An imprinted transcript, antisense to Nesp, adds complexity to the cluster of imprinted genes at the mouse Gnas locus

Stephanie F. Wroe*, Gavin Kelsey†, Judith A. Skinner*, Dorothy Bodle‡, Simon T. Ball*, Colin V. Beechey*, Josephine Peters‡*, and Christine M. Williamson*

*Mammalian Genetics Unit, Medical Research Council, Harwell, Didcot, Oxfordshire, OX11 0RD, United Kingdom; and †Laboratory of Developmental Genetics and Imprinting, The Babraham Institute, Cambridge, CB2 4AT, United Kingdom

Communicated by Mary F. Lyon, Medical Research Council, Oxon, United Kingdom, January 12, 2000 (received for review August 24, 1999)

The Gnas locus in distal mouse chromosome (Chr) 2 is emerging as a complex genomic region. It contains three imprinted genes in the order Nesp-Gnasxl-Gnas. Gnas encodes a G protein α-subunit, and Nesp and Gnasxl encode proteins of unknown function expressed in neuroendocrine tissue. Together, these genes form a single transcription unit because transcripts of Nesp and Gnasxl are alternatively spliced onto exon 2 of Gnas. Nesp and Gnasxl are expressed from opposite parental alleles, with Nesp encoding a maternal-specific transcript and Gnasxl encoding a paternal-specific transcript. We now identify a further imprinted transcript in this cluster. Reverse transcription–PCR analysis of Nesp expression in 15.5-days-postcoitum embryos carrying only maternal or paternal copies of distal Chr 2 revealed an isoform that is exclusively paternally expressed. Strand-specific reverse transcription–PCR showed that this form is an antisense transcript. The existence of a paternally expressed antisense transcript was confirmed by Northern blot analysis. The sequence is contiguous with genomic sequence downstream of Nesp and encompasses Nesp exons 1 and 2 and an intervening intron. We propose that Nesp is an additional control element in the imprinting region of mouse distal Chr 2; it adds further complexity to the Gnas-imprinted gene cluster.

Genomic imprinting is a phenomenon whereby genes are differentially expressed according to parental origin (1). Most imprinted genes in the mouse are located within nine imprinting regions distributed across six autosomes [C.V.B. and B. M. Cattanach (Medical Research Council Mammalian Genetics Unit, Harwell, Oxfordshire, U.K.); World Wide Web Site: Genetic and Physical Imprinting Map of the Mouse; http://www.mgu.har.mrc.ac.uk/anomaly/anomaly.html]. One of the first described imprinting regions was distal chromosome (Chr) 2 (2). Mice with two maternal copies of the region (MatDp.dist2) are hypoactive; they have long, flat-sided bodies and die within a few hours of birth. By contrast, mice with two paternal copies of the region (PatDp.dist2) have an opposite phenotype because they are hyperactive; they also are edematous, have short, square bodies, and survive for several days after birth (2, 3). It was shown from genetic studies that the phenotypes must be due to at least two imprinted genes, one of which is maternally imprinted and the other which is paternally imprinted (4).

Using representational difference analysis, based on parent-of-origin methylation differences, we recently have identified two oppositely imprinted transcripts, Nesp and Gnasxl, at the Gnas locus in distal Chr 2 that are candidates for the imprinting phenotypes (5, 6). Nesp is paternally imprinted/maternally expressed, and Gnasxl is maternally imprinted/paternally expressed. Both determine proteins found in neuroendocrine tissues although their functions are unknown (7, 8). Remarkably, Nesp, Gnasxl, and Gnas are all part of the same transcription unit, as Nesp and Gnasxl transcripts splice onto Gnas exon 2 (5). The human homologues, NESP55 and XL s, have been shown to be imprinted and in a similar manner to the mouse (refs. 9 and 10, respectively). The Gnas/GNAS1 locus is the first example of which we are aware of a cluster of imprinted genes in which two oppositely imprinted transcripts share the same exons.

The order of genes in the Gnas cluster is Nesp-Gnasxl-Gnas with 15 kb separating Nesp and Gnasxl, whereas Gnasxl lies 30 kb upstream of Gnas (5, 6). Nesp is associated with a 2.8-kb region of paternal methylation, and Gnasxl is associated with a 5.5-kb region of maternal methylation that extends 3.3 kb upstream of the extra-large exon. There was no evidence of parental-specific methylation (5) associated with a Gnas promoter (11) despite the existence of good biochemical and clinical evidence that Gnas/GNAS1 shows maternal-specific expression in a subset of tissues (12, 13).

