Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 10038–10043, August 1998
Genetics

The human *GNAS1* gene is imprinted and encodes distinct paternally and biallelically expressed G proteins

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Edited by Stuart H. Orkin, Harvard Medical School, Boston, MA, and approved June 18, 1998 (received for review April 29, 1998)

ABSTRACT **The *GNAS1* gene encodes the α subunit of the G protein G_s, which couples receptor binding by several hormones to activation of adenylate cyclase. Null mutations of *GNAS1* cause pseudohypoparathyroidism (PHP) type Ia, in which hormone resistance occurs in association with a characteristic osteodystrophy. The observation that PHP Ia almost always is inherited maternally has led to the suggestion that *GNAS1* may be an imprinted gene. Here, we show that, although $G_s\alpha$ expression (directed by the promoter upstream of exon 1) is biallelic, *GNAS1* is indeed imprinted in a promoter-specific fashion. We used parthenogenetic lymphocyte DNA to screen by restriction landmark genomic scanning for loci showing differential methylation between paternal and maternal alleles. This screen identified a region that was found to be methylated exclusively on a maternal allele and was located ≈35 kb upstream of *GNAS1* exon 1. This region contains three novel exons that are spliced into alternative *GNAS1* mRNA species, including one exon that encodes the human homologue of the large G protein XLαs. Transcription of these novel mRNAs is exclusively from the paternal allele in all tissues examined. The differential imprinting of separate protein products of *GNAS1* therefore may contribute to the anomalous inheritance of PHP Ia.**

Hormone signaling through many cell surface receptors is coupled to cyclic AMP generation, via the heterotrimeric GTP-binding protein G_s, which stimulates adenylate cyclase. The α subunit of G_s ($G_s\alpha$) is encoded by the *GNAS1* gene, located on chromosome 20q13.11 and originally reported to consist of 13 exons (1). Heterozygous null mutations of *GNAS1*, with reduced red cell $G_s\alpha$ activity, are found in patients with pseudohypoparathyroidism type Ia (PHP Ia) (2–4). However, analysis of published pedigrees has indicated that PHP Ia almost always is inherited maternally (5), suggesting that *GNAS1* might be an imprinted gene. Despite this clinical observation, we have shown previously that *GNAS1* is biallelically expressed in a wide range of human fetal tissues (6). Although monoallelic expression of *GNAS1* confined to particular cell types or developmental stages has not been excluded rigorously, to date the only molecular evidence in favor of imprinting of the gene is from studies of murine *Gnas1*. A difference in intensity of *in situ* hybridization signals in the renal glomerulus was found between mice with paternal or maternal uniparental disomy for the region of chromosome 2 containing *Gnas1*, suggesting a predominantly paternal origin of glomerular *Gnas1* transcripts (7).

Here, in contrast, we show that human *GNAS1* is indeed imprinted but that this imprinting is promoter-specific. Novel *GNAS1* exons ≈35 kb upstream of the originally described exon 1 lie within a region of allele-specific methylation. Transcripts from this region are alternatively spliced onto exon 2, yielding mRNA species that are derived exclusively from the paternal allele, including one that encodes a protein homologous to the large $G_s\alpha$-related protein XLαs of the rat (8). The XLαs promoter is active only on a paternal allele, even in the same tissue samples in which transcription from the major promoter (upstream of exon 1) is biallelic.

MATERIALS AND METHODS

Restriction Landmark Genomic Scanning. The screening method using human parthenogenetic material will be detailed elsewhere. In brief, peripheral blood DNA from patient F.D. (9) or from age-matched normal Scottish females was analyzed by RLGS-M (10) by using the enzyme combination *Asc*I—*Eco*RV–*Mbo*I. The A20 spot, identified by being absent (methylated) in F.D. DNA but present at haploid intensity in all controls, was cloned into pBluescriptII (Stratagene) as described (11).

DNA Sequencing. Sequencing was performed by using [^{33}P]-ddNTP or dye-terminator cycle sequencing kits (both Amersham). IMAGE cDNA clone 359933 was identified by BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/) with the A20 genomic clone. It then was obtained from the MRC Human Genome Mapping Project (HGMP) Resource Centre and completely sequenced.

Genomic Cloning. The RPCI1 human PAC library (from the HGMP Resource Centre) was screened by PCR for the presence of *GNAS1* exon 7. Four clones (60o16, 63n2, 110p14, and 309f20) were isolated. One (309f20) was positive on rescreening for the presence of exon A20. Subcloned *Bam*HI and *Sac*I–*Asc*I fragments containing this exon were sequenced.

