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

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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, rather than maternally, 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 *Nespas* 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 are also 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 XLαs, 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 *Nespas* 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.

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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′-AGTGGAGGCAC-CTCTCGGA-3′, nucleotides 85–103, GenBank accession no. AF125315), NR3 (5′-CTCTGGCTCTGCAGAGAGT-3′, nucleotides 354–372, accession no. AF125315), and R7 (5′-TTAGTGACGCCGGATGGGGA-3′, nucleotides 997–978, accession no. AF175305) are shown in Fig. 2*c*. 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′-ACCAGTCACTCACTCAGCGT-3′, nucleotides 711–730, accession no. AF175305) and R7, are shown in Fig. 2*c*. All PCR products were probed with a 229-bp PCR product derived from cDNA extending from F2 (5′-CAAGGAGGAAAACAG-GCAGC-3′, nucleotides 883–902, accession no. AF175305) to exon 2 of *Gnas* (5′-CTCCGTTAAACCCATTAACATGCA-3′, nucleotides 205–182; ref. 19); the primers are shown in Fig. 2*c*.

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-^{32}P]dCTP (NEN) by using Megaprime (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-^{32}P]UTP (Amersham Pharmacia). Sense and antisense riboprobes for *Nesp* were prepared as described previously (5) by transcription with T3 polymerase from clones 317 and 330 (opposite orientation), which contain a 423-bp genomic fragment extending from exon 1 to exon 2 of *Nesp*. The actin riboprobe was generated from pTRI-β-actin-mouse template DNA (Ambion). Hybridization was done overnight by using ULTRAhyb (Ambion), and blots were washed by using Ambion's Low and High Stringency Wash Buffers. The CLONTECH Northern was hybridized under standard Express Hyb conditions (CLONTECH).

Sequencing. A genomic phage lambda clone for the *Nesp* 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 *Xho*I fragment was subcloned into the *Sal*I 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.

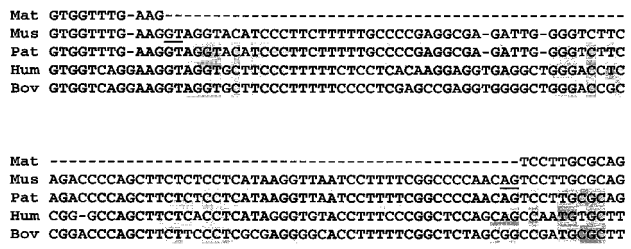

Fig. 1. Multiple alignment of mouse 95-bp intron and flanking *Nesp* sequence with human and bovine sequence. The intron is upstream of the *Nesp* ORF. Mat, maternally expressed mouse transcript (accession no. AF175305); Mus, mouse genomic (accession no. AF125315); Pat, paternally expressed mouse transcript (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 *Nesp*.

Results

Identification of a Paternally Expressed *Nesp* Transcript. *Nesp* had been identified as a candidate imprinted gene by the isolation of a differentially methylated *Hpa*II 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 *Nesp* ORF. Sequence alignments showed that bovine NESP55 cDNA (accession no. U77614; ref. 7) and human NESP cDNA (accession no. AJ009849; ref 9) were unspliced forms (Fig. 1). Therefore, to determine whether there were unspliced *Nesp* 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 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. 2*a*, 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. 2*a*, 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).

Sequence alignment of the 1,083-bp paternally expressed transcript (derived from primers NL3 to R7) with bovine NESP55 cDNA (accession no. U77614) and human NESP 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 *Nesp* transcript (accession no.

