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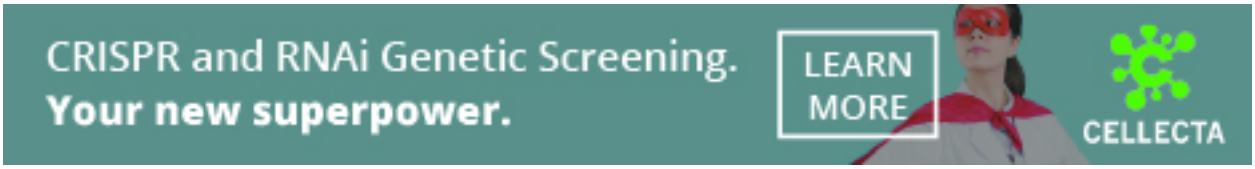

Letter

A Comprehensive Transcript Map of the Mouse *Gnas* Imprinted Complex

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The recent publication of the FANTOM mouse transcriptome has provided a unique opportunity to study the diversity of transcripts arising from a single gene locus. We have focused on the *Gnas* complex, as imprinting loci themselves provide unique insights into transcriptional regulation. Thirteen full-length cDNAs from the FANTOM2 set were mapped to the *Gnas* locus. These represented one previously described transcript and 12 putative new transcripts. Of these, eight were found to be differentially expressed from either the maternal or paternal allele. Two clones extended Nespas in the 3′ direction, providing evidence of antisense transcription spanning a 30-kb genomic region from a single allele. The transcripts were summarized into six transcriptional units, Nespas, Nesp, Gnasxl, F7, exon 1A, and Gnas. The resolution of the Gnas transcript map by the FANTOM2 clones revealed a pattern of alternate splicing. In addition to the transcripts described previously as splicing onto exon 2 of *Gnas*, each new sense transcript had an alternate short 3′UTR independent of *Gnas*. Both spliced and unspliced variants of the new imprinted sense transcripts were found. Whereas the functional significance of these alternate transcripts is not known, the availability of the FANTOM clones has provided remarkable insights into the repertoire of transcripts in the *Gnas* complex locus.

[Supplemental material is available online at www.genome.org. The sequence of the clones described in this paper have a RIKEN accession no., and will be assigned GenBank nos. once the data are released.]

Distal Chromosome 2 was one of the first imprinted regions identified in mouse (Cattanach and Kirk 1985). Mice with two maternal copies of the region and no paternal copies [MatDp(dist2)] fail to survive for more than a few hours after birth, are small, and hypoactive. In contrast, mice with two paternal copies but no maternal copies [PatDp(dist2)] are hyperactive, edematous, and usually die within a few days of birth. In recent years, the *Gnas* complex locus has been shown to lie within the region (Williamson et al. 1996) and to be imprinted (Kelsey et al. 1999; Peters et al. 1999). Some features of the PatDp(dist2) and MatDp(dist2) mice are recapitulated in mice with maternal and paternal inheritance, respectively, of a targeted deletion of *Gnas* exon 2 (Yu et al. 1998), and also in *Oed* mice (Cattanach et al. 2000), which have a missense mutation in *Gnas* exon 6 (Skinner et al. 2002). These observations strongly suggest that *Gnas* contributes to the PatDp(dist2) and MatDp(dist2) phenotypes. Mice with maternal inheritance of the *Gnas* exon 2 knockout, however, and also those with a missense mutation in *Gnas* exon 6, are not hyperactive at birth and variably survive, unlike PatDp(dist2) mice, suggesting that these effects are attributable to other imprinted genes or transcripts at the *Gnas* locus that exclude exon 2 or exon 6. In addition, mice with paternal inheritance of the *Gnas* exon 6 missense mutation show postnatal growth retardation (Skinner et al. 2002), as do those mice with paternal inheritance of the *Gnas* exon 2 knockout that survive the neonatal period (Yu et al. 1998). Therefore, the death of MatDp(dist2) within a few hours of birth may also be attributed to other imprinted genes or transcripts at the *Gnas* locus that exclude exon 2 or exon 6.