Methylation Analysis. A 46,XX parthenogenetic lymphoblastoid cell line was established from patient F.D. and used to prepare genomic DNA for Southern blotting by standard methods (9).

RT-PCR Analysis. Collection of first trimester fetal material and genotyping for a *Fok*I polymorphism in *GNAS1* exon 5 were described (6). For reverse transcription, RNA samples and 500 nM primer dGAGCTCGAGTCGACATCGA$(T)_{17}$ in water were incubated at 85°C for 5 min and then placed on ice. The reverse transcription components then were added, giving a 30-μl reaction of 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP, 100 units of

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: PHP, pseudohypoparathyroidism; RLGS-M, restriction landmark genomic scanning for methylation.
Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AJ224867 (cDNA clone 359933) and AJ224868 (XLαs-containing genomic region)].
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Superscript II (GIBCO/BRL), and 39 units of RNAguard (Pharmacia). This was incubated for 60 min at 42°C, 15 min at 50°C, and 15 min at 95°C. This RT mix (2 μl) was amplified in a 50-μl reaction containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 300 nM each primer, 200 μM each dNTP, and 25 units/ml recombinant *Taq* polymerase. Cycling conditions were 5 min hot start 94°C, followed by 40 cycles (94°C, 64°C, 72°C for 45 s, 45 s, 60 s). All reactions included a common downstream exon 6 primer (dCCTTGGCATGCTCATAGAATTC). The upstream primer was located in exon 2 (dACCATTGTGAAGCAGATGAGGAT), exon 1 (dCCATGGGCTGCCTCGGGAACA), or the XLαs exon (dGGATGCCTCCGCTGGTTTCAG). PCR product (15 μl) was digested with 1 unit of *Fok*I (New England Biolabs) for 2 h at 37°C, followed by heat inactivation at 65°C for 15 min, ethanol precipitation, and analysis on 2% agarose gels.

RESULTS

Identification of *GNAS1* in a Screen for Imprinted Genes. That *GNAS1*, despite its biallelic transcription (6), might indeed be imprinted was indicated to us by its identification in a screen for unknown imprinted genes. We devised an approach to screening for differentially methylated loci, by using DNA from an individual (F.D.) whose peripheral blood leucocytes are parthenogenetic and isodisomic (9). By Southern blotting for known imprinted loci (*SNRPN*, chromosome 15q11-q13; *IGF2R*, chromosome 6q26), we first confirmed our prediction that F.D. lymphoblastoid cell DNA should lack paternal-specific methylation patterns (Fig. 1). This suggested that screening for methylation differences between F.D. and normal DNA then might be used to identify unknown imprinted human genes. Unlike other human parthenogenetic sources (ovarian teratomas) in which uniparental methylation patterns are not faithfully retained (12, 13), use of parthenogenetic leukocyte or lymphoblastoid cell DNA was expected to allow precise matching for cell type and age with normal DNA samples (14).

By using restriction landmark genomic scanning (15–17), we compared DNA from F.D., from six age-matched, normal Scottish females, and from a complete hydatidiform mole. A differentially methylated spot (A20), absent from F.D. but present at haploid intensity in all control females, was cloned and sequenced. Database searching showed that this 232-nt *Mbo*I–*Asc*I fragment (corresponding to nucleotides 1,661–1,892 of deposition AJ224868) contained a single 91-nt exon, as indicated by the fact that this 91-nt region also forms part of an IMAGE cDNA clone, 359933 (827-k06).

When completely sequenced, cDNA clone 359933 unexpectedly proved to be derived from the *GNAS1* gene; it consists of: (*i*) a 376-nt upstream region, which further database searching showed to be homologous to part of the 5′ portion of the mRNA encoding the rat Gsα-related protein XLαs (8); (*ii*) the 91-nt A20 exon; (*iii*) another previously unidentified 67-nt exon, A21; (*iv*) *GNAS1* exons 2 and 3; and (*v*) a variant terminal exon (3N) and polyadenylation site, located in intron 3 and homologous to the novel GsαN1 terminal exon described in the rat (18).