Parent-specific methylation is the simplest explanation for monoallelic expression of Nesp and Gnasxl because both genes carry methylation marks, with the expressed allele being unmethylated. An expression competition model (14, 15) in which methylation regulates the availability in cis of shared regulatory elements also could account for the opposite imprinting of Nesp and Gnasxl. Antisense transcripts can act as regulatory elements. We describe a maternally imprinted antisense transcript of Nesp that, we predict, regulates expression of genes within the Gnas cluster. This imprinted antisense transcript adds further complexity to the cluster of imprinted genes in the distal imprinting region of mouse Chr 2.

Methods

Distal Chr 2-Duplication/Deficient Mice. Mice with maternal duplication of distal Chr 2 (MatDp.dist2) and the reciprocal paternal duplication (PatDp.dist2) were generated by intercrossing heterozygotes for the reciprocal translocation T(2;8)26H (16). The duplication offspring were identified by typing for the marker D2Mit226 (17). These mice and wild-type siblings were used for expression analysis.

Reverse Transcription–PCR (RT-PCR) Analysis. For RT-PCR analysis, approximately 1 µg of poly(A)+ RNA, isolated by using the FastTrack 2.0 mRNA isolation kit (Invitrogen), was reverse-transcribed by mouse murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) using oligo(dT)15.

Abbreviations: Chr, chromosome; dpc, day postcoitum; RT-PCR, reverse transcription–PCR; RACE, rapid amplification of cDNA ends.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF175305 (partial cDNA of maternal Nesp transcript), AF173359 (partial cDNA of paternal Nesp transcript), AJ251480, and AJ245856 (genomic sequences between Nesp and Gnasxl)].

To whom reprint requests should be addressed. E-mail: j.peters@har.mrc.ac.uk.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.050015397. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.050015397.
primer (Promega). Conditions for PCR were 25 cycles of 1 min each at 94°C, 55°C, and 72°C by using Thermoprime Plus DNA polymerase (Advanced Biotechnologies, Columbia, MD). The positions of the primers NL3 (5′-AGTGAGGCGCACCCTTCCGGGTA-3′, nucleotides 85–103, GenBank accession no. AF125315), NR3 (5′-CTCTGGCTCTGCAGAGGT-3′, nucleotides 354–372, accession no. AF125315), and R7 (5′-TTATGGACCCCGATGGGGA-3′, nucleotides 997–978, accession no. AF175305) are shown in Fig. 2. 5′ Rapid amplification of cDNA ends (RACE)-PCRs were performed on poly(A)⁺ RNA from 15.5-days-postcoitum (dpc) PatDp.dist2 embryos by using the SMART RACE kit (CLONTECH). PCR products were subcloned by using the Invitrogen TA cloning kit.

**Strand-Specific RT-PCR.** Poly(A)⁺ RNA, isolated as described above, was treated with RNase-free DNase I by using the Message Clean kit (GenHunter Corporation, Nashville, TN). Each sample was set up in duplicate for reverse transcriptase and reverse transcriptase reactions. Strand-specific primers and 1 μg of oligo(dA) [(dA)₈₀; Genosys, The Woodlands, TX] were added to 0.15 μg of poly(A)⁺ RNA, and the mixture was heated at 70°C for 10 min. The (dA)₈₀ oligonucleotide was added to all samples, except those with oligo(dT)₁₅ primer, to trap any oligo(dT) that might have copurified with the poly(A)⁺ RNA (18). First-strand cDNA was synthesized at 50°C for 50 min by using either sense or antisense primers with Superscript II (200 units; Life Technologies). The enzyme was inactivated at 80°C for 45 min. First-strand cDNA was amplified by PCR as described above. The relative position of the reverse transcriptase primers, R7 and NL3 (specific for the sense and antisense transcripts, respectively), and primers for subsequent PCR, F1 (5′-CCCCGTCCAATCTAATGCCTG-3′, nucleotides 711–730, accession no. AF175305) and R7, are shown in Fig. 2c. All PCR products were probed with a 229-bp PCR product derived from cDNA extending from F2 (5′-CAAGGAGGAAACAGGCAGC-3′, nucleotides 883–902, accession no. AF175305) to exon 2 of Ngn (5′-CTCCGTTAAMACCATATCGC-3′, nucleotides 205–182; ref. 19); the primers are shown in Fig. 2c.

