). A second nested PCR was performed on 2 μl of the original product in a total reaction volume of 20 μl with GNAS4F1 (5′-labeled with FAM)/GNAS6R1, using Megamix PCR premix (Microzone). Ten microliters of the PCR product was digested with *Fok*I (NEB) and separated on a 4% polyacrylamide gel on an ABI 377 DNA Sequencer.

Amplification and Sequencing of NESP55, Exon 1A, and Gsα Transcripts

NESP55, exon 1A, and Gsα transcripts were amplified from cDNA in all patients by using the primer combinations shown in table 2 and figure 1*b*. Primers and conditions are shown in table 1. In some patients, reamplification of the original NESP55 PCR by using a second nested primer set, NESPF2/GNAS11R2, was necessary. One and a half microliters of original NESP55 PCR product was reamplified in a total volume of 30 μl. All PCRs from cDNA were performed using Hot Star *Taq* polymerase (Qiagen), following the manufacturer's instructions. Exon 1A is GC rich, and all PCRs required the use of Q buffer (supplied with Hot Star *Taq* polymerase) and, also, the addition of 5% dimethyl sulfoxide in the sequencing reaction. All PCR products were sequenced using BigDye (Applied Biosystems) with a 96°C denaturing temperature and a 55°C annealing temperature, under conditions recommended in the kit. Sequencing in the forward direction was performed using NESPF2, EX1ARNAF, and nested primers GNAS4F1 (5′-TGA GAG GGC AAC CAA AGT G-3′) and GNAS9F1 (5′-CAT GTT TGA CGT GGG TGG

Table 2

Primer-Set Combinations for Gsα, NESP55, and Exon 1A cDNA Amplifications

Patient	Primer Set for		
	Gsα	NESP55	Exon 1A
AHO-17	MET1F/GNAS11R2	NESP/GNAS11R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS10R1
AHO-10	MET1F/GNAS11R2	NESP/GNAS11R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS10R1
AHO-15	MET1F/GNAS11R2	NESP5/GNAS13R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS11R2
AHO-29	MET1F/GNAS11R2	NESP5/GNAS13R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS11R2
AHO-55	MET1F/GNAS11R2	NESP/GNAS11R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS11R2
AHO-33	MET1F/GNAS11R2	NESP/GNAS11R2, re-PCR with NESPF2/GNAS11R2	EX1ARNAF/GNAS11R2
AHO-11	MET1F/GNAS11R2	NESP5/GNAS11R2	EX1ARNAF/GNAS10R1
AHO-278	MET1F/GNAS11R2	NESP5/GNAS10R1	EX1ARNAF/GNAS10R1
AHO-13	MET1F/GNAS11R2	NESP5/GNAS11R2	EX1ARNAF/GNAS11R2

Note.—For primer sequences and conditions, see table 1.

C-3′), and the reverse strands were sequenced with the original PCR primer.

Results

GNAS1 *Mutation Screening*

Mutations were identified in nine patients from whom lymphoblastoid cell lines were available. Their clinical features are summarized in table 3, and mutations are summarized in table 4. All mutations are numbered according to the cDNA sequence reported by Kozasa et al. (1988) (see the Genome Database [GDB ID GDB:120628]).

AHO-17.—This patient with AHO and PHP has been reported previously (Oude Luttikhuis et al. 1994). He has a de novo 43-bp deletion (c.278del43bp), extending from exon 4 across the splice-donor junction to intron 4, that is predicted to result in exon 4 skipping, causing a frameshift and premature termination six codons downstream in exon 5.

AHO-10.—This patient with AHO and PHP has been reported previously (Yu et al. 1995). He has the common 4-bp deletion (c.565-568delGACT) in exon 7; this deletion is predicted to result in a frameshift and premature protein truncation. It has been shown by others that this mutation is not expressed in mRNA, presumably because of NMD (Weinstein et al. 1992). The mutation was not present in his clinically normal mother. His father was deceased but had no known features of AHO.

AHO-15.—This patient with AHO and PHP has a G→A substitution (c.586-1G→A) at the splice-acceptor site of intron 7. Samples from her parents were not available. This mutation has not been reported previously. The mutation was predicted either to result in exon 8 skipping with a frameshift and premature termination in exon 9 or to form a weak novel splice-acceptor site 1 base downstream, resulting in a single-nucleotide deletion with a frameshift and premature termination in exon 8.

AHO-29.—This patient has AHO and evidence of resistance to TSH, suggesting that she may have the PHP form. Her mother and younger sister also have features of AHO. She was found to have a c.725C→T transition predicted to result in the nonconservative substitution of threonine by isoleucine at amino acid residue 242 in exon 10. This amino acid lies within one of the three switch regions of Gsα that are involved in conformational changes between the active and inactive states (Pennington 1994). The mutation segregated with the AHO phenotype in the family and was not found in 300 unrelated chromosomes screened.

