Human Molecular Genetics, 2000, Vol. 9, No. 5 835–841

An imprinted antisense transcript at the human *GNAS1* locus

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Received 17 December 1999; Revised and Accepted 14 January 2000 DDBJ/EMBL/GenBank accession nos AJ251759–AJ251761

Recent studies of the *GNAS1* gene have shown a highly complex imprinted expression pattern, with paternally, maternally and biallelically derived protein products, raising questions regarding how such transcriptional complexity is established and maintained. *GNAS1* was originally identified as the gene encoding an important and widely expressed signal transduction protein, the α subunit of the stimulatory G protein G_s. Partial $G_s\alpha$ deficiency results in the hormone resistance syndrome, pseudohypoparathyroidism type 1a. $G_s\alpha$ is encoded by exons 1–13 of *GNAS1* and, in most tissues at least, expression of this transcript is biallelic. Two large upstream exons, however, have monoallelic expression patterns, and in each case their transcripts splice onto *GNAS1* exon 2. The most 5′ of these is maternally expressed, and encodes neuroendocrine secretory protein 55 (NESP55), whose coding region does not overlap with that of $G_s\alpha$. The other exon, 14 kb further 3′, is paternally expressed, and encodes XLαs (extra large αs-like protein), translated in-frame with $G_s\alpha$ exons 2–13. This close proximity of two oppositely imprinted promoters suggested the likelihood of important regulatory interactions between them, and to investigate this possibility we have performed a search for other transcripts in the region. Here we show that the maternally methylated region upstream of the XLαs exon gives rise to a spliced polyadenylated antisense transcript, which spans the upstream NESP55 region. This antisense transcript is imprinted, and expressed only from the paternal allele, suggesting that it may have a specific role in suppressing *in cis* the activity of the paternal NESP55 allele.

INTRODUCTION

Recent studies in both human and mouse have shown that *GNAS1*, located on human chromosome 20q13 and mouse distal chromosome 2, is a complex imprinted gene. As originally described (1), *GNAS1* consisted of 13 exons, encoding the α subunit of the stimulatory guanine nucleotide-binding protein G_s. G_s is required for coupling of the ligand-activated forms of many seven-transmembrane-domain hormone receptors to the intracellular generation of cAMP. Consequently, null mutations of *GNAS1* cause the hormone resistance syndrome pseudohypoparathyroidism type 1a (PHP1a) (2–4). This syndrome shows an anomalous autosomal dominant inheritance pattern, with maternal transmission being required for full phenotypic manifestation (5,6). However, despite these clinical pointers towards *GNAS1* being an imprinted gene, human $G_s\alpha$-encoding transcripts were shown to be biallelically derived in a range of fetal tissues (7).

Targeting of the murine *Gnas* locus provided fresh evidence that this gene was indeed imprinted, since paternally and maternally inherited heterozygous mutations result in different phenotypes (8). These bear little resemblance, however, to the PHP1a phenotype, so that the relevance of this animal model to human *GNAS1* function remains unclear.

Recent studies of human *GNAS1* have shown, however, that the locus is much more complex than previously realized. As well as $G_s\alpha$, this locus also encodes two other distinct polypeptides, XLαs (9) and NESP55 (10). These proteins are encoded by alternatively spliced messages that initiate from either of two novel upstream exons, located ~35 kb (XLαs) and 49 kb (NESP55) upstream of exon 1. All these alternative first exons splice onto the acceptor site of exon 2, within the $G_s\alpha$-encoding body of the gene (11,12).

Each of the novel upstream exons is embedded within a differentially methylated region, a common feature of imprinted genes. By analysis of a *Fok*I polymorphism within the *GNAS1* transcript, we also showed that both upstream exons are monoallelically expressed. Despite their proximity to each other, however, these two exons are oppositely imprinted, as the XLαs transcript is expressed from its unmethylated paternal allele and the NESP55 transcript from its unmethylated maternal allele.