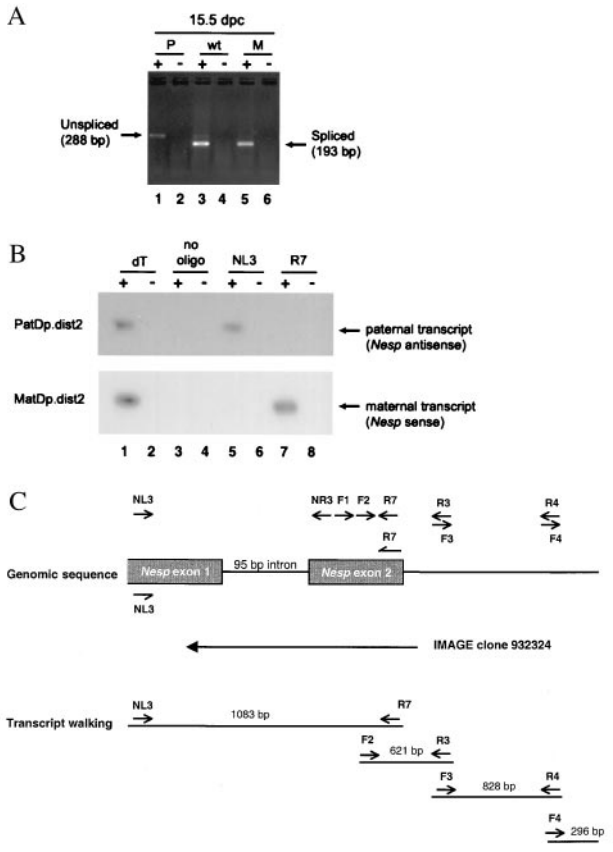

Fig. 2. Identification of an imprinted antisense transcript for *Nesp*. (*A*) RT-PCR demonstrating the differential expression of *Nesp* transcripts in 15.5-dpc embryos. Oligo(dT)-primed cDNA was derived from 15.5-dpc PatDp.dist2 (P), MatDp.dist2 (M), and wild-type sib (wt) embryos. Twenty-five cycles of amplification were performed by using the primers NL3 and NR3. The arrows indicate the *Nesp* 193-bp spliced and 288-bp unspliced PCR products. RT reactions were carried out in the presence (+) and absence (−) of reverse transcriptase. (*B*) Southern hybridization of strand-specific RT-PCR products demonstrating that the paternally expressed unspliced isoform lies antisense to *Nesp*. The first lane is a control RT, using oligo(dT) to show the presence of a product, and the third lane is a negative control for RT without exogenous primer to show the absence of priming by endogenous factors in the RNA preparations. Primer NL3 is specific for the antisense transcript, and R7 is specific for the sense strand. PCR was performed by using primers F1 and R7, which lie 3′ of the intron in *Nesp* exon 2; 35 cycles were required to detect the less abundant paternal transcript in PatDp.dist2, and 25 cycles were required for the more abundant maternal transcript in MatDp.dist2. The probe was a 229-bp PCR product derived from cDNA extending from primer F2 to exon 2 of *Gnas*. (*C*) Diagram showing the approximate position of the primers on genomic sequence (accession nos. AJ245401 and AJ251480). The reverse transcriptase primers (half-arrowheads) specific for the antisense and sense are shown above and below the map, respectively; PCR primers are designated by normal arrows; primer sequence is given in *Methods*. The approximate position of the IMAGE clone is shown together with PCR products from transcript walking by RT-PCR. The 296-bp product was isolated by RACE. The figure is not to scale.

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 *Nesp* (Fig. 2*c*). Analysis of the IMAGE clone sequence (accession nos. AI561892 and AA530580) revealed that the transcript extends at least 198 bp downstream of *Nesp* exon 2, and this is contiguous with the genomic sequence (AJ245401). In the reverse orientation there are no consensus donor and acceptor splice sites around the 95-bp intronic sequence, thus suggesting the paternally expressed isoform may be derived from an antisense transcript.

Orientation of the Paternally Expressed Transcript with Respect to the Maternally Expressed *Nesp* Transcript. Strand-specific RT-PCR was performed on RNA derived from PatDp.dist2 15.5-dpc embryos to determine whether the paternally expressed isoform was derived from a sense or antisense transcript. MatDp.dist2 material was also tested to confirm the strand specificity of the primers. Poly(A)$^+$ RNA was reverse-transcribed by using primers specific for each strand; primer NL3 designed from the 5′ end of *Nesp* (Fig. 2*c*) was specific for the antisense strand, and primer R7, designed from the 3′ end of *Nesp*, was specific for sense. The strand-specific cDNA was amplified with primers F1 and R7, designed from the 3′ end of *Nesp* as shown in Fig. 2*c*. In PatDp.dist2 embryos that express the unspliced form of *Nesp*, the expected PCR product of 287 bp was detected in the oligo(dT) and NL3-primed cDNA as shown in Fig. 2*b* (lanes 1 and 5, respectively). No amplification was detected in the sample with oligo(dA) alone and in the R7-primed PatDp.dist2 cDNA (Fig. 2*b*, lanes 3 and 7, respectively). These results show that the paternally expressed transcript is obtained when an antisense-specific primer is used, indicating that it is derived from an antisense transcript. The opposite was observed with MatDp-.dist2 embryos that express the spliced form of *Nesp*; a PCR product was detected in the oligo(dT) and R7-primed cDNA as shown in Fig. 2*b* (lanes 1 and 7, respectively) but no amplification was detected in the sample with oligo(dA) alone (Fig. 2*b*, lane 3) and the NL3-primed MatDp.dist2 cDNA (lane 5). This shows that the maternally expressed *Nesp* transcript is found only when a sense-specific primer is used, indicating that this product is derived from a sense transcript. Similar PCR results were obtained by using cDNA reverse transcribed with other sense and antisense primers across the *Nesp* exons (data not shown). Because the antisense transcript overlaps exons 1 and 2 of *Nesp*, we have designated this transcript *Nespas* (*Nesp* antisense).