AHO-55.—This patient with AHO and PHP has a c.776A→T substitution predicted to result in the nonconservative substitution of glutamate by valine at amino acid 259 in exon 10. The mutation was not present in either of his clinically normal parents and was not found in 300 unrelated chromosomes screened. This residue is highly conserved across all Gα subunits and lies within the third switch region of Gsα (Pennington 1994). The same amino acid substitution has been reported previously and has been shown to result in impaired adenylyl cyclase stimulation (Ahmed et al. 1998; Warner et al. 1999).

AHO-33.—No mutations were identified in this patient with AHO and PHP, after screening *GNAS1* exons 2–13 and adjacent splice sites by SSCP and DHPLC and sequencing of exon 1. She was heterozygous (+/−) for the common *Fok*I polymorphism in exon 5 in genomic DNA. However, analysis of Gsα cDNA from her lymphoblast cell line showed that she was only expressing her − allele. Her father was clinically normal and was shown to be homozygous for the *Fok*I − allele. Her mother was deceased but was not known to have had AHO. It was assumed that this patient had an unidentified mutation on her maternally derived + allele, resulting in NMD. Subsequent screening of the NESP55 transcript resulted in the identification of a single-nucleotide substitution (IVS4:−11A→G), within intron 4, that causes retention of intron 4 and predicted premature termination 21 codons into the intron-derived sequence (fig. 2).

AHO-11.—This patient with AHO and PPHP, con-

Table 3

Clinical Features of the Patient Cohort

Patient (AHO Features[a])	Age Seen (years)	PTH Axis[b]			PTH-Stimulation Testing	Thyroid[c]	Family History	Clinical Classification
		Calcium	Phosphate	PTH				
AHO-17[d] (Ob, Br, LD, Os)	10	2.09 mmol/liter (2.1–2.6)	2.61 mmol/liter (.8–1.4)	361 ng/liter (5–45)	No	Hypothyroidism	No	PHP Ia
AHO-10[e] (Ob, Br, LD, Os)	31	4.6 mg/dl	8.1 mg/dl	…	No	Normal	No	PHP Ia
AHO-15 (SS, Ob, Br, Os)	31	Investigated outside the United Kingdom; presented with tetany and basal ganglia calcifications; treated with vitamin D	Normal	Affected daughter	PHP Ia			
AHO-29 (Ob, Br, LD)	13	2.49 mmol/liter (2.1–2.6)	1.61 mmol/liter (.8–1.4)	…	No	T4: 11.6 pM (11–31); TSH: 3.5 mU/liter (.2–5)	Affected mother and sister	Probable PHP Ia
AHO-55 (Ob, Br, LD)	4	1.57 mmol/liter	3.56 mmol/liter	24 pmol/liter (1–7)	No	Congenital hypothyroidism	No	PHP Ia
AHO-33 (Ob, Br, LD)	10	1.31 mmol/liter	2.92 mmol/liter	811 ng/liter (10–55)	No	Normal T4 and TSH	No	PHP Ia
AHO-11[f] (SS, Ob, Br, LD, Os)	14	Normal	Normal	Normal	Normal cyclic adenosine 3′,5′-monophosphate response	Normal	No	PPHP
AHO-278 (SS, Ob, Br, LD)	32	Normal	Normal	Not done	No	Normal	Daughter with AHO	Probable PPHP
AHO-13 (SS, Br)	40	Normal	Normal	Not done	No	Hypothyroidism diagnosed at age 31 years	Three affected children with hypothyroidism and hypocalcemia	Probable PPHP

[a] SS = short stature; Ob = obesity; Br = brachydactyly; Os = ectopic ossifications; LD = learning difficulties.

[b] Normal ranges (in the same units as the corresponding actual values) are given in parentheses.

[c] T4 and TSH.

[d] Reported previously (Oude Luttikhuis et al. 1994).

[e] Reported previously (Yu et al. 1995).

[f] Reported previously (Wilson et al. 1994).

Table 4

Results of NESP55, Exon 1A, and Gsα cDNA Analysis

Patient (Clinical Phenotype)	*GNAS1* Mutation[a] (Location)	cDNA Result for		
		NESP55	Exon 1A	Gsα
AHO-17 (PHP Ia)	c.278del43bp (exon 4)	Exon 4 skipped allele only	Normal allele only	Normal allele only
AHO-10 (PHP Ia)	c.565-568del4bp (exon 7)	565del4bp allele only	Normal allele only	Normal allele only
AHO-15 (PHP Ia)	c.586-1G→A (intron 7)	586delG allele only	Normal allele only	Normal allele only
AHO-29 (PHP Ia)	c.725C→T; T242I (exon 10)	T242I allele only	Normal allele only	Normal/T242I allele
AHO-55 (PHP Ia)	c.776A→G; E259V (exon 10)	E259V allele only	Normal allele only	Normal/E259V allele
AHO-33 (PHP Ia)	IVS4:−11A→G	Intron 4 retained allele only	Normal allele only	Normal allele only
AHO-11 (AHO)	c.432+1G→A (intron 5)	Normal allele only	Exon 5 skipped allele only	Normal/exon 5 skipped allele
AHO-278 (AHO)	c.432+1G→A (intron 5)	Normal allele only	Exon 5 skipped allele only	Normal/exon 5 skipped allele
AHO-13 (AHO)	c.737T→C; F246S (exon 10)	Normal allele only	F246S allele only	Normal/F246S allele

[a] Numbered according to the cDNA sequence reported by Kozasa et al. (1988) (see the Genome Database [GDB ID GDB:120628]).

firmed by PTH-stimulation testing, has been reported previously (Wilson et al. 1994). She has a de novo single-nucleotide substitution (c.432+1G→A), affecting the intron 5 splice-donor site, that is predicted to result in exon 5 skipping, confirmed by analysis of the Gsα and exon 1A cDNA transcripts (fig. 3*b*). Exon 5 skipping is not associated with any frameshift.