**Southern Hybridization.** DNA was transferred onto charged nylon membranes (Hybond N⁺; Amersham Pharmacia) by alkaline transfer. PCR products were radiolabeled with 25 μCi of [α-³²P]dCTP (NEN) by using Megaprim (Amersham Pharmacia). The Southern filters were hybridized by using Church and Gilbert hybridization buffers (20).

**Northern Blot Analysis.** Poly(A)⁺ RNA was treated with DNase I, and Northern blots were prepared by using the NorthernMax–Gly kit (Ambion, Austin, TX). Riboprobes were made by using the Strip-EZ RNA labeling kit (Ambion) and [α-³²P]UTP (Amersham Pharmacia). Sense and antisense riboprobes for Nespr had been identified as a candidate imprinted gene by the isolation of a differentially methylated HpaII fragment with a spliced maternally expressed transcript that lacks a 95-bp intron present in genomic DNA (5, 6). The intron is upstream of the Nespr ORF. Sequence alignments showed that bovine NES55 cDNA (accession no. U77614; ref. 7) and human NESPR cDNA (accession no. AJ009849; ref. 9) were unspliced forms (Fig. 1). Therefore, to determine whether there were unspliced Nespr isoforms in the mouse, the oligonucleotide primers NL3 and NR3 were designed across the 95-bp intron (Fig. 2) and RT-PCR analysis was carried out on whole 15.5-dpc embryos. As expected, a 193-bp spliced RT-PCR product was observed in the MatDp.dist2 (lane 5) and wild-type sib cDNA (lane 3) but not in the PatDp.dist2 cDNA (Fig. 2a, lane 1). In addition, a 288-bp unspliced RT-PCR product of lower intensity and exclusive paternal expression was observed. This band was seen in the PatDp.dist2 (Fig. 2a, lane 1) and wild-type sib cDNA (lane 3) but not in the MatDp.dist2 cDNA (lane 5). Genomic DNA contamination in the RNA samples, which could account for the 288-bp unspliced form, was ruled out by the absence of the product in the controls without reverse transcriptase. Sequencing of the MatDp.dist2 and PatDp.dist2 RT-PCR products confirmed that the 95-bp intron is absent in the 193-bp maternal RT-PCR product but present in the 288-bp paternal RT-PCR product. Similar results were obtained by using newborn MatDp.dist2 and PatDp.dist2 tissues obtained using another Chr 2 translocation, T(2;19)68H (refs. 5 and 21; data not shown).

**Sequencing.** A genomic phage lambda clone for the Nespr locus was isolated from a library of 129/SvJ mouse DNA in Lambda FIX II (Stratagene; library no. 946309) by hybridization with the CpG island clone M1/1 (ref. 6; RZPD clone EDIUP123NO611Q4 at http://www.rzpd.de). A 14.5-kb XhoI fragment was subcloned into the SalI site of pDELTA 2 (Life Technologies), and a series of nested deletions was prepared according to the manufacturer’s instructions. Sequencing was done with SP6 and T7 promoter primers as described below, and sequences were assembled by using the GAP4 program of the STADEN package. The ABI Prism dichlororhodamine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) was used for sequencing. All sequencing products were electrophoresed on an ABI 377 (Perkin–Elmer) automated sequencer. Sequences were analyzed for similarity by using the BLAST program accessed at http://www.ncbi.nlm.nih.gov/BLAST.

**Results**

**Identification of a Paternally Expressed Nespr Transcript.** Nespr had been identified as a candidate imprinted gene by the isolation of a differentially methylated HpaII fragment with a spliced maternally expressed transcript that lacks a 95-bp intron present in genomic DNA (5, 6). The intron is upstream of the Nespr ORF. Sequence alignments showed that bovine NES55 cDNA (accession no. U77614; ref. 7) and human NESPR cDNA (accession no. AJ009849; ref. 9) were unspliced forms (Fig. 1). Therefore, to determine whether there were unspliced Nespr isoforms in the mouse, the oligonucleotide primers NL3 and NR3 were designed across the 95-bp intron (Fig. 2) and RT-PCR analysis was carried out on whole 15.5-dpc embryos. As expected, a 193-bp spliced RT-PCR product was observed in the MatDp.dist2 (lane 5) and wild-type sib cDNA (lane 3) but not in the PatDp.dist2 cDNA (Fig. 2a, lane 1). In addition, a 288-bp unspliced RT-PCR product of lower intensity and exclusive paternal expression was observed. This band was seen in the PatDp.dist2 (Fig. 2a, lane 1) and wild-type sib cDNA (lane 3) but not in the MatDp.dist2 cDNA (lane 5). Genomic DNA contamination in the RNA samples, which could account for the 288-bp unspliced form, was ruled out by the absence of the product in the controls without reverse transcriptase. Sequencing of the MatDp.dist2 and PatDp.dist2 RT-PCR products confirmed that the 95-bp intron is absent in the 193-bp maternal RT-PCR product but present in the 288-bp paternal RT-PCR product. Similar results were obtained by using newborn MatDp.dist2 and PatDp.dist2 tissues obtained using another Chr 2 translocation, T(2;19)68H (refs. 5 and 21; data not shown).