Structure and Methylation of the Imprinted Region of *GNAS1*. In the rat, XLαs is a 78-kDa protein encoded by an

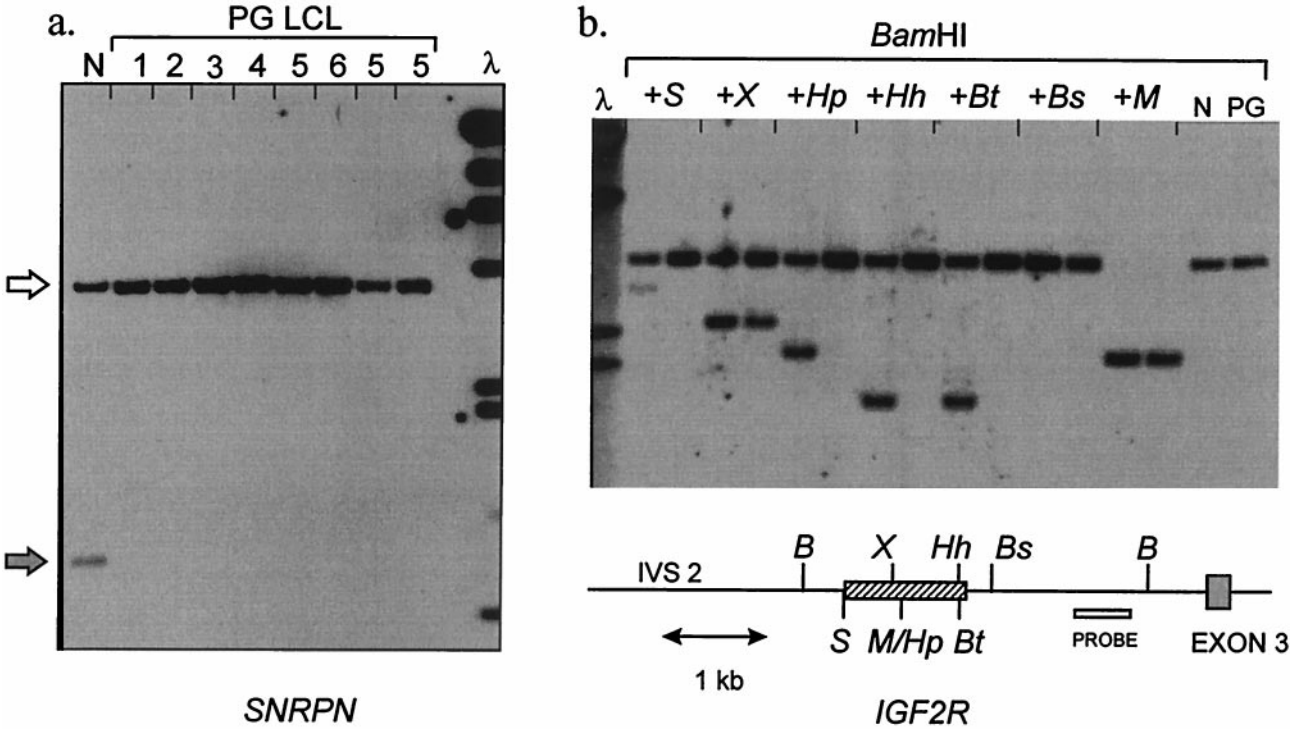

FIG. 1. Uniparental methylation pattern at imprinted loci in human parthenogenetic LCL. (*a*) Methylation of *SNRPN* (chromosome 15q11-q13). The probe used for Southern blotting is KB17, a standard diagnostic reagent for Prader–Willi syndrome. DNAs are digested with *Xba*I + *Not*I. The paternal allele (gray arrow) is unmethylated, yielding a 0.9-kb fragment from normal DNA (N). 1–6, six subcultured lines of parthenogenetic lymphoblastoid cells (LCL) established from patient F.D. Only the methylated 4.2-kb maternal band (white arrow) is present. λ, λ*Hin*dIII marker; 23, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. (*b*) Methylation of *IGF2R*. The probe (open box) is fragment *Bx* of clone pE3UP (25), which detects a 3.2-kb *Bam*HI fragment. Pairs of lanes contain normal (N) or parthenogenetic (PG) LCL DNA digested with *Bam*HI, alone or plus a second enzyme as indicated: *B*, *Bam*HI; *S*, *Sac*II; *X*, *Xho*I; *M*/*Hp*, *Msp*I/*Hpa*II; *Hh*, *Hha*I; *Bt*, *Bst*UI; *Bs*, *Bss*HII. For each enzyme only the site nearest the probe is shown. The cross-hatched box indicates the region of allele-specific methylation; the *Hpa*II, *Hha*I, and *Bst*UI sites are methylated only on a maternal allele; the *Sac*II site in addition is methylated incompletely on a paternal allele; the *Xho*I is methylated incompletely on a maternal allele and unmethylated on a paternal allele. These results concur with previous data (25) and indicate faithful allele-specific methylation across this region in the PG LCL DNA. λ, λ*Hin*dIII marker; 6.6, 4.4, 2.3, 2.0 kb.

mRNA in which variant 5′ sequences are spliced onto *Gnas1* exon 2; its expression appears specific to certain endocrine secretory cells and neurons (8). In human cDNA clone 359933, the XLαs-like sequences are separated from *GNAS1* exon 2 by exons A20 and A21. As discussed below, this cDNA is unlikely to encode a functional protein, both because it is truncated at its 3′ end and because the A20-A21 region shifts the frame between XLαs and exon 2 and introduces a stop codon in frame with the exon 2–3 ORF. Nonetheless, the presence of both XLαs-homologous and exon 2–3 sequences indicated that cDNA 359933 represented a genuine *GNAS1* transcript. To examine the underlying genomic structure, PAC clones containing *GNAS1* were isolated, one of which (309f20) also contained the A20 exon.