This arrangement, which is shown in Figure 1, is noteworthy for the very close proximity of two oppositely methylated and oppositely expressed promoter regions (the NESP55–XLαs intron is ~12 kb). This in turn raises a number of questions concerning the mechanism by which such a mutually exclusive expression pattern is established and maintained. One specific possibility is that mutual suppression *in cis* between the NESP55 and XLαs promoters might be exerted, as a consequence of transcription originating at one promoter and extending across the other promoter region. Precedent for such a mechanism comes from studies of the murine *Igf2r* locus, at which paternally derived

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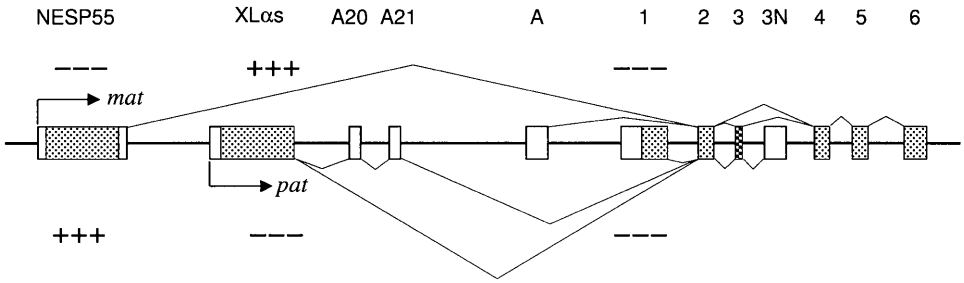

Figure 1. Schematic representation of the complex splicing patterns previously observed for *GNAS1* transcripts (sense transcripts). The diagram is not to scale. Coding regions are shaded; exons 7–13 have been omitted. The names or numbers of individual exons are indicated at the top. The methylation status of CpG-rich regions (where it has been investigated) is shown as – – – (unmethylated) or +++ (methylated), either above or below the line drawing, to refer to the maternal or paternal allele, respectively. Transcription from NESP55 is maternal, and from XLαs paternal, as indicated by the arrows. Transcription from exon I is predominantly (at least) biallelic. The allele of origin of transcripts containing exon A has not been examined. A20 and A21 are short exons found in a minority of XLαs transcripts; their inclusion disrupts the open reading frame. Exon 3 (dark shading) is alternatively spliced (an in-frame exon-skipping event) to yield alternative isoforms of $G_s\alpha$. Further isoform diversity is generated by the use of two alternative splice acceptor sites at the intron 3–exon 4 boundary (data not shown). Exon 3N is spliced onto by some truncated transcripts; it contains a termination codon and polyadenylation site.

antisense transcripts originating within a differentially methylated region in intron 2 are implicated in *cis*-suppression of the sense promoter, resulting in a maternal pattern of *Igf2r* expression (13). Such a mechanism operating at *GNAS1* might therefore implicate antisense transcription from the XLαs region in suppressing NESP55 promoter activity.

In this report, we demonstrate that there is indeed a third imprinted transcript derived from the *GNAS1* locus, which fulfils some of the expectations raised above. This is a spliced, paternally expressed antisense transcript, arising from a discrete site within the differentially methylated XLαs region, and traversing the oppositely imprinted upstream NESP55 exon. The observations suggest the differentially methylated antisense promoter region as a prime target for experimental manipulations aimed at understanding the regulation of *GNAS1* imprinting.

RESULTS

To investigate possible regulatory interactions between the NESP55 (maternally expressed) and XLαs (paternally expressed) regions of *GNAS1*, we first characterized the genomic region containing these two exons. If important regulatory elements and/or transcripts exist in this region, it is possible that they would be conserved between human and mouse, both of which show a similar pattern of promoter-specific imprinting at this locus (11,12,14). We therefore sequenced the entire NESP55–XLαs region in both human and mouse. These two sequences (GenBank accession nos AJ251760, 20 924 bp, and AJ251761, 21 927 bp) display conservation across the whole of the inter-exonic region. Outside the NESP55 and XLαs exons, the highest degree of conservation (87% identity over 285 bp) occurs in a region ~3.5 kb upstream of the XLαs exon. This highly conserved element lies at the upstream end of a 5.8 kb CpG-rich region, that also encompasses XLαs itself and ~1 kb of sequence downstream (Fig. 2).

The antisense transcript

Next, database searching using the 21 kb human genomic sequence was performed. This resulted in identification of an expressed sequence tag (EST T84962) matching the sequence of a region 1.0–1.3 kb 3′ to the NESP55 exon. The corresponding cDNA clone (IMAGE 111764) was obtained and completely sequenced. Comparison with the genomic sequence showed that this cDNA (shown as clone 3 in Fig. 3b) represented a portion of a spliced polyadenylated message that was transcribed in the antisense direction relative to NESP55.

To characterize this antisense transcript further, reverse transcription–polymerase chain reaction (RT–PCR) and 5′-RACE were performed using RNA from a normal female lymphoblastoid cell line. This allowed us to characterize a series of alternatively spliced transcripts, up to 1.1 kb in length (GenBank accession no. AJ251759). It should be noted that these RT–PCR experiments do not rely on strand-specific priming to demonstrate the antisense nature of the transcript. This is because the transcripts are all spliced, and comparison with the genomic sequence clearly demonstrates that they could have originated only from an antisense primary transcript.