Primers were designed from genomic sequence between *Nesp* and *Gnasxl* (accession no. AJ245401) to allow walking along the antisense transcript by strand-specific RT-PCR. PCR products from NL3 strand-specific cDNA were generated by primers F2 (Fig. 2*c*, nucleotides 2374–2393 of accession no. AJ245401) and R3 (nucleotides 2994–2975) and by F3 (nucleotides 2975–2994) and R4 (nucleotides 3802–3783) to give products of 621 bp and 828 bp, respectively, which were sequenced. These products extended the sequence of *Nespas* beyond the IMAGE clone by 1.4 kb. Although attempts to define the transcriptional start site by RACE were unsuccessful, sequence analysis of 5′ RACE products generated by the gene-specific primer F4 (nucleotides 3783–3806) and the RACE primer gave a further 296 bases of sequence, thus providing a total of 2.2 kb of sequence extending from primer NL3 for *Nespas*, and this is contiguous with genomic sequence (accession no. AJ245401 and AJ251480) and appears to lack coding potential. Genomic sequence (AJ251480 and AJ245856) further 5′ to *Nespas* gave an excellent match on the complementary strand to Aa955517/Aa955518.em_est5 (rat cDNA, source undefined, 89% over 240 bp by BESTFIT) and AI047184.em_est6 (mouse ES cell cDNA, 99% over 494 bp by BESTFIT), and work is in progress to determine whether they are part of the paternally expressed *Nespas* transcript.

Expression Pattern of *Nespas* by Northern Blot Analysis. Northern blot analyses were performed to confirm the existence of an antisense transcript to *Nesp* by a nonamplifying method. A riboprobe extending from exon 1 into exon 2 of *Nesp*, specific for antisense, revealed a smear and bands in most mouse tissues on a Northern blot (CLONTECH). These were strongly expressed in heart (Fig. 3*a*), consistent with the isolation of IMAGE clone 932324 from a heart library. The *Nespas*-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. 3*b*). That the