A 2.5-kb region containing XLαs, A20, and A21 exons was sequenced. The XLαs-homologous region is encoded in a single large exon lying ≈35 kb upstream of exon 1 (Fig. 2). A 1.9-kb *Sac*I–*Asc*I fragment was used as probe in Southern blotting of lymphoblastoid cell DNA to assess the methylation status of the *Not*I, *Bss*HII, and *Asc*I sites in this region (Figs. 2 and 3). Normal DNA yielded bands indicating the presence of both methylated and unmethylated alleles at all three restriction sites. Parthenogenetic DNA, in contrast, was completely methylated at all three sites. This confirms that the XLαs-A20 region of *GNAS1* is differentially methylated, the paternal allele being unmethylated and the maternal methylated. In contrast to the differential methylation of the XLα region, Southern blotting showed that *Not*I and *Ngo*MI sites in the vicinity of exon 1 (Fig. 2) were unmethylated on both maternal and paternal alleles (data not shown).

Monoallelic Expression of XLαs-Containing Transcripts. To determine whether the differential methylation of *GNAS1* reflects monoallelic expression, RT-PCR analysis of fetal RNA samples was performed. Previously, we had used RNA from first trimester fetuses, informative for a *Fok*I polymorphism in exon 5, to show that *GNAS1* mRNA (amplified by using primers in exons 4 and 5) was derived equally from both alleles in all tissues surveyed (6). RNA samples from seven heterozygous fetuses now were reanalyzed by RT-PCR between exons 2 and 6, followed by *Fok*I digestion. This assay, which examines total expression of all of the major *GNAS1* species, regardless of which exon is spliced in front of exon 2, confirmed biallelic expression in all samples (Fig. 4, all pairs of lanes labeled 2). When, however, RT-PCR was performed by using an upstream primer in the XLαs exon rather than exon 2, *Fok*I digestion revealed that all of the XLαs-containing transcripts were derived from the paternal allele (Fig. 4, pairs of lanes labeled XL). Identical results were obtained for several fetuses, both those with a $+^{m}/-^{p}$ genotype (Fig. 4, fetuses DM0909, HM1709) in which the XL RT-PCR products were completely *Fok*I-resistant and those with a $-^{m}/+^{p}$ genotype (Fig. 4, fetuses LR1907, SN3010, MC2601) in which