The antisense RNA species identified in these experiments are shown in Figure 3. Five antisense exons were identified, four of which lie between the NESP55 and XLαs exons, whereas the final exon, containing the polyadenylation signal for the transcript, lies ~19 kb upstream of NESP55 (Fig. 3a). These antisense transcripts are a heterogeneous population (Figs 3b and 4b), due to the occurrence of exon skipping and the use of alternative splice sites within exons (Fig. 3c). Despite this, two of the three 5′-RACE products characterized initiated at exactly the same nucleotide. The largest potential open reading frame in the largest transcript would encode a 97 amino acid polypeptide lacking homology to any described protein. This, in conjunction with the heterogeneity of the antisense transcripts, suggests that they lack coding potential.

The highly conserved 285 bp genomic region previously identified in human and mouse genomic sequence comparisons was now seen to comprise the first 100 bp of antisense exon I and the region immediately upstream of it (Fig. 2a). This suggests that conservation of the antisense promoter and at least part of its transcript may be imposed by functional constraints.

Imprinting of the antisense transcript

Figure 2b summarizes the relationship between the exons of the antisense *GNAS1* transcript and the CpG-rich regions of the human and mouse loci. As mentioned above, a large CpG-rich region (shown in Fig. 2b by the presence of multiple *Hha*I and *Hpa*II restriction sites) that includes the XLαs exon extends

a.

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Human CGTGGTCTTCCAGGCCTAGCCGCCATACACCCGCCCCCCACCGGCTTCCAACCACCCC....AGCAGCACCTCTTCGGGCGTTCCAACGCGGCGCCCCCT 11646
Mouse CGTGGCCTTCTAGGCCTGGCCGCCATACGCCCGCCCGCCAACCGCTTCCAACCAACCCAGCAAGCACAACCTCTTTGGGCGTTCCAACGCGGCGCCCCCT 13135

      *
Human ATTGGACCTCCCACCCATATCCGGTTTCCGGTCTGACGCCCCCTGCTCATTCGCCAGGCAGCCTTGATTGGCATGACCCCTGCCCAATGGCTGACCCGGC 11746
Mouse ATTGGACCTCCCACCCATATCCGGTTTCCGGTCCGAAGCCCCCTGCTCATTCGCCAGGCATCCTTGATTGGCGTGACCCCAGCCCAATAGCTGACCTGTC 13235

Human GCTGGGGTCGGAGGGGAGTGAGTTGGT.GTTGGGACCCAGTTGGGCCATTCAGTGGATGCTCAAGTGGTTAAGCCGTCTTGCGTGGGTGT 11835
Mouse GCTGGGGTCGGAGGGGAGTGGGCTAGTGGTTGAGACCCAGTTGGCCTATTCGGTGTATGTTCAAACCGTTTCGCCATTTTGAGTGTGTGT 13325
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b.

