Parental origin of transcription from the human GNAS1 gene

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Abstract

Variation in the phenotypic expression of Albright's hereditary osteodystrophy (AHO) determined by the parent of transmission, suggests that the human Gs α gene (GNAS1), in which mutations occur in AHO, may be under imprinted control. GNAS1 is also known to map to a chromosomal region (20q13.11) showing syntenic homology with the imprinted mouse region 2E1-2H3. To establish if GNAS1 is indeed imprinted, we have examined the parental origin of GNAS1 transcription in human fetal tissues. Of 75 fetuses genotyped, at gestational ages ranging from 6 to 13 weeks, 13 heterozygous for a *FokI* polymorphism in exon 5 of GNAS1 were identified whose mothers were homozygous for one or other allele. RNA from up to 10 different tissues from each fetus was analysed by RT-PCR. In all cases expression from both parental alleles was shown by *FokI* digestion of RT-PCR products and quantification of the resulting fragments. No tissue specific pattern of expression was discerned in these experiments.

If genomic imprinting regulates the expression of the human GNAS1 gene, our data suggest that the effect must either be subtle and quantitative, or be confined to a small subset of specialised hormone responsive cells within the target tissues.

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lation of adenylate cyclase following binding of PTH to its receptor. Null mutations of the gene encoding the Gs α subunit (GNAS1) have been shown in patients with AHO,^{10,11} who may also show defective response to other hormones which act via the adenylate cyclase-cAMP system.¹²⁻¹⁴

AHO is an autosomal dominant disorder, with sex modification accounting for the 2:1 female to male preponderance.¹⁵ An explanation for the differing phenotypes resulting from GNAS1 mutation, and for the preponderance of maternally inherited PHP cases, has recently been offered. Genomic imprinting of GNAS1 has been suggested, following a review of published cases in which inheritance of the condition from the mother was always seen in PHP, whereas paternal inheritance produced PPHP.¹⁶ Imprinting of this locus would be consistent with the chromosomal localisation of GNAS1 at 20q13.11, a region showing syntenic homology with the imprinted murine region 2E1-2H3.^{17,18}

To date, four murine genes have been shown to be imprinted.¹⁹⁻²² Three of their human homologues are similarly modified, H19,^{23,24} IGF2,^{25,26} and SNRPN,²⁷ while both IGF2R and IGF1R escape imprinting.^{28,29} For each gene, allelic origin has been assigned to mRNA by the use of intragenic polymorphisms; presence or absence of imprinting has usually been considered as an all or none effect. We have now used similar methodology to assign parental origin to GNAS1 cDNA PCR products; allelic expression was quantified in three ways. Our experiments, examining up to 10 different tissue types across a seven week gestational spectrum, have failed to confirm that GNAS1 is under imprinted control in early human embryogenesis. However, it has become apparent that subtle imprinting effects, such as small quantitative differences between paternal and maternal expression, or imprinting confined to a single cell type in one tissue, cannot be ruled out by this type of analysis.

Materials and methods

TISSUE SAMPLES

With informed maternal consent and ethical committee permission, fetal tissue was collected from surgical terminations of pregnancy at 6 to 12 weeks' gestation, together with a maternal blood sample. Fetal tissues were separated from maternal blood clot and decidua, and individual organs isolated under the dissecting microscope. They were washed in ice cold phosphate buffered saline and immediately homogenised in 4 mol/l guanidinium isothiocyanate. Peripheral blood lymphocytes were isolated from maternal blood.

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Albright's hereditary osteodystrophy (AHO) is characterised by obesity, brachydactyly, rounded facies, short stature, and subcutaneous calcification.¹ Two phenotypic variants exist. In pseudohypoparathyroidism (PHP), hypocalcaemia and hyperphosphataemia, refractory to the administration of exogenous parathormone (PTH), are seen. This results from end organ resistance to the action of PTH. AHO is also seen without these biochemical changes, a situation assigned the term pseudopseudohypoparathyroidism (PPHP).² It is now generally accepted that these two entities are variants of the same disorder, since both may appear in the same family^{3,4} and a patient's biochemical status may even change with age.^{5,6}

The function of the renal PTH receptor-adenylate cyclase signalling pathway is disturbed in these patients.⁷ Reduced expression or altered function of an ubiquitous signalling protein, Gs, has been shown in the erythrocytes of patients with AHO.^{8,9} Gs, a heterotrimer of α , β , and γ subunits, mediates stimu-

phocyte RNA was similarly prepared, after separation of 20 ml venous blood from 20 adult volunteers on Lymphoprep (Nycomed) gradients. These samples were paired with mouth-wash samples (10 ml tap water) from their parents.