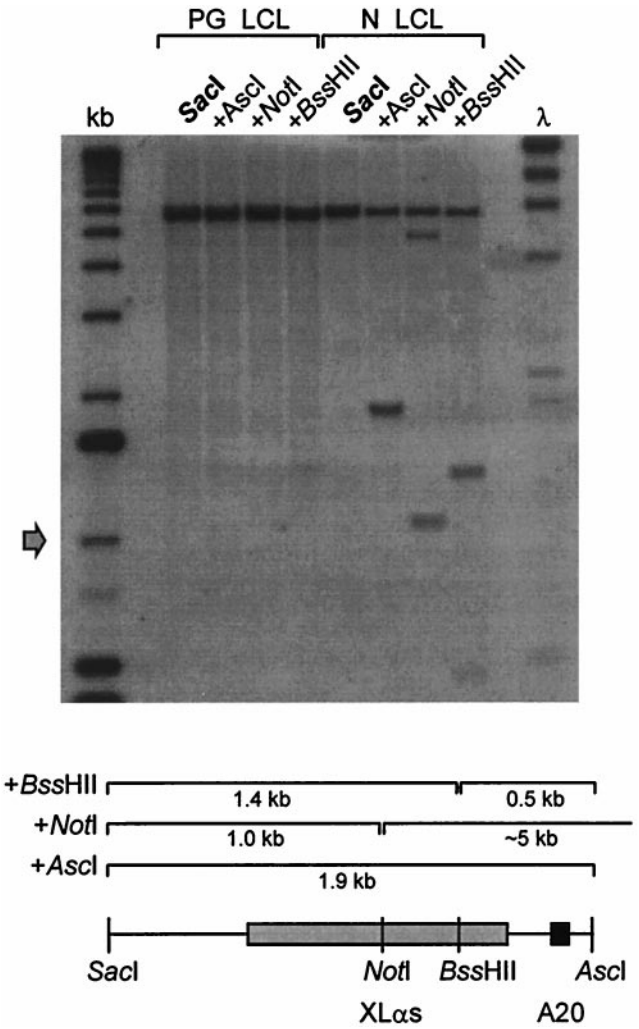

FIG. 3. Differential methylation in the XLαs region of *GNAS1*. The probe used was a 1.9-kb *Sac*I–*Asc*I genomic fragment containing both the XLαs and A20 exons (shaded boxes). Genomic DNA from parthenogenetic (left) or normal (right) lymphoblastoid cell DNA was digested with *Sac*I, alone or in combination with *Asc*I, *Not*I, or *Bss*HII. *Sac*I generates a fragment of ≈6 kb; the expected fragment sizes derived from additional cleavage at the methylation sensitive sites are indicated on the diagram. λ, λ*Hin*dIII marker; 23, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. kb, 1-kb ladder (GIBCO/BRL); upwards from arrow 1.0, 1.6, 2.0, 3.1, 4.1, 5.1, 6.1, 7.1 kb.

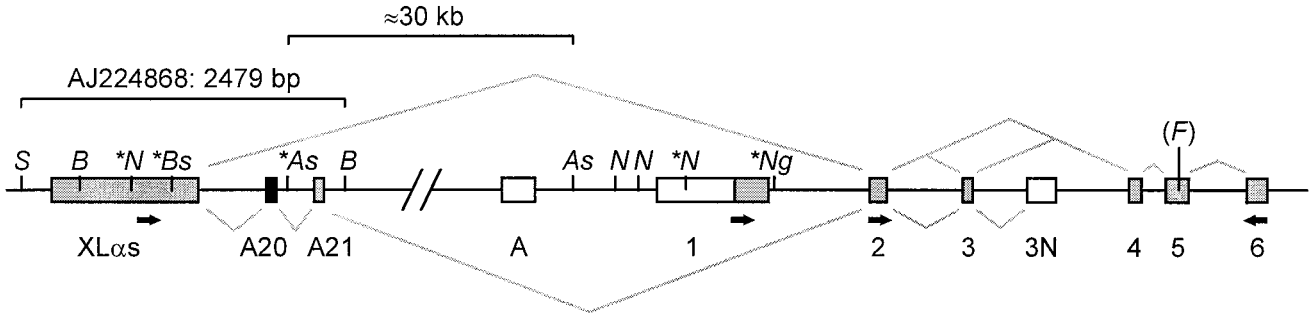

FIG. 2. Organization of the XLαs region of *GNAS1*. The map is not to scale, and not all restriction sites are shown, with the exception of *Asc*I and *Not*I. Grey lines above the exons represent the splicing patterns observed in the majority of XLαs-containing RT-PCR products and below the line the splicing pattern seen in the 359933 cDNA clone. Italic letters indicate restriction sites: *S*, *Sac*I; *B*, *Bam*HI; *N*, *Not*I; *As*, *Asc*I; *Ng*, *Ngo*MI; *Bs*, *Bss*HII; (*F*), *Fok*I (polymorphic site). Asterisks indicate those sites whose methylation status was assessed by Southern blotting (see text). The distance between the two *Asc*I sites is ≈30 kb. The 2.5-kb genomic region that was sequenced is indicated. The A20 exon is black; other exons are gray or white for untranslated regions. The exon labeled A has been described (26). Small black arrows indicate approximate positions of primers used for RT-PCR.