AHO-278.—This patient has AHO with no history of any symptomatic endocrine abnormality and has normal baseline calcium and thyroid function. He was ascertained through his daughter, who has osteoma cutis. His mutation is identical to that in patient AHO-11, to whom he is unrelated.

AHO-13.—This patient with AHO has three children with AHO and PHP Ia, all of whom developed hypothyroidism and hypocalcemia requiring treatment within the first few years of life. She consented only for her samples to be included in the study. She was reported to have developed hypothyroidism at age 31 years, but there was no information available regarding her thyroid antibodies. She was normocalcemic and had never had symptomatic hypocalcemia. She was found to have a c.737T→C transition predicted to result in the nonconservative substitution of phenylalanine by serine at amino acid 246 in exon 10. In Gα subunits across all species, this residue is either phenylalanine or tyrosine (Pennington 1994). The mutation was not found in 300 unrelated chromosomes screened.

Imprinting of NESP55 and Exon 1A Transcripts Is Conserved in Lymphoblastoid Cell Lines

Imprinting of NESP55 and XLαs has been demonstrated in a range of human fetal tissues (Hayward et al. 1998; Hayward and Bonthron 2000), and paternal expression of exon 1A has been demonstrated in cDNA extracted from whole blood (Liu et al. 2000). However, skewed imprinting and loss of imprinting in lymphoblasts have occasionally been reported (Antequera et al. 1990; Kubota et al. 1996; Hannula et al. 2001). For that reason, we tested whether imprinting of NESP55 and exon 1A was conserved in lymphoblastoid cell lines.

Genomic DNA from normal control lymphoblastoid cell lines and normal fetal tissue samples was typed for a common *Fok*I restriction-enzyme polymorphism (c.393T/C; I131I) in exon 5 of *GNAS1*. Seven lymphoblastoid cell lines and two normal fetal tissue samples were found to be heterozygous (+/−) (data not shown). cDNA from these samples was used to amplify the NESP55, exon 1A, and Gsα transcripts in separate reactions, using the appropriate 5′ exon forward primer and a reverse primer in exon 10 of *GNAS1* (giving three distinct products). The authenticity of the transcripts was confirmed by sequencing from exon 2 back to the 5′ exon. The three amplicons were subsequently reamplified using nested exonic primers (a labeled forward primer in exon 4 and a reverse primer in exon 6), to generate smaller fluorescently labeled products encompassing the *Fok*I polymorphism. The products were digested with *Fok*I and were sized. *Fok*I digestion of the initial PCR products was not attempted, because of the complexity introduced by alternative splicing of exon 3 and because of the presence of additional *Fok*I sites. As expected, Gsα transcripts were biallelically expressed in both lymphoblasts and fetal tissue. As predicted, the NESP55 and exon 1A transcripts were found to be monoallelically expressed in all the normal lymphoblast and fetal samples. In addition, the NESP55 and exon 1A transcripts were confirmed to be oppositely imprinted (fig. 4).

Determining the Parental Origin of GNAS1 *Mutations*

The NESP55 and exon 1A transcripts were amplified from lymphoblastoid cDNA in separate reactions, using a forward primer in the unique 5′ exon and a reverse primer in either exon 10 or exon 11 of *GNAS1* (according to the mutation position). The NESP55 product was reamplified using nested primers, since the initial NESP55 PCR product was weak (possibly because of low copy