NUCLEIC ACID PURIFICATION AND cDNA SYNTHESIS

Maternal and fetal DNA were extracted from leucocytes and fetal tissues respectively by proteinase K/SDS digestion, phenol/chloroform extraction, and ethanol precipitation. The acid guanidinium isothiocyanate/phenol/chloroform extraction method was used to isolate total RNA from lymphocytes and fetal tissues.³⁰

cDNA synthesis was performed using approximately 1 µg of total RNA. This was reverse transcribed with 100 U of MMLV reverse transcriptase (Gibco/BRL), priming with 0.1 µg random hexanucleotides (Pharmacia) in a 30 µl reaction volume. Other reaction components were: 50 mmol/l Tris HCl pH 8.3, 55 mmol/l KCl, 3 mmol/l MgCl₂, 100 µmol/l each dNTP, 20 mmol/l DTT, and 10 U human placental ribonuclease inhibitor (Gibco/BRL). Samples were incubated at 37°C for 60 minutes and then the reverse transcriptase was denatured at 95°C for five minutes; 15 µl of this cDNA mix was then used for PCR amplification. Negative controls, to which no reverse transcriptase was added, were also amplified.

POLYMERASE CHAIN REACTION

A single pair of primers was designed to amplify both genomic and cDNA. The sequences were GNAS-4: 5'-AGAAGG-CAACCAAAGTGCAGGACA (sense) and GNAS-5: 5'-AGGGAAGTCAAAGTCAG-GCACGTT (antisense).

Genomic DNA amplification was performed using 0.5 µg DNA template, in the presence of 1 × PCR buffer (50 mmol/l KCl, 10 mmol/l Tris HCl pH 8.3, 1.5 mmol/l MgCl₂, 100 µg/ml gelatin), 0.25 µmol/l each primer, 0.1% Triton X-100, 1 U *Taq* polymerase (Perkin-Elmer Cetus), and 250 µmol/l each dNTP.

PCR cycling was as follows: 94°C/30 seconds, 65°C/30 seconds, 72°C/60 seconds, for 35 cycles, and final extension at 72°C/10 minutes. This amplified a fragment of 256 bp (fig 1). cDNA amplification was performed by adding 15 µl of the cDNA synthesis product above (including dNTPs) to 35 µl of a mix composed of 1 × PCR buffer, primers, *Taq* polymerase, and gelatin, as above. PCR cycling was as for genomic DNA but for 30 cycles only, and generated a 173 bp product (fig 1). End labelling of primer GNAS-4 was performed with $\gamma^{32}\text{P}$ -ATP (5000 Ci/mmol, Amersham International plc) in the presence of T4-polynucleotide kinase before addition to the cDNA PCR mix.

ANALYSIS OF ALLELIC EXPRESSION

A *FokI* polymorphism in exon 5^{31,32} was used to genotype DNA and to assign parental origin to each allele in heterozygotes; 3 µl of PCR product was digested with 10 U *FokI* (New England Biolabs) at 37°C for six hours. Predicted cleavage product sizes were 205/51 bp for genomic DNA and 122/51 for cDNA. On 3% agarose gels the small cleavage fragment (51 bp) cannot be resolved from primer-dimer complexes.

³²P labelled fragments were separated on 10% polyacrylamide gels and visualised by ethidium bromide staining. The bands were excised and added to 10 ml Scintan EX (BDH) for direct scintillation counting. Alternatively, the intact gel was fixed in 10% methanol, 10% acetic acid, and 5% glycerol, dried onto Whatman 3MM paper, and scanned on a Molecular Dynamics model 425E-130 phosphorimager after 20 minute and 40 minute exposures. The band densities were then computer analysed (Imagequant, Molecular Dynamics).

In addition, PCR primer GNAS-4 was fluorescein labelled during synthesis, using Fluorprime (Pharmacia). *FokI* digestion products were then analysed on an automated laser fluorescence sequencer (ALF, Pharmacia) and peak areas representing each allele were integrated using the manufacturer's software.