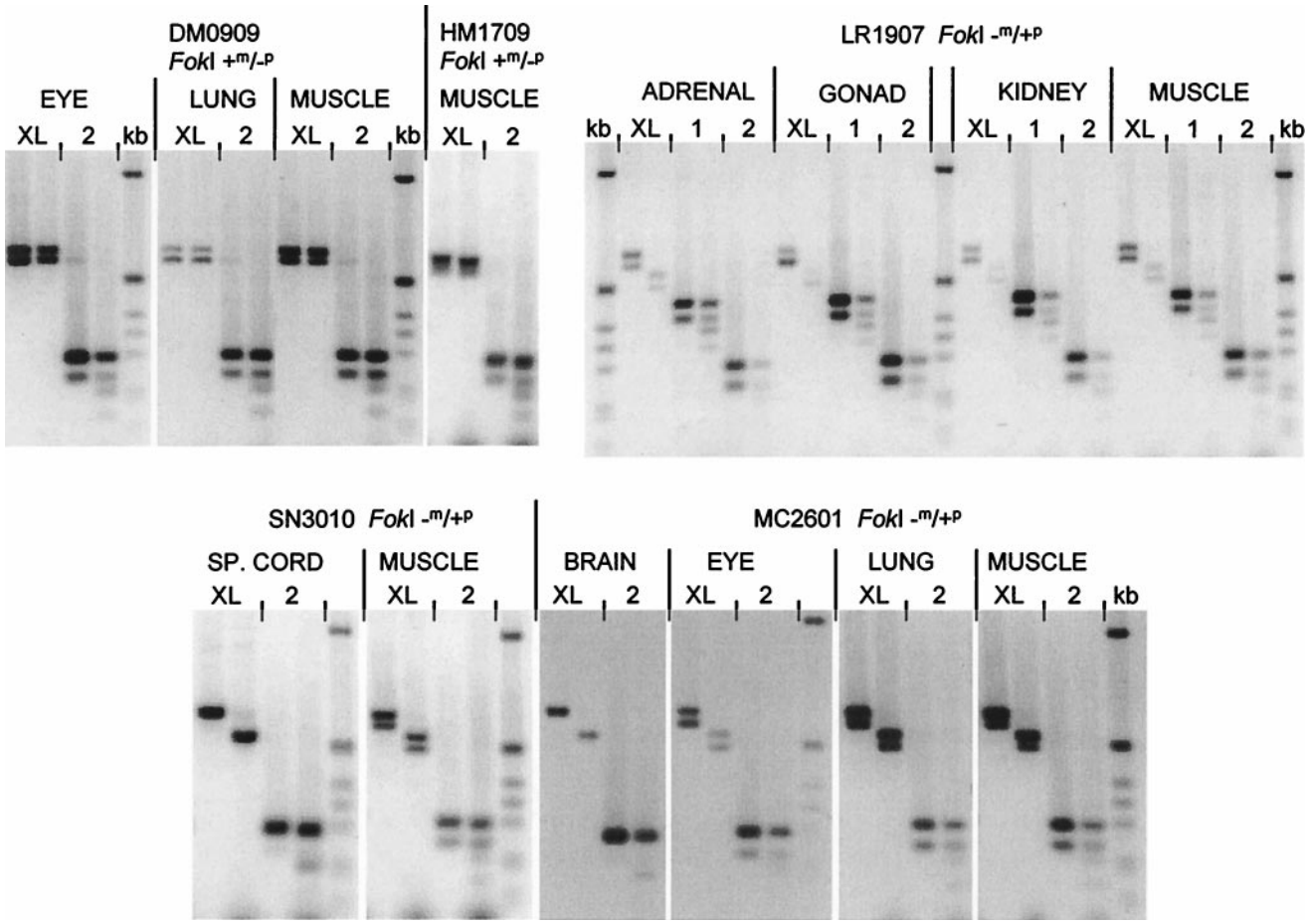

FIG. 4. Exclusively paternal expression of XLαs-containing *GNAS1* transcripts. All experiments used a downstream PCR primer in exon 6; the location of the upstream primer is indicated (either in the XLαs exon, exon 1 or exon 2) above each pair of lanes. Each pair of lanes contains undigested (*Left*) or *Fok*I-digested (*Right*) RT-PCR product. The two bands seen in most undigested samples result from alternative splicing of exon 3. In fetuses DM0909 and HM1709, the paternal allele is lacking a *Fok*I site (genotype $+^{m}/-^{p}$). The XLαs-derived RT-PCR products (XL) are completely *Fok*I-resistant. The total *GNAS1* transcripts, in contrast (2), are partially cleaved by *Fok*I, indicating their biallelic origin. In fetus LR1907, the genotype is $-^{m}/+^{p}$. The XLαs-derived products are digested to completion by *Fok*I, again demonstrating their exclusively paternal origin. Also, in this panel, exon 1-specific transcription has been analyzed separately, showing biallelic expression from the promoter upstream of exon 1. Fetuses SN3010 and MC2601 are again *Fok*I $-^{m}/+^{p}$. RT-PCR products are digested to completion if XLαs-derived but only partly when the exon 2 primer is used. kb, 1-kb ladder: 1,018, 517/506, 396, 344, 298, 220, 201, 154, 134 bp.

the XL RT-PCR products were cleaved to completion by *Fok*I. The XLαs mRNA was derived from the paternal allele in all tissues and in all seven fetuses examined. Exon 1-specific products, in contrast, were derived from both alleles (Fig. 4, LR1907, lanes labeled 1). Thus, the biallelic expression seen using the exon 2 primer does not simply reflect a mixture of transcripts derived from one paternally active (i.e., XLαs exon) and one maternally active (i.e., exon 1) promoter. Some of the ethidium-stained cleavage products of exon 2-exon 6 products in Fig. 4 appear faint. However, careful quantitation by ^{32}P-labeling and phosphorimaging or fluorescent primer labeling has shown that, within the limits of experimental error, the "pooled" *GNAS1* transcripts represented by these RT-PCR products are derived equally from maternal and paternal alleles (6).