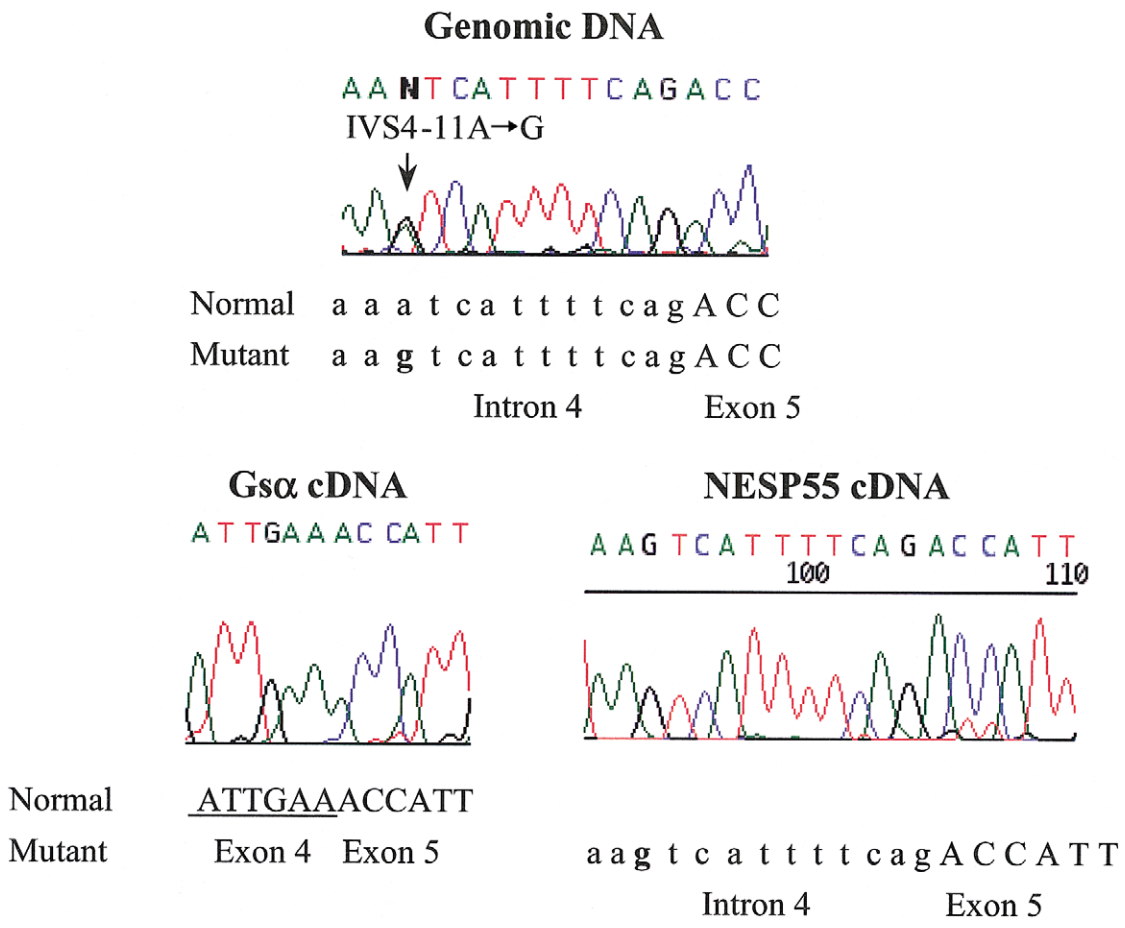

Figure 2 Sequence data from patient AHO-33. A heterozygous IVS4:−11A→G mutation was present in genomic DNA. Gsα cDNA amplification products showed only normal transcript, owing to NMD of the mutant transcript, whereas amplification of the NESP55 transcript revealed retention of intron 4. The base substitution is depicted in boldface. Intronic and exonic sequences are given in lowercase and uppercase, respectively. Exon 4 sequence is underlined.

number in lymphoblasts). The NESP55 and exon 1A transcripts were then sequenced using internal primers spanning the mutation but avoiding the inclusion of exon 3 because of the complexity introduced by the alternative splicing. The results are summarized in table 4.

In two patients, the parental origin of the mutation was known. Patient AHO-29, with borderline low thyroxine, had a clinically affected mother in whom the mutation was also demonstrated. The mutation was confirmed to be present in the NESP55 transcript but not the exon 1A transcript. The converse was found in patient AHO-11, in whom the mutation was found in the exon 1A transcript while the NESP55 sequence was normal (fig. 3*b*). In this patient, a diagnosis of PPHP has been confirmed, in the past, by normal PTH-stimulation testing. In addition, her mutation has previously been shown to be paternally derived, using the nearby *Fok*I polymorphism, for which she was informative (Wilson et al. 1994).

The results for the remaining patients are summarized in table 4. In each patient, the mutation was found in either the NESP55 transcript or the exon 1A transcript but not both. In the five patients with de novo mutations who have confirmed PHP Ia, the paternally derived exon 1A allele showed only normal sequence, whereas the maternally derived NESP55 allele carried only the mutated sequence.

In two normocalcemic adults with AHO, neither of whom had any history of hypocalcemia, the mutations were found to be of paternal origin, since they were present in the exon 1A transcript but not the NESP55 transcript.

Protein-Terminating Mutations That Were Undetectable in the Gsα Transcript Were Detected in NESP55 cDNA

In patient AHO-17, the mutation was predicted to result in skipping of exon 4 and a frameshift. Analysis