**Sequencing**

The 1,083-bp paternally expressed transcript (derived from primers NL3 to R7) with bovine NES55 cDNA (accession no. U77614) and human NESPR cDNA (accession no. AJ009849) showed that there is sequence conservation within the region of the 95-bp intron (Fig. 1). Apart from the 95-bp intron, the sequence of the paternally expressed transcript (accession no. AF173359) matched with exons 1 and 2 of the maternally expressed Nespr transcript (accession no. U77614).

**Fig. 1.** Multiple alignment of mouse 95-bp intron and flanking Nespr sequence with human and bovine sequence. The intron is upstream of the Nespr ORF. Mat, maternally expressed mouse transcript (accession no. AF175305); Mus, mouse genomic (accession no. AF122315); Pat, paternally expressed mouse transcript (accession no. AF173359); Hum, human sequence (accession no. AJ009849; ref. 9); and Bov, bovine mRNA (accession no. U77614; ref. 7). Bases in common between the mouse paternal transcript and at least one other species are shaded. Putative splice donor and acceptor sites are underlined in the mouse genomic sequence (Mus) and are not present in the antisense orientation with respect to Nespr.
AF175305; alignment not shown). A mouse IMAGE clone 932324 (accession no. AI561892) contains the 95-bp intron and represents a sequence apparently transcribed in the opposite orientation with respect to Nesphi (Fig. 2c). Analysis of the IMAGE clone sequence (accession nos. AI561892 and AA530580) revealed that the transcript extends at least 198 bp beyond the IMAGE clone by a nonamplifying method. A riboprobe extending from exon 1 into exon 2 of Nesphi, specific for antisense, revealed a smear and bands in most mouse tissues on a Northern blot (CLONTECH). These were strongly expressed in heart (Fig. 3a), consistent with the isolation of IMAGE clone 932324 from a heart library. The Nesphi-specific riboprobe recognized both a smear and a 4.4-kb band in the heart of PatDp.dist2 and wild type but not in MatDp.dist2, confirming the paternal-specific expression of antisense (Fig. 3b).

Expression Pattern of Nesphi by Northern Blot Analysis. Northern blot analyses were performed to confirm the existence of an antisense transcript to Nesphi by a nonamplifying method. A riboprobe extending from exon 1 into exon 2 of Nesphi, specific for antisense, revealed a smear and bands in most mouse tissues on a Northern blot (CLONTECH). These were strongly expressed in heart (Fig. 3a), consistent with the isolation of IMAGE clone 932324 from a heart library. The Nesphi-specific riboprobe recognized both a smear and a 4.4-kb band in the heart of PatDp.dist2 and wild type but not in MatDp.dist2, confirming the paternal-specific expression of antisense (Fig. 3b). That the
antisense appeared as a smear on a Northern blot suggests that it could be either an unusually large RNA that is degraded in preparation using standard methods or a collection of differently sized RNAs. It therefore is possible that the antisense bands detected in Fig. 3a and b are nothing more than artifacts resulting from the presence of ribosomal RNA bands that act to concentrate an RNA smear above and below the 28S and 18S bands. However, the band in skeletal muscle (Fig. 3a) is probably a genuine transcript because there is no evidence of a smear in this tissue. The Northern blot in Fig. 3b showed an inverse correlation between the expression of the sense and antisense transcripts. A clear dosage effect was seen with MatDp.dist2, which expressed a double dose of the sense transcript (Nesp) and no antisense transcript (Nespas) whereas with PatDp.dist2 there was no sense transcript but enhanced expression of antisense. In wild type, expected to have one dose of sense and one of antisense, there was intermediate expression. The detection of both Nesp and Nespas transcripts from opposite parental alleles in heart supports a proposal that antisense controls expression of the sense transcript from the paternal allele.