The XLαs-exon 6 RT-PCR products were considerably shorter than the 783 bp predicted from the sequence of the 359933 cDNA clone. Sequencing of these transcripts confirmed that, in them, as in the rat XLαs mRNA, the XLαs exon is spliced directly onto exon 2, resulting in a mRNA with a continuous ORF homologous to that of the rat mRNA. RT-PCR products containing A20 or A21 were not detected in these experiments, suggesting that most XLαs-containing transcripts do not contain the A20/A21 exons. Because A20/A21-containing transcripts also do not have a continuous ORF, they may be aberrant or functionally unimportant splice forms. Additional RT-PCR experiments using a primer specific for A20 did nonetheless show that A20-containing transcripts also are derived exclusively from the paternal allele (not shown).

In almost all RT-PCR experiments, a doublet band consisting of two products differing in size by 45 bp was obtained (Fig. 4). These derive from alternatively spliced mRNAs (1) including or excluding exon 3. Both XLαs- and $G_s\alpha$-encoding transcripts show this alternative splicing. In some individual RNA samples, however (including all three spinal cords examined), only the larger exon 3-containing mRNA was detected (Fig. 4, SN3010 spinal cord, MC2601 brain).

Sequence Conservation and Repetitive Elements in the XLαs Region. Attempts to map the 5′ end of the XLαs-containing transcripts by RACE were unsuccessful, probably because of the GC-richness of this region. However, RT-PCR products diagnostic of XLαs-exon 2 spliced mRNA could be generated by using upstream primers as much as 1.1 kb from the 3′ end of the XLαs exon. (nucleotides 375–1,482 of accession number AJ224868; data not shown). These experiments indicated that the XLαs exon is >1.1 kb in size. It may extend even further 5′ because the reading frame remains open beyond the start of our sequence (1,482 nt upstream of the 3′ end of the XLαs exon). In Fig. 5, the translated human ORF

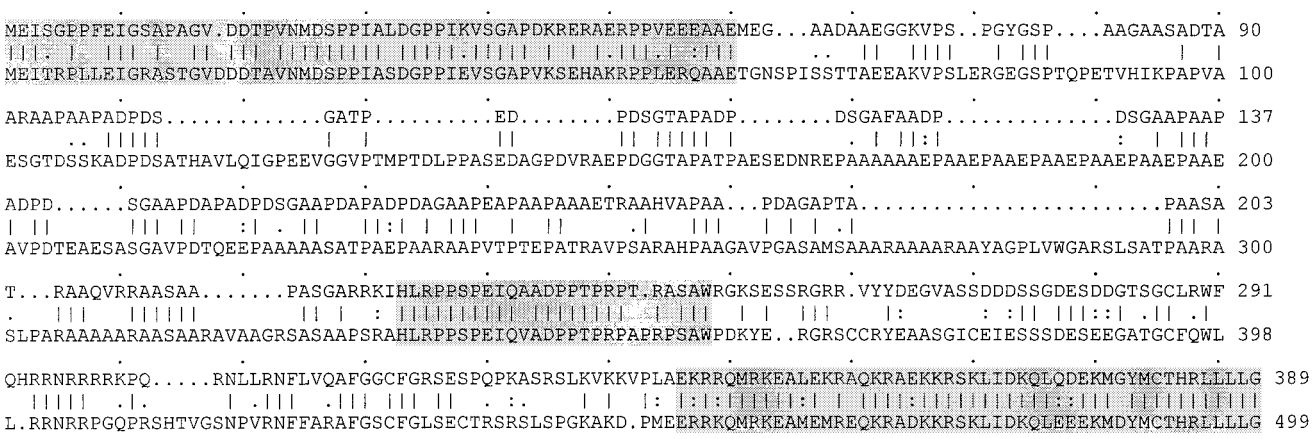

FIG. 5. Comparison between human (*Upper*) and rat (*Lower*) XLαs peptide sequences. Extensive homology is confined to the three shaded regions, whereas, as discussed in the text, the central repetitive region aligns poorly between species. Translation of the human exon has been started at position 318 relative to the *Sac*I site (AJ224868). However, the true translation initiation site has not been defined experimentally either for human or rat XLαs (8).