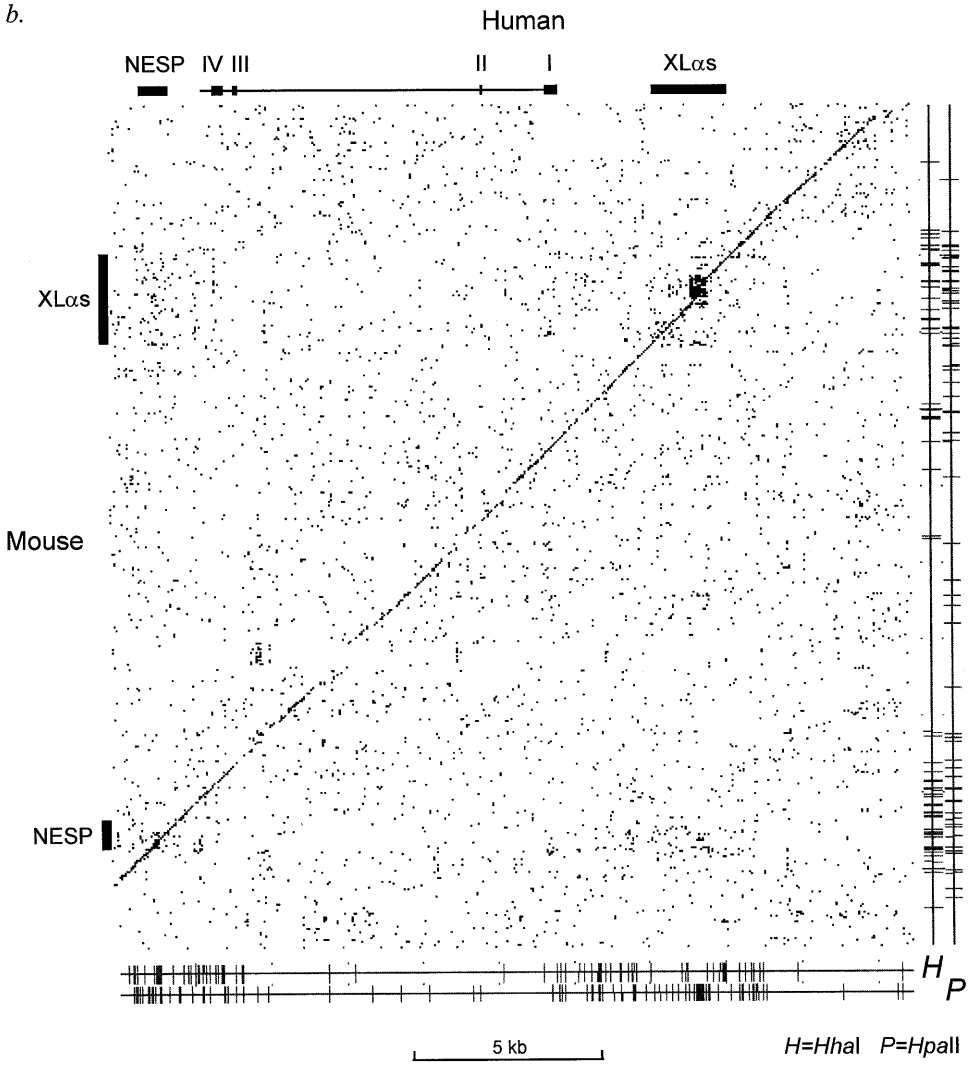

Figure 2. (**a**) Alignment of the most highly conserved segment of sequence between NESP55 and XLαs. Subsequent analysis of cDNA clones showed that the underlined segment comprises part of antisense exon I. The antisense start site (asterisk) is seen to lie at the centre of a particularly well conserved sequence block. (**b**) Dotplot comparison of the entire genomic sequences of the mouse (vertical) and human (horizontal) regions encompassing the NESP55 and XLαs exons. The comparison was generated using the GCG COMPARE program. The filled rectangles represent the positions of the NESP55, XLαs and human antisense (I, II, III and IV) exons. The positions of *Hpa*II (*P*) and *Hha*I (*H*) restriction sites are marked on the vertical right side (mouse) and horizontal lower side (human) to show the extent of the CpG-rich regions in both species.

upstream as far as the location of the first antisense exon. We showed previously that the XLαs exon and the region immediately downstream of it were methylated exclusively on the maternal allele (11). Therefore, we next assessed by Southern blotting whether the most upstream part of this CpG-rich region, adjacent to the first antisense exon, was also differentially methylated. Figure 5 shows that this region is indeed methylated on the maternal allele, in a fashion similar to that of XLαs. The presence of a single non-imprinted *Nar*I site 3 kb upstream of antisense exon I could indicate either that some CpGs scattered within the region are not imprinted or alternatively that the differentially methylated region has a bipartite structure.