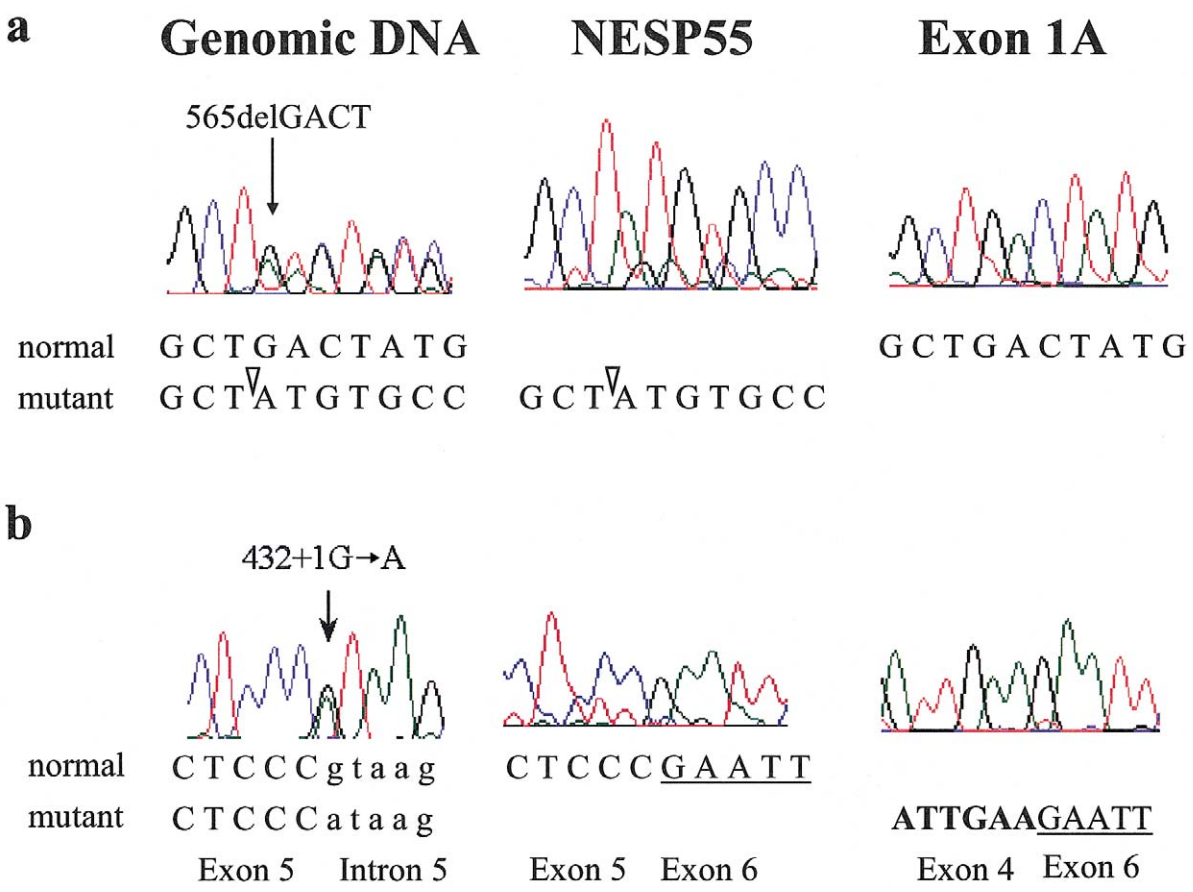

Figure 3 Sequence data showing genomic DNA and NESP55 and exon 1A cDNA products. *a,* Patient AHO-10. The heterozygous c.565-568delGACT deletion is present in genomic DNA, as denoted by the inverted triangle. Only deleted sequence is present in the NESP55 transcript, and only normal sequence is present in exon 1A, indicating that the *GNAS1* mutation is maternally derived. *b,* Patient AHO-11. The heterozygous c.432+1G→A intron 5 splice-site mutation is present in genomic DNA. In NESP55 cDNA, normal exon 5 splicing has occurred. In exon 1A cDNA, skipping of exon 5 has occurred, confirming that the *GNAS1* mutation is paternally derived. Exonic sequence is given in uppercase, and intronic sequence is given in lowercase. Exons 4, 5, and 6 sequences are given in boldfaced, normal, and underlined text, respectively.

of the Gsα mRNA showed only normal sequence, consistent with reduced expression or NMD affecting the mutant allele. Analysis of the NESP55 mRNA by using nested amplification from the unique 5′ exon to exon 11 showed only an abnormally short product, the sequencing of which confirmed skipping of exon 4.

In patient AHO-10, who has the common c.565-568GACT deletion, we confirmed that the mutant Gsα mRNA was not detectable but that the 4-bp deletion was present in the NESP55 transcript (fig. 3*a*).

In patient AHO-15, with a mutation affecting the intron 7 splice-acceptor site, only normal Gsα transcript was present. Analysis of the NESP55 transcript revealed that the weak novel splice-acceptor site 1 nt downstream was used, resulting in a single-base deletion, which in the Gsα transcript would be predicted to cause a frameshift and premature termination eight codons downstream in exon 8 (fig. 5).