(starting at nucleotide 318 of AJ224868) is aligned with the peptide sequence predicted by a full length rat cDNA (8). There are well conserved regions at the N- and C-terminal extremes of the XLαs segments. The central region aligns much less well, but there are nonetheless qualitative similarities between the two species in this region, which in both human and rat contains a repetitive domain that interrupts the conserved unique regions. (There is also an additional insertion of ≈300 nt in the rat sequence.) In this repetitive region, the rat XLαs protein has a tetrapeptide repeat based on EPAA (single letter amino acid code). The predicted human protein has a tripeptide repeat motif, which is again rich in proline, alanine, and an acidic amino acid (aspartate rather than glutamate). There is also a higher order repeat of 9 or 12 amino acids (Fig. 6). The overall ratio of D+E:P:A in this region (20:22:42) is virtually identical to that in the rat EPAA repeat region.

Although the function of the repeated elements of XLαs is undefined, there are interesting parallels with the structure of the $p57^{KIP2}$ protein, encoded by another imprinted gene, *CDKN1C* (19, 20). Murine $p57^{KIP2}$ contains a proline-rich domain that overlaps with an acidic domain containing multiple tetrapeptide repeats based on the motif EPVE. In human $p57^{KIP2}$, these domains are replaced by a simpler repeat, based on PAPA (21, 22). As in XLαs, the functions of these acidic or proline/alanine rich repeats and the reason for their poor conservation between human and rodent, are unclear. However, because the XLαs region is GC-rich and repetitive, like several other regions of differential methylation in imprinted genes, it is possible that selection to maintain these features operates at the DNA rather than the protein level (23).

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74 SPGYGSPAAGAA
     SADTAARAAPAA
     PADPDSGAT
     PEDPDSGTA
     PADPDSGAF
     AADPDSGAAPAA
     PADPDSGAAPDA
     PADPDSGAAPDA
     PADPDAGAAPEA
     PAAPAAAETRAAHVA
     PAAPDAGA PTA
     PAASATRAAQVR
     RAASAAPASGARRKI 225
```

FIG. 6. Internal repeat motifs in the human XLαs exon. Almost the whole of the central poorly conserved region is included in this repeat, which begins at residue 74 and ends at residue 225, immediately before the second highly conserved region (shaded in Fig. 5).

DISCUSSION

Based on studies of multi-generation families with PHP/PPHP, only maternal inheritance appears to result in the full blown PHP syndrome, with hormone resistance (5). This observation is supported strongly by the pedigree data presented in a more recent study of a large number of families (even though its authors did not favor the imprinting hypothesis) (24). That maternal inheritance of *GNAS1* mutations may be required for development of PHP Ia suggests the existence of maternally expressed *GNAS1* transcripts. It is therefore unexpected that we have now demonstrated specific paternal expression from a *GNAS1* promoter. It remains possible that other, maternally derived *GNAS1* transcripts with distinct functions could exist or even that there is maternal-only transcription from exon 1 at discrete developmental stages or in subpopulations of hormone-responsive cells. Despite this, the possibility that XLαs is involved in the pathogenesis of PHP Ia also merits further investigation. However, for this to be consistent with the maternal inheritance pattern of PHP, one would have to explain how a paternally inherited mutation, resulting in both reduced $G_s\alpha$ and loss of XLαs, could have a less severe effect than a maternal one, resulting in reduced $G_s\alpha$ activity alone. Additional studies of the biochemical and physiological function of XLαs will be needed before definite conclusions can be drawn.

In the mouse, *in situ* hybridization has suggested preferential expression of the paternal *Gnas1* allele but confined to the renal glomerulus (7). We, in contrast, have shown here that promoter-specific imprinting of *GNAS1* in human fetal tissues is not tissue-specific. It is possible that a re-evaluation of murine *Gnas1* imprinting in such a way as to distinguish different transcript species would show evolutionary conservation of the paternal origin of XLαs-containing transcripts. If so, it also may be possible to test hypotheses about the relationship between XLαs and PHP Ia by introducing germline mutations into the murine XLαs exon.

This work was supported by the Ludovici Bequest to the University of Edinburgh; by Wellcome Trust Project Grant 053701 to D.T.B.; by Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation, Special Coordination Funds and a Research Grant for the Genome Exploration Research Project from the Science and Technology Agency of the Japanese Government, and by a Grant-in-Aid for Scientific Research

on Priority Areas and Human Genome Program from the Ministry of Education and Culture, Japan to Y.H. V.M. is supported by a scholarship from CONACYT (Mexican National Council for Science and Technology).

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