Discussion

Previous results have shown that the imprinting at the Gnas/GNAS1 locus in mice and humans is complex (5, 9, 10). Three genes, Nesp, Gnasxl, and Gnas, were found to be part of the same transcription unit, and two of these, Nesp and Gnasxl, show exclusive monoallelic expression. For Nesp, only the maternally derived allele is expressed, and Gnasxl expression is just from the paternally derived allele (5, 9, 10). The results presented here now indicate that the situation is even more complex; there is expression of Nesp antisense from the paternally derived allele.

Six imprinted genes with antisense transcripts are now known (22–29). For two of these genes, Igf2 and ZNF127/Zfp127, both the sense and antisense transcripts are expressed from the paternal allele (23, 26, 27), but for the remaining four, UBE3A, Igf2r, KvLQT1/Kvlqt1, and now Nesp, the antisense transcript is expressed from the opposite allele to the sense, protein-encoding transcript (5, 23–25, 28, 29). Furthermore, these four genes are paternally imprinted/maternally expressed and their antisense transcripts are maternally imprinted/paternally expressed. These four would conform to the “expression competition” model of genomic imprinting whereby expression of the antisense transcript from the paternal allele represses the expression.
of the sense transcript from the same allele (14, 15). For the other two genes, Igf2 (23) and ZNF127/Zfp127 (26, 27), in which the overlapping sense and antisense transcripts are expressed from the paternal allele, the regulation of imprinted gene expression is likely to require a different mechanism (29).

The finding of an antisense transcript of Nesp has implications for the regulation of the Gnas cluster. If Nesp is postulated to repress the expression of Nesp in cis from the paternal allele, Nespas can be regarded as an “imprinter” and Nesp as the imprinted target. It could lead to nonexpression of the sense transcript by any of the currently proposed methods: occlusion of the sense promoter, inactivation of the paternal allele by localized heterochromatinization, and competition for shared transcription factors or enhancers by the sense and antisense promoters (15, 30). One noted feature has been that the Nespas transcript appears to be less abundant than the Nesp transcript. The Nespas transcript therefore either may be less stable or only weakly expressed but, even so, still able to regulate the silencing of the sense Nesp transcript from the paternal allele.

Although the position of the 5′ end of Nespas is not yet defined, it must lie downstream of the Nesp promoter. The differentially methylated region that is downstream of Nesp and upstream of Gnasxl is a candidate for the 5′ end of Nespas and its promoter; it is also the candidate region for the Gnasxl promoter (Fig. 4). From this region, which is unmethylated on the paternal allele, there may be bidirectional transcription from this allele to lead to the production of both Gnasxl and Nespas transcripts. The situation is different for the maternal allele. The region is maternally methylated, and this can account for nonexpression of Gnasxl and Nespas from the maternally derived chromosome. An expression competition model in which methylation regulates the availability of shared regulatory elements (14, 15) also could account for the expression of Nesp and lack of expression of Gnasxl from the maternal allele.

The function of the protein products of Nesp and Gnasxl are not yet known. NESP55, however, is a neuroendocrine secretory protein originally identified in bovine chromaffin cells and resembles the chromogranin-like polypeptides (7). A more extensive examination of NESP55 mRNA in rat brain revealed a significant overlap with noradrenergic, adrenergic, and serotonergic transmitter systems (31). The product of Gnasxl has conserved regions for guanine nucleotide binding and is tightly membrane-associated at the trans-Golgi network. It therefore may function in secretory vesicle formation (8). Both Nesp and Gnasxl are expressed in neuroendocrine tissue (7, 8) and may function in a common pathway in which it is important that their expression is mutually exclusive and monoallelic. Nespas is a candidate gene for the imprinting phenotypes associated with PatDp.dist2 and MatDp.dist2 (2, 3). PatDp.dist2 will have a double dose of the Nespas transcript whereas MatDp.dist2 will lack Nespas transcripts.

We are grateful to B. M. Cattanach for useful comments on the manuscript and a referee for helpful suggestions. We also thank A. Ford, K. Glover, and S. Thomas for figures and E. Prescott for animal husbandry. The animal studies described in this paper were carried out under the guidance issued by the Medical Research Council in “Responsibility in the Use of Animals for Medical Research” (July 1993) and Home Office Project License Number 30/1518. G.K. is a senior fellow of the Medical Research Council. J.A.S. is funded by the National Kidney Research Fund, Grant R12/1/97.