Figure 1 Diagram of part of the *GNAS1* gene, showing the positions of the polymorphic *FokI* site and the PCR primers GNAS-4/GNAS-5. Exons 4 and 5 are indicated by rectangles. Genomic (256 bp) and cDNA (173 bp) PCR products are easily distinguished by 3% agarose gel electrophoresis.

IMPRINTING OF THE IGF2 GENE

To provide a positive control, the allelic origin of IGF2 expression was also studied, using an *Apa*I polymorphism (allele frequencies 0.47/0.53); PCR primers and conditions were as previously reported.^{26 33} RT-PCR product (3 µl) was digested for six hours with 10 U *Apa*I (Gibco/BRL).

Results

DISCRIMINATION BETWEEN PARENTAL ALLELES OF GNAS1

Genomic DNA from 75 fetuses was typed for a C/T polymorphism in exon 5 of GNAS1 by *Fok*I digestion of the 256 bp PCR product (fig 1). Thirty-nine fetuses were heterozygous. To assign a parental identity to each allele, maternal genomic DNA, obtained from peripheral blood, was typed. Since paternal DNA was not available, the only informative situation for these experiments was one in which the mother was homozygous at the *Fok*I site. Thirteen such mother-fetus pairs were identified and analysed further.

BIALLELIC EXPRESSION OF GNAS1

Total RNA was isolated from available fetal organs and reverse transcribed before PCR amplification. Up to 10 tissue types were analysed from each fetus (table 1), the material spanning 6 to 13 weeks of gestation. The RT-PCR product (173 bp) was easily distinguishable from the 256 bp genomic DNA product (figs 1 and 2A), providing a control for any genomic DNA contamination of the RNA. Such contamination was in fact never seen. After *Fok*I digestion, visual assessment of ethidium stained 3% agarose gels indicated roughly equivalent biallelic expression in all fetal tissues at all gestational ages (fig 2A). IGF2 expression by comparison, in the same tissues from fetuses DM09 and LR19, showed exclusive paternal transcription, consistent with previous reports^{25 26} (fig 2B).

QUANTIFICATION OF ALLELIC EXPRESSION

Three methods were used to quantify the relative expression of each parental allele. Firstly, cDNA-PCR was performed in the presence of ³²P end labelled oligonucleotide primer GNAS-4 (fig 1). The relative expression of each allele was then quantified by excision of the bands from an acrylamide gel and scintillation counting, or by phosphorimager analysis of the fixed dried gel (fig 3).

Finally, an alternative non-radioactive technique, using a fluorescein labelled primer (again GNAS-4) and automated laser fluorescence (ALF), was used. The amounts of digested and undigested cDNA-PCR product were estimated by computer integration of the peak areas corresponding to each fragment (fig 4).

As shown in detail in tables 2 to 4, all three analytical methods suggested a slight predominance (ratio ~1.2) of expression from the maternal GNAS1 allele. However, this effect did not reach statistical significance for the data generated from fluorescein labelling and automated peak integration (table 4). It is further discussed below.

GNAS1 EXPRESSION BY PERIPHERAL BLOOD LYMPHOCYTES

Following the above experiments, a second source of RNA was studied. In this experiment, DNA and RNA were extracted from peripheral blood from persons in one generation, and mouthwash DNA was later obtained from the parental generation. This was designed to eliminate any bias introduced by possible maternal blood/tissue contamination of our fetal samples. Twenty persons were genotyped and in those heterozygous for the *Fok*I polymorphism (n=13), the relative expression of each parental allele in lymphocyte RNA was measured as above. Only then were the parents of these 13 persons genotyped. This showed seven informative persons in whom one or both parents were homozygous,

Table 1 Range of tissues examined in 13 informative fetuses. Gestational ages are indicated in weeks. The fetuses are separated into two groups to allow for the detection of possible systematic errors owing to incomplete restriction enzyme digestion; their mothers are homozygous either for presence (n=7) or absence (n=6) of the *Fok*I restriction site