In patient AHO-33, no mutation had been found on screening of genomic DNA; however, she was heterozygous for the common *Fok*I polymorphism, and only her paternally derived Gsα − allele could be amplified from lymphoblast cDNA. To confirm that the *Fok*I + allele was maternally derived, we amplified exons 4–6 of her NESP55 cDNA by using nested PCR. The resulting product was abnormally large, and sequencing revealed that intron 4 had been retained. The only demonstrable alteration in the exon 4/intron 4/exon 5 sequence was a single-nucleotide substitution (−11A→G) at position IVS4. In retrospect, the sequence change had resulted in an altered DHPLC elution peak of the amplicon encompassing exons 4 and 5. This had been discounted as being unlikely to be pathological after sequencing and analysis using a neural network splice-predictor program (see the Berkeley Drosophila Genome Project Web site), which did not predict any alteration

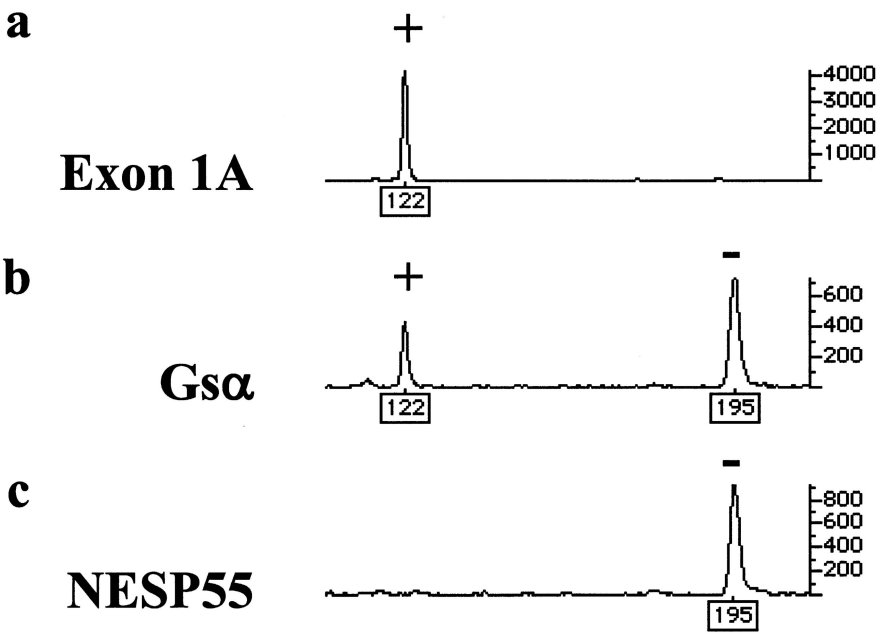

Figure 4 Imprinting of *GNAS1* and overlapping transcripts in normal lymphoblast cell lines. The transcripts were amplified using an upstream primer in the unique exon and a downstream primer in exon 10 of *GNAS1*. Because of alternative splicing of exon 3 and multiple *Fok*I restriction-enzyme cut sites, nested amplification from exons 4–6 was performed on each transcript prior to *Fok*I digestion. The *Fok*I − allele (c.393T) gave a 195-bp product, and the *Fok*I + allele (c.393C) gave two products, of 122 and 73 bp (only the 122-bp product was visualized). An electropherogram demonstrating the fragments from one cell line (sized on an ABI 377 sequencer) is shown. *a*, Monoallelic expression of exon 1A, with only the + allele being present. *b*, Biallelic expression of Gsα, with − and + alleles being present. *c*, Monoallelic expression of NESP55 (opposite to exon 1A), with only the − allele being present.

of the 3′ splice site. No other sequence variant to explain the inclusion of intron 4 could be found, and the IVS4: −11A→G change has not been found in 70 control chromosomes. The inclusion of intron 4 in the Gsα transcript would be predicted to result in premature truncation 21 codons downstream.

Discussion

Determining the Parental Origin of GNAS1 *Mutations*

We have exploited two oppositely imprinted overlapping mRNA transcripts, to determine the parental origin of *GNAS1* mutations in nine patients with AHO. This approach has previously been used to demonstrate the parental origin of activating *GNAS1* mutations in pituitary adenomas (Hayward et al. 2001). Using the maternally expressed NESP55 and paternally expressed exon 1A transcripts, we were able (1) to confirm the parent of origin in two patients for whom it was known (one maternal and one paternal) and (2) to demonstrate a maternal origin in five patients with PHP Ia. The present study included two adults with AHO whom we had classified as having likely PPHP, given that they were normocalcemic and had no history of hypocalcemia. One had developed hypothyroidism, at age 31 years, for which she was treated with thyroxine. Her three affected children all developed hypothyroidism between 1.5 and 4.75 years of age and hypocalcemia between 2.5 and 10.75 years of age. Her hypothyroidism could be a mild manifestation of PHP Ia or could be coincidental; in the absence of PTH-infusion testing, it is difficult to be sure. Nevertheless, it is of interest that both of these individ-

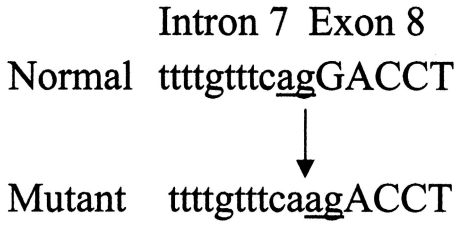

Figure 5 Effect of the c.586-1G→A mutation (patient AHO-15). The G→A substitution obliterates the normal AG acceptor site in intron 7 (*underlined*) and causes NMD of the mutant Gsα transcipt. Analysis of the NESP55 transcript reveals that exon skipping does not occur. Instead, the novel splice site created 1 nt downstream is used, resulting in deletion of a single G nucleotide with a frameshift and premature termination in exon 8. Consensus splice-acceptor sequence is defined as NCAGG (Shapiro and Senapathy 1987). Intronic and exonic sequences are given in lowercase and uppercase, respectively.

uals had mutations originating on their paternal allele and that neither had manifested any endocrine dysfunction in childhood or early adulthood, when the onset is typical. In fact, neither had come to medical attention prior to the diagnosis of AHO in their children. These results are in keeping with the observed parental-origin effects in familial AHO (Davies and Hughes 1993; Wilson et al. 1994).