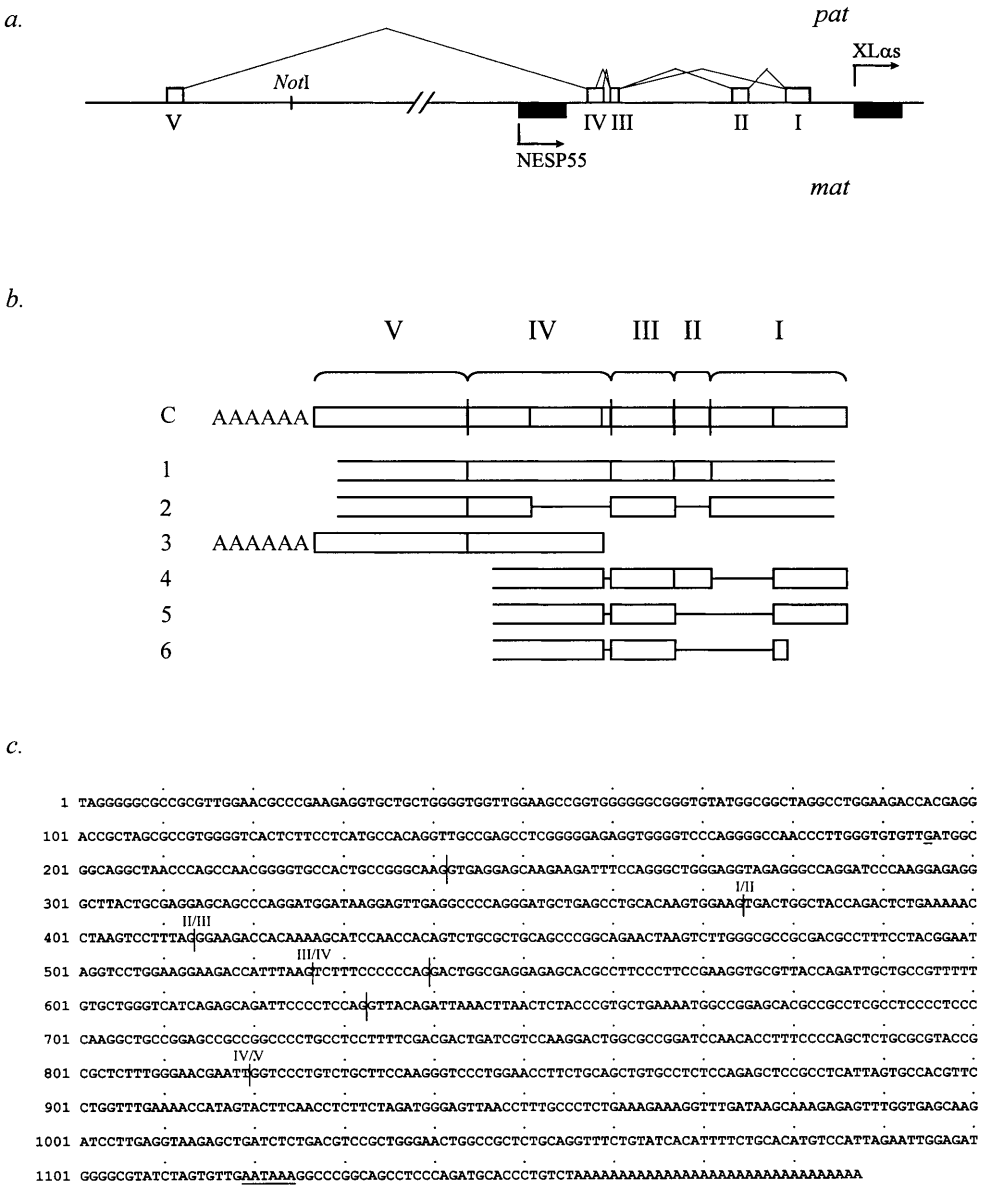

Figure 3. (**a**) Diagram of the genomic region containing the antisense exons (open rectangles) and the NESP55 and XLαs exons (filled rectangles). The direction of sense transcription is shown by an arrow and the respective active chromosome is indicated by *pat* (paternal-specific trancription) or *mat* (maternal-specific transcription). Some of the splicing patterns observed for the antisense transcript are shown above. (**b**) Schematic diagram of the various species of antisense transcript characterized. C represents the complete consensus antisense transcript. The positions of exons I–V are delineated by lines extending outside the rectangle whereas the positions of alternative splice sites within each exon are indicated by shorter lines confined within the rectangle. The poly(A) tail is represented by AAAAAA. Numbers 1–6 represent the individual transcripts characterized. On these diagrams, the use of a specific primer is indicated by an open-ended rectangle, and closed rectangles represent either the 5′ end of a 5′-RACE product or the end of IMAGE clone 111764 (clone 3). Clones 1 and 2 were generated by using primers 88F3 + 88R8; clone 3 is IMAGE clone 111764; clones 4, 5, and 6 are 5′-RACE products made using primer 88F6. (**c**) Sequence of the antisense transcript. This is the consensus sequence compiled from all the sources and shown in (b) as C. The positions of the splice junctions are indicated by vertical lines and a label (I/II, II/III, III/IV, IV/V), and the alternative splice sites shown in (b) are indicated by unlabelled vertical lines. Two of three 5′-RACE clones initiated at the first nucleotide of this sequence; the 5′ end of the other was at nucleotide 195 (underlined).

Distinguishing between these possibilities will require a more detailed analysis of the methylation pattern across the whole region.