Fetus no	Gestation (weeks)	Tissues										
		Heart	Sp cord	Muscle	Kidney	Lung	Gut	Eye	Brain	Adrenal	Stomach	Gonad
Maternal allele upper (uncut) (n=6)												
MC2601	10	+		+		+	+	+	+			
CA2505	6			+					+			
LF0101	10	+	+	+	+		+		+			
FB1103	12	+		+	+	+	+	+		+		
SN3010	8	+	+	+								
LR1907	13	+	+	+	+		+	+	+	+	+	+
Maternal allele lower (cut) (n=7)												
NG1003	6								+			
DB2807	8		+	+					+			
HW3004	11	+	+	+				+				
EH1311	8		+	+		+						
DM0909	9			+		+	+		+			
MT1509	7		+	+								
HM1709	10	+	+	+		+	+					

Table 2 Ratio of expression of GNAS1 alleles (maternal/paternal), as measured by direct scintillation counting, phosphorimager analysis, or automated laser fluorescence (ALF). Each figure is the mean of between two and six experiments. Persons are again divided into those in whom the mother is homozygous for presence of the FokI site or for absence of the site. No systematic bias in favour of the larger fragment was seen, suggesting that FokI digestion was complete and that failure to digest heteroduplexes was not a significant problem with this enzyme

	Tissue										
	Heart	Sp cord	Muscle	Kidney	Lung	Gut	Eye	Brain	Adrenal	Stomach	Gonad
Maternal allele upper (uncut)											
Scintillation:											
Mean ratio mat/pat (SEM)	1.204 (0.03)	1.343 (0.137)	1.168 (0.057)	1.11 (0.062)	1.15 (0.2)	1.22 (0.09)	1.22 (0.014)	1.34 (0.113)	1.04 (0.028)	0.91 (—)	1.04 (—)
Phosphorimaging:											
Mean ratio mat/pat (SEM)	1.332 (0.157)	1.596 (0.361)	1.185 (0.147)	0.967 (0.039)	1.155 (0.134)	1.18 (0.068)	1.303 (0.045)	1.34 (0.18)	0.8 (0.24)	1 (—)	0.93 (—)
ALF:											
Mean ratio mat/pat (SEM)	1.31 (0.148)	1.22 (0.08)	1.28 (0.09)	1.25 (0.068)	1.11 (0.028)	1.17 (0.162)	1.3 (0.184)	1.436 (0.146)	0.97 (0.17)	1.29 (—)	1.17 (—)
Maternal allele lower (cut)											
Scintillation:											
Mean ratio mat/pat (SEM)	1.03 (0.028)	1.154 (0.03)	1.085 (0.049)		1.07 (0.043)	0.95 (—)	1.16 (0.028)	1.25 (0.063)			
Phosphorimaging:											
Mean ratio mat/pat (SEM)	1.215 (0.021)	1.322 (0.168)	1.096 (0.033)		1.09 (0.141)	1.06 (—)	1.385 (0.035)	1.52 (0.158)			
ALF:											
Mean ratio mat/pat (SEM)	1.29 (0.127)	1.28 (0.13)	1.03 (0.063)		1.04 (0.004)	1.12 (0.27)	1.18 (—)	2.1 (0.214)			

Table 3 Ratio of expression of *GNAS1* alleles (maternal/paternal) in adult lymphocytes. Seven informative persons are represented. Scintillation counting (single experiment), phosphorimager analysis, and ALF (mean of six experiments) showed no significant predominance of maternal to paternal expression

	MB2906	JJ0603	LC1303	EG3011	CM3110	KM0903	LF0105
Scintillation:							
Ratio mat/pat	0.87	1.16	1.26	1.41	0.85	1.1	1.88
Phosphorimaging:							
Mean ratio mat/pat (SEM)	1.23 (0.073)	0.95 (0.066)	1.04 (0.089)	1.24 (0.19)	1.08 (0.061)	1.0 (0.102)	1.1 (0.13)
ALF:							
Mean ratio mat/pat (SEM)	0.99 (0.062)	0.87 (0.075)	1.35 (0.106)	1.4 (0.122)	1.5 (0.101)	1.07 (0.095)	1.15 (0.069)

Figure 3 Phosphorimager analysis of a 10% polyacrylamide gel, showing biallelic expression of *GNAS1* following *FokI* digestion of RT-PCR products from fetus LR 19. Ten tissue types are shown. Below each lane are the values obtained by quantification of each band, expressed as a ratio of maternal (upper) : paternal (lower) allele.

allowing assignment of the origin of each allele. No statistically significant difference in parental allelic expression could be shown by scintillation counting, phosphorimaging, or automated laser fluorescence (table 3).