This method allows for the determination of parental origin in any patient with a confirmed mutation in exons 2–13 of *GNAS1*. It is not applicable to patients with mutations in exon 1, since that exon is not present in any of the reported overlapping imprinted transcripts. It is of potential value in patients who are heterozygous for the common *Fok*I polymorphism in exon 5 (i.e., in 50% of the cohort that we studied), who can quite simply be screened for the presence of NMD indicative of a truncating mutation within that transcript. Parental origin can be determined by typing of the NESP55 and exon 1A transcripts for the *Fok*I polymorphism. We believe that, in the absence of PTH-stimulation testing, this technique could be useful for clinicians faced with a young patient with a de novo mutation and AHO who is normocalcemic and euthyroid at first presentation, since it would predict the likelihood of associated PHP Ia and would help guide the frequency of monitoring for hypocalcemia and hypothyroidism.

There have been relatively few reports of paternal transmissions of AHO, probably because normocalcemic, euthyroid patients are less likely to come to medical attention, biasing the ascertainment. In the few paternal transmissions that have been reported, there is one instance in which the offspring was found to have PTH resistance on stimulation testing (although all baseline hormone measurements were normal), indicating that the parental-origin effect may not be absolute (Schuster et al. 1994). Use of the technique that we report allows the parental origin to be determined in most patients with sporadic disease. It could potentially increase the number of patients with AHO who have confirmed paternally derived *GNAS1* mutations for endocrine evaluation. In turn, this could help quantify the predictive value of knowing the parental origin in determining the endocrine phenotype in AHO, given that the effect may not be absolute.

The Effects of Intronic Mutations on Splicing

Three of the mutations we report resulted in altered mRNA splicing detectable in the NESP55 transcript. Splice sites are characterized by a consensus nucleotide sequence at the 5′ and 3′ ends of the intron and a further consensus branch-site sequence upstream of the 3′ acceptor site. These sites are recognized by small nuclear ribonuclear proteins that bind, forming a multisubunit RNA-protein complex known as the "spliceosome." This facilitates cleavage at the 5′ end of the intron and formation of a lariat at the branch site, after which the 3′ end of the intron is cleaved and the two exon ends are ligated (Ohshima and Gotoh 1987). Branch sites have a consensus sequence of Y81 NY100 T87 R81 A100 Y94 (where "Y" denotes pyrimidine, "R" denotes purine, and "N" denotes any nucleotide) (Krainer and Maniatis 1988), although definitive identification is often difficult. Within the branch site, the adenine residue is thought to be essential for lariat formation and successful splicing (Ohshima and Gotoh 1987).

In patient AHO-17, deletion of the intron 4 splice-donor site was expected to result in exon 4 skipping. This was confirmed to have occurred in the NESP55 transcript.

In patient AHO-33, the intronic mutation (IVS4: −11A→G) 11 nt upstream of the intron 4 splice-acceptor site is of particular interest. We were unable to test whether it was de novo, because the mother of the patient was deceased. Prior to our analysis of the Gsα and NESP55 transcripts, testing with a splice-predictor program had indicated that it was not likely to be pathogenic. However, it resulted in retention of intron 4 in the NESP55 transcript, possibly as a result of altering the branch site. Nucleotides −10 to −16 upstream of the 3′ acceptor site of intron 4 may represent a branch site, although the sequence does not conform exactly to the consensus (fig. 2). It is possible that the IVS4:−11A→G change disrupts the conserved adenine nucleotide, causing failure of the spliceosome to bind or incorrect formation of the lariat during splicing with resulting retention of intron 4 in the mature mRNA (fig. 6).

In patient AHO-15, the 586-1G→A mutation affects the conserved guanine residue in the 3′ acceptor site of intron 7. The effect of most splice-acceptor site mutations is skipping of the entire exon, which in the Gsα transcript would have resulted in exon 8 skipping with a frameshift and premature protein truncation in exon 9. Analysis with a neural network splice-predictor program (see the Berkeley Drosophila Genome Project Web site) showed that the

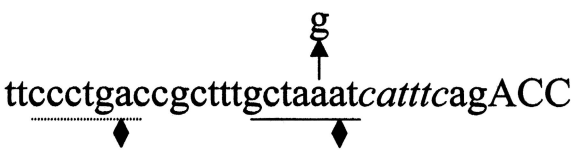

Figure 6 Splice-recognition sequences within *GNAS1* intron 4. The putative branch site GCTAAAT (*underlined*) is disrupted by the IVS4:−11A→G mutation (*arrow*). A second possible upstream branch site, CCCTGAC, is also shown (*dotted underline*). The diamond symbol denotes the conserved adenine nucleotide within the branch sites. The unusually short polypyrimidine tract CATTTC is given in italics. Exon 5 sequence is given in uppercase.

normal 3′ acceptor site of intron 7 had a score of 0.78 (where 1.0 is the most likely splice-acceptor site). While the 586-1G→A change could create an immediately adjacent novel 3′ splice site, the predicted score for this site was 0.28 (fig. 5). Analysis of the NESP55 mRNA revealed that splicing does indeed occur at this adjacent site, resulting in deletion of a single G nucleotide (c.586delG), a frameshift, and predicted premature protein truncation in exon 8, rather than exon 9.