In order to investigate whether the antisense transcript also has an imprinted expression profile we searched for polymorphisms within its five exons. Sequencing of all five exons (1.1 kb total) in DNA from six unrelated individuals yielded only one polymorphism: a C→T transition in exon I. Screening a collection of fetal DNAs for informative heterozygotes showed that this polymorphism was very rare, occurring only once in 51 fetuses. RT–PCR of the antisense transcript was performed using RNA from this heterozygous fetus. The sequences of these RT–PCR products and of a genomic PCR product spanning the same region are shown in Figure 4a. Although the genomic sequence contains both alleles, RT–PCR products from muscle and spinal cord contain only the T allele, indicating that the antisense transcript is expressed from only one chromosome. Unfortunately, there was no parental DNA available for this heterozygous fetus, so we were

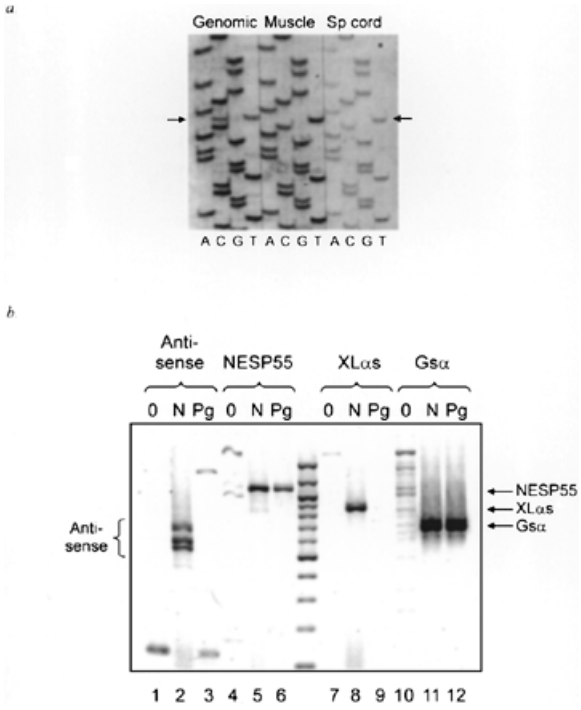

Figure 4. (**a**) Sequence of genomic DNA or cDNA-derived PCR products corresponding to a C/T polymorphism within antisense exon I. The polymorphic site (nucleotide 94 in Fig. 3c) is indicated by an arrow. Both alleles are represented in genomic DNA but only the T allele in cDNA from fetal muscle and spinal cord. (**b**) PCR products derived from total RNA of either a normal lymphoblastoid cell line (N) or the parthenogenetic cell line (Pg), reverse-transcribed using the RACE2 primer. The negative control (0) contained no target material. Antisense PCR used primers 88F6 + 88R8; NESP55 primers NESP5 + GNAS10R; XLαs primers XL1F + GNAS10R, and $G_s\alpha$ (exon I) GNAS1 + GNAS10R. The downstream primer GNAS10R is in exon 10 of *GNAS1*. The antisense RT–PCR products are heterogeneous, as noted from previous cDNA cloning experiments, but are in the size range expected (indicated by a bracket).