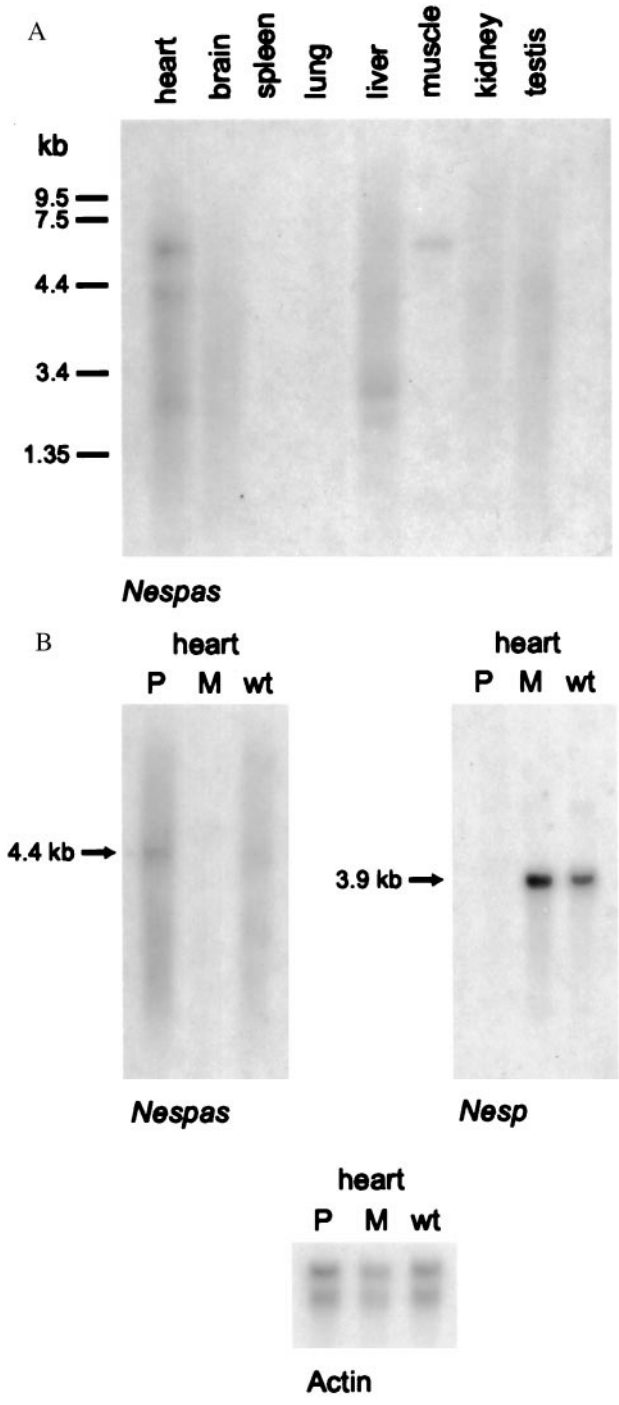

Fig. 3. Northern blot analysis of *Nespas* expression. (*A*) CLONTECH multiple tissue Northern blot from adult mouse probed with *Nespas*-specific riboprobe. The position of the RNAs in the 0.24- to 9.5-kb RNA ladder (Life Technologies) is shown to the left of the blot. (*B*) Northern blot of approximately 2 μg of poly(A)$^+$ RNA extracted from the heart of newborn PatDp.dist2 (P), MatDp.dist2 (M), and wild-type (wt) offspring from the T26H intercross. The *Nespas*-specific riboprobe recognized a smear and a 4.4-kb band in PatDp.dist2 and wt (overnight exposure) whereas the *Nesp*-specific riboprobe hybridized to a discrete, 3.9-kb transcript in MatDp.dist2 (4-h exposure). Actin was included as a loading control (30-min exposure).

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

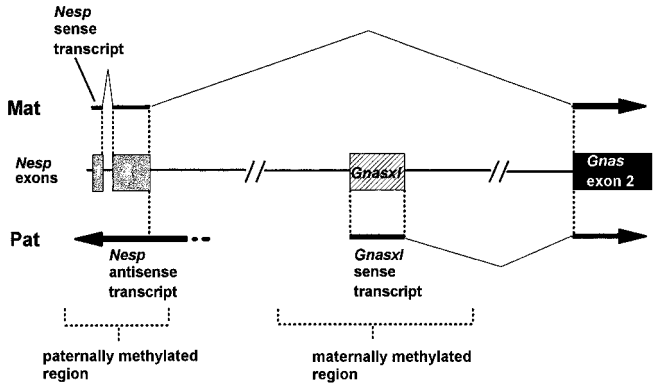

Fig. 4. Summary of oppositely imprinted transcripts at the *Gnas* locus in distal mouse Chr 2. Gene order was determined previously (5, 6); the orientation with respect to centromere and telomere is not known. Shaded blocks show exons exclusive to *Nesp*, the hatched block represents the exon specific for *Gnasxl*, and the solid block represents *Gnas* exon 2. Mat and Pat refer to maternally and paternally derived alleles, respectively. The maternally and paternally methylated regions represent the approximate positions of differentially methylated *Hpa*II sites identified by methylation-sensitive representational difference analysis (6). The maternally expressed *Nesp* transcript that extends into *Gnas* exon 2, the paternally expressed *Gnasxl* transcript that also extends into exon 2, and the paternally expressed transcript that lies antisense to *Nesp* are represented by arrows showing the direction of transcription; the *Gnas* transcripts are not shown. The figure is not to scale.

detected in Fig. 3 *a* 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. 3*a*) is probably a genuine transcript because there is no evidence of a smear in this tissue. The Northern blot in Fig. 3*b* 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 *Nespas* is postulated to repress the expression of *Nesp* in *cis* from the paternal allele, *Nespas* can be regarded as an "imprintor" 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.

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