Although these splice events were the only ones we detected, we cannot exclude the possibility that other aberrantly spliced transcripts resulted that failed to amplify sufficiently for us to detect with the method used.

The Effects of Protein-Truncating Mutations on NMD

NMD is a mechanism that protects cells from mutations that yield truncated, potentially deleterious proteins. In mammalian cells, it appears to be largely dependent on pre-mRNA splicing during which a complex of proteins involved with the splicing or subsequent steps of mRNA transport becomes associated 20–24 nt upstream of the exon-exon junctions (where they are known as "destabilizing elements"). These proteins are stripped from the mRNA with the first passage of the ribosome during translation. If translation terminates prematurely, then proteins from the downstream destabilizing complex can interact with proteins in the normal translational termination complex and other proteins, to activate a decapping protein and subsequent rapid degradation of the mRNA by intracellular 5′→3′ exonucleases (for review, see Maquat 2002). The normal NESP55 transcript is unusual in having so many introns within its 3′ UTR. It is believed that the destabilizing elements do not bind 3′ to the constitutive termination codon. This is likely to explain the stability of the normal NESP55 transcript.

In patients in whom we found protein-truncating mutations, only normal Gsα transcript was found. The simplest explanation is that the mutations resulted in NMD of the mutant transcript, but we cannot exclude the possibility that low levels of mutant Gsα transcript were obscured by preferential amplification and detection of the normal allele. In each instance, we were able to consistently detect a mutant NESP55 transcript that, had the Gsα transcript been processed in the same way, would have been expected to result in protein truncation. It is likely that the mutant NESP55 transcript was not subject to NMD because the mutations lay within the 3′ UTR. Nevertheless, it may be that our ability to detect the mutant NESP55 transcripts was more sensitive as a result of the monoallelic expression, rather than any increased stability compared to the mutant Gsα transcript. To detect the NESP55 transcript, even in the normal control lymphoblast cell lines, we found that it was necessary to perform a nested amplification, which is likely to reflect a low copy number in lymphocytes but may also have improved our detection of mutant NESP55 as compared to Gsα transcripts.

A Useful Model System for Analyzing the Effects on mRNA of Sequence Changes That Usually Result in NMD

It is well recognized that the classification of sequence changes as missense, frameshift, or splicing mutations can be inaccurate when based on inspection of the sequence alone. When possible, analysis of the mutant mRNA is desirable. Although the observation of NMD in many instances indicates the introduction of a premature termination codon, it obscures the precise effect that the mutant sequence has on the mRNA and its processing. In the patients with splicing and protein-truncating mutations whom we studied, the effects of the mutations were revealed through analysis of the NESP55 transcript. On the basis of our findings, we believe that the *GNAS1* overlapping imprinted gene cluster represents an interesting model system for detailed study of the effects that altered intronic and exonic sequences have on mRNA splicing, since any mutation downstream of *GNAS1* exon 1 is likely to be detectable in either the NESP55 or the exon 1A transcript, despite NMD of the Gsα transcript.

Genotype-Phenotype Correlations in AHO

GNAS1 mutations have the potential to affect the overlapping transcripts differently, depending on whether they occur in exon 1 or exons 2–13 and whether they occur on the maternal allele or the paternal allele. Mutations of exons 2–13 might be expected to have additional manifestations, given that they affect the overlapping transcripts as well. It seems unlikely that mutations of the maternal allele will exert any additional effects through alterations of NESP55. It is not yet clear whether paternally derived mutations in exons 2–13 will affect the function of the exon 1A transcript, but they would be expected to impair XLαs. Careful molecular and clinical characterization of patients may reveal some genotype-phenotype correlations in future, but, to date, there has been no obvious effect, other than that of parental origin on endocrine dysfunction.

Acknowledgments

We are grateful to the Birth Defects Foundation for funding S.J.R.'s post and this work, which was also funded, in part, by a pump-priming grant from the Institute of Child Health, London. Many in the patient cohort were identified during a previous study, during which L.C.W. was funded by a Medical Research Council Training Fellowship. Fetal tissue samples were kindly provided by Diana Blaydon. We would like to thank the clinicians who sent samples for *GNAS1* mutation

screening and the patients and their families who participated in this study.

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/splice.html (for neural network splice-predictor program)

Genome Database, The, http://gdbwww.gdb.org/ (for GDB ID GDB:120628)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AHO)

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