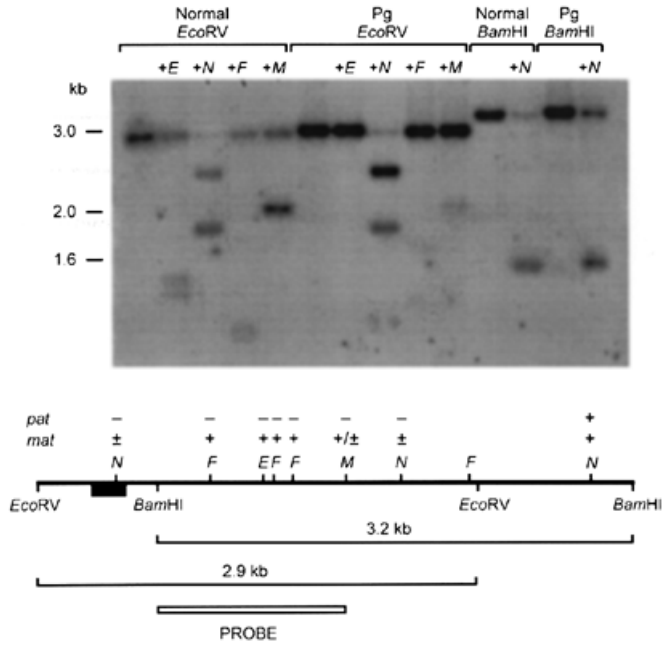

Figure 5. Methylation analysis of the *GNAS1* antisense region. A restriction map is shown of the genomic region around and upstream of the antisense exon I (black rectangle). DNA was digested with either *Eco*RV or *Bam*HI, singly or in double digests with a methylation-sensitive enzyme. The methylation-sensitive restriction sites are indicated as follows: *N*, *Nar*I; *F*, *Fsp*I; *E*, *Eag*I; *M*, *Mlu*I. Methylation at each site is indicated by +, lack of methylation by –, and partial methylation by ±. The blot shows a comparison between normal (biparental) DNA and parthenogenetic (Pg) DNA derived from the patient FD (34). The *Mlu*I site is almost completely methylated on the maternal allele, but a very faint cleavage product band is present in lane 10, indicating a small proportion of unmethylated chromosomes. The interpretation of partial methylation at the left and middle *Nar*I sites is a little more complicated. Briefly, the *Bam*HI digest allows assessment of the middle but not the left *Nar*I site; partial cleavage in the parthenogenetic DNA clearly indicates that this middle site is partially methylated. In the *Eco*RV digest, the left and middle *Nar*I sites are examined. From the 2.9 kb *Eco*RV fragment, cleavage at only the left, only the middle, or both of these *Nar*I sites is predicted to yield bands of 2.4, 2.4 and 1.9 kb, respectively. In parthenogenetic DNA (lane 8), there is a minor proportion of the 1.9 kb band, indicating that the left site is at least partially unmethylated. However, there is also a faint uncleaved 2.9 kb band, suggesting that on some chromosomes both the left and middle *Nar*I sites are methylated. Together, these observations indicate incomplete methylation of the left, as well as the middle, *Nar*I site. As expected, most of the double digest fragments from parthenogenetic DNA are in the 1.9 kb bands (cut at one *Nar*I but not at the other). In normal DNA, a greater proportion of the fragments is cut to 1.9 kb, suggesting that the paternal allele is largely (at least) unmethylated at both sites.

unable to determine from this experiment alone the inheritance pattern of the polymorphism and hence which allele was expressed.

To determine the direction of the imprinting of the antisense transcript, we tested for the presence of the NESP55, XLαs, $G_s\alpha$ and antisense transcripts in RNA from a normal female lymphoblastoid cell line (LCL) and from the parthenogenetic lymphoblastoid cell line FD (11,12). Figure 4b shows that although the normal cell line expresses all four transcript species, the parthenogenetic cell line, as predicted by our previous data, expresses the maternally derived NESP55 transcript but the paternally derived XLαs transcript is undetectable. The antisense transcript, like XLαs, is easily detected in normal LCL (Fig. 4b, lanes 2 and 8) but undetectable by RT–PCR in the parthenogenetic cell RNA (lanes 3 and 9). This indicates that, like XLαs, the antisense transcript is expressed from the paternal chromosome only.

DISCUSSION

Although differential methylation is a consistent marker for imprinted regions, other factors are becoming implicated in the regulation of imprinted genes. These include chromatin conformation (15–17), the presence of chromatin boundary elements (18,19), histone acetylation and deacetylation (20) and the presence of non-coding RNA transcripts (13,21–27). Non-coding RNA transcripts are also intimately involved in the hyperactivation of the single X chromosome in male *Drosophila* (28) and in the inactivation of one X chromosome in female mammals (29,30).

With the exception of the H19 transcript (27), non-coding imprinted transcripts seem to be located at least partly within protein-coding imprinted genes and are transcribed in the antisense direction relative to the coding transcript. These antisense transcripts can be spliced and relatively short (1–2 kb), or unspliced and extremely large (>60 kb) (22,25), but a common feature seems to be that they traverse one or more sense exons of the gene with which they are associated.

We postulated that the close proximity (12 kb) of oppositely imprinted promoters at the *GNAS1* locus might require specific regulatory interactions between the two. In particular, we wondered whether the XLαs region might have bidirectional promoter activity, with a paternally derived antisense transcript exerting a *cis*-suppressive effect on NESP55 transcription. The present report shows that such an imprinted antisense transcript does indeed exist, though the antisense promoter region appears likely to be separate from the XLαs promoter. Careful site-directed manipulation of this region will be required to determine whether the functions of the antisense and XLαs promoters can in fact be separated.