Discussion

The pattern of inheritance of Albright’s hereditary osteodystrophy has suggested some form of parental control of *GNAS1* transcription.¹⁶ From the available evidence it was anticipated that were this gene imprinted, maternal monoallelic expression might be demonstrable in one or more tissues. However, our three analytical methods showed biallelic expression in all fetal tissue types examined and at all gestations.

For some imprinted genes, privileged tissues may fail to silence the imprinted allele.^{21 28} This implies that control of imprinting can operate in a tissue specific manner. Furthermore, in individual tissues, some imprinted genes may also show incomplete transcriptional repression.³⁶⁻³⁸ The mechanism underlying the latter observation could be either biallelic expression by certain cell types within a tissue, or alternatively partial silencing of one allele in all cells of such tissues. Both of these observations suggest that imprinting is not necessarily an “all or nothing” phenomenon. An analogy which may be drawn is that of partial escape from X inactivation by certain genes, including the human steroid sulphatase gene.³⁹ A measure of the ratio of female to male steroid sulphatase activity (XX dosage:XY dosage) yields a range of 1.42, 1.7, or 1.8:1^{34 35 40} rather than the 1:1 or 2:1 values expected for complete inactivation or non-inactivation of this gene respectively.

If *GNAS1* is under imprinted control, such control is unlikely to operate in a simple global way. In erythrocytes of patients with PHP type 1a, Gs activity is reduced to about half normal, rather than being 0 or 100% as expected for a completely imprinted gene. This suggests the possibility of cell type specific or subtle quantitative imprinting effects. It was for this reason that we surveyed a wide range of different tissues and quantitated relative levels of expression as far as was possible.

Both of our ³²P based assays suggested a small preponderance of maternal over paternal expression (ratio ~1.2) in virtually all tissues examined (tables 2 and 3). A similar but non-significant trend was seen using the fluorescein labelling protocol; this assay disappointingly

Figure 4 Analysis of allelic expression of *GNAS1* by automated laser fluorescence. Peaks 1-4 represent the fluorescent primer peak, primer-dimer complexes, and digested (122 bp) and undigested (173 bp) *GNAS1* products respectively. Lanes 1 and 2 show results obtained from fetuses homozygous for cut and uncut fragments. Lanes 3-7 represent the heterozygous fetus LR 19 (kidney, muscle, brain, gut, adrenal) and show the equivalence of maternal and paternal peak sizes.

displayed a rather greater degree of random variability. Although a slight maternal predominance of transcription would provide a possible explanation for the more severe phenotype observed on maternal transmission of PHP/PPHP, we do not consider that our results prove this point. There is a risk that contamination of the fragmented fetal tissues by maternal blood or tissue could give rise to a similar effect, despite the careful washing which was performed. To address this possibility, the analysis of adult peripheral blood lymphocyte *GNAS1* expression was undertaken. Not only was this experiment free from possible maternal contamination effects, but it was also performed blinded, the RT-PCR quantification being performed before the parental genotypes were revealed. Table 3 shows that a reproducible difference in expression levels between the maternal and paternal alleles was not obtained in this experiment.

If *GNAS1* imprinting is a tissue specific phenomenon, it should be possible from the phenotypic features of AHO to predict which tissues might be involved. These would include PTH target tissues and other endocrine organs in which adenylate cyclase was the second messenger and whose function is disrupted in AHO, namely kidney (proximal tubule), thyroid, gonad, and bone. Why such embryologically diverse tissues should share a common transcriptional control mechanism is, however, difficult to explain. We have been unable to show a target tissue specific effect at the whole organ level, having examined kidneys from three fetuses and one pair of fetal ovaries. It remains possible, though, that imprinting is limited to a specific subset of hormone sensitive cells. If so, examining the whole fetal kidney might not show such an effect, as the PTH sensitive proximal renal tubular epithelial cells would form a tiny proportion of the total tissue.

In summary, we have found no compelling experimental evidence that *GNAS1* is under imprinted control, despite the strong clinical evidence supporting such an effect. Prospective study of further AHO pedigrees, combined with mutational analysis, may shed further light on the underlying postulate of imprinted inheritance of PHP and PPHP.

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