These results add another level to the complex expression profile of the *GNAS1* locus. Antisense transcripts have been described in five other imprinted genes; *UBE3A* (21), *KVLQT1* (called *LIT1*) (22,25), *Igf2r* (13), *Igf2* (23) and *Zfp127/ZFP127* (24,26). Interestingly, all of these antisense transcripts, like the *GNAS1* antisense transcript, are expressed from the paternal chromosome, though whether this paternal exclusivity merely reflects the small number of genes that have been analysed remains to be seen. The transcriptional start sites of *LIT1* (22,25) and *Igf2r* antisense (13) have been localized to differentially methylated regions (DMRs) within introns of their respective sense genes, and we have now shown that this is also the case for the *GNAS1* antisense transcript. The removal of the *Igf2r* DMR2 from a YAC transgene (13) eliminated the production of *Igf2r* antisense transcripts and released expression of the sense *Igf2r* transcript from the paternal chromosome. Analysis of Beckwith–Wiedemann syndrome (BWS) patients showed that loss of imprinting of the *LIT1* transcript was the most common genetic alteration in BWS (22). In both these examples, dysregulation of an antisense transcript appears to lead to a functional effect on the sense (protein-coding) gene. Our current efforts are therefore directed at assessing a comparable regulatory role for the *GNAS1* antisense transcript. Its potential importance is suggested by the observation that it initiates in a 285 bp region that is highly conserved in the mouse. In addition, the paucity of polymorphic variation within the human antisense exons, and the discrete 5′ ends of antisense transcripts, further support the idea that this non-coding RNA is under selective constraints.

MATERIALS AND METHODS

Identification of genomic clones and sequence analysis

The isolation of the human P1-derived artificial chromosome (PAC 309F20) containing the *GNAS1* locus has been described elsewhere (11). The mouse NESP55–XLαs region was isolated as follows: after comparison of the human NESP55 sequence and the partial mouse EST described by Ischia *et al.* (10) an RT–PCR product was generated from murine RNA using primers whose sequence was conserved in the NESP55 exon (NESP10) and in exon 2 (GNAS2R). This product was used to probe the RPCI21 mouse PAC library (HGMP Resource Centre, Hinxton, UK) and two positive clones were identified: 583L07 and 653B05. 583L07 was used to generate genomic subclones.

For sequencing, subclones were generated using standard methods and sequenced using random transposon insertion (GPS-1 kit; New England Biolabs) to generate sequencing templates. Sequencing was performed using the ThermoSequenase radio-labelled terminator cycle sequencing kit and [^{33}P]dideoxynucleotides from Amersham Pharmacia Biotech (Uppsala, Sweden) and by BigDye terminator sequencing on an ABI 377 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Sequence assembly and comparisons were performed using the Genetics Computer Group (Madison, WI) package, hosted at the MRC HGMP site (http://www.hgmp.mrc.ac.uk). Database searching was performed using BLAST (31) hosted at NCBI (http://www.ncbi.nlm.nih.gov).

PCR and RT–PCR

RT was performed with Superscript RT-II (Gibco BRL, Gaithersburg, MD) using RNA isolated using the AGPC method (32). Standard RT used a primer containing a $d(T)_{17}$ tract (RACE2) whereas 5′-RACE used an antisense specific primer (88F5), under conditions previously described (33). PCR used primers RACE3+88F6 to amplify the 5′-RACE transcripts (40 cycles of 94°C for 25 s, 62°C for 25 s and 72°C for 3 min) and 88R8+88F3 or 88R8+88F6 to amplify other antisense transcripts ['hot start' and then six cycles of 94°C for 30 s, 66°C for 30 s (–0.5°C per cycle) and 72°C for 90 s, and 34 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 90 s]. RT–PCR of specific *GNAS1* transcripts (using primers NESP5/XL1F/GNAS1F + GNAS10R) used the same PCR conditions. PCR products were cloned into the Easy-T vector (Promega, Madison, WI). Primer sequences were:
RACE2, dGAGCTCGAGTCGACATCGA(T_{17});
RACE3, dGAGCTCGAGTCGACATCGATTT;
NESP5, dTCGGAATCTGACCACGAGCA;
NESP10, dGAGCTCGCCATAATTACAACG;
XL1F, dGGATGCCTCCGCTGGTTTCAG;
GNAS1F, dCCATGGGCTGCCTCGGGAACA;
GNAS2R, dGGATCCTCATCTGCTTCACAAT;
GNAS10R, dCACGAAGATGATGGCAGTCAC;
88F3, dGCCCCATCTCCAATTCTAAT;
88F5, dCAGGAACGTGGCACTAATGAG;
88F6, dGATCAGTCGTCGAAAAGGAGG;
88R8, dGAAGAGGTGCTGCTGGGGTG.

Methylation analysis

Southern blotting using standard methods was performed on parthenogenetic and matched normal 46,XX lymphoblastoid cell genomic DNA as described previously (11,12,34).

ACKNOWLEDGEMENTS

We are very grateful to Dr L. Strain for the Southern blot shown in Figure 5. This work was supported by the Wellcome Trust (grant no. 053701).

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