The *Gnas* locus is extremely transcriptionally complex through the use of alternative first exons, alternative splicing of downstream exons, and antisense transcripts. Four alternative first exons are known—Nesp, Gnasxl, Gnas exon 1a, and Gnas exon 1—each of which splices onto exon 2 of *Gnas* itself (Peters et al. 1999; Liu et al. 2000). Nesp is expressed exclusively from the maternal allele, and Gnasxl and Gnas exon 1a are oppositely paternally expressed. Although the Gnas exon 1 transcript is mainly bi-allelically expressed, it shows maternal specific expression in some tissues (Yu et al. 1998). *Gnas* has 13 exons, but transcripts are known that terminate in a neural exon following exon 3 (Crawford et al. 1993). A number of transcripts antisense to Nesp have been described that are collectively called Nespas and may have a regulatory function (Li et al. 2000; Wroe et al. 2000; Williamson et al. 2002). The discovery of new transcripts at the *Gnas* locus provides candidates for a complete functional analysis of this region and greater understanding of the MatDp(dist2) and PatDp(dist2) phenotypes.

RESULTS

The FANTOM2 data set were screened for clones lying in the *Gnas* imprinting cluster. Thirteen putative *Gnas* clones were identified through the FANTOM2 annotation process, and de-

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fined by the FANTOM2 genome mapper as part of the known *Gnas* locus (The FANTOM Consortium 2002). Only one of the FANTOM clones had been described previously and corresponded to Gnas exons 2–13 (data not shown). The other 12 FANTOM clones appeared to represent new transcripts in the *Gnas* imprinting cluster and their genomic location and exon structure are shown in Figure 1. All splicing followed the GT/AG rule. Supplementary Table 1 summarizes the tissues from which ESTs associated with each transcript originated.

Strand-specific RT-PCR was performed on RNA derived from 15.5-dpc embryos with maternal duplication [MatDp(dist2)] or paternal duplication [PatDp(dist2)] of the imprinting cluster to confirm the existence of the new transcripts and to establish their imprinting status on the sense or antisense strand. The strand-specific reverse transcription primers, together with the PCR primers for each clone are listed in Supplementary Table 2. The integrity of the RNA was confirmed by reverse transcription with oligo dT and amplification with *Hprt* primers (Fig. 2A). Classification of embryos was confirmed by expression analysis. Therefore expression of maternal-specific Nesp coupled with lack of expression of paternal-specific Gnasxl confirmed the MatDp(dist2) embryos. Conversely, lack of expression of Nesp, together with expression of Gnasxl, confirmed the PatDp(dist2) embryos (Fig. 2B,C).

Extending the Length of Nespas

FANTOM cDNA clones, D330038P10 of 629 bp and D030028H20 of 3.8 kb derived from 13-dpc embryonic heart and 9-dpc embryonic whole body libraries respectively, were designated F1 and F2, respectively (Fig. 1). They gave an excellent sequence match at the 5′ end of Nesp. The match to the BAC genomic sequence (accession no. AL593857) was on the complementary strand, and the association of each clone with ESTs previously orientated as antisense to Nesp, suggested that F1 and F2 are derived from antisense transcripts. An imprinted antisense transcript, Nespas, had been identified previously. F1 and F2 were mapped 15 kb and 1.87 kb, respectively, from the 3′ end of a Nespas poly (A) signal. Following strand-specific reverse transcription and amplification (see Fig. 1 and Supplementary Table 2), a band of the expected size was amplified in PatDp(dist2) but not MatDp(dist2) (Fig. 2D,E), confirming that F1 and F2 represent antisense transcripts that are paternally expressed. PCR from F1 to exon IV of the Nespas spliced form (Williamson et al. 2002) revealed the presence of a paternal-specific product (Fig 2L). Sequencing identified two novel exons, the first lying between F1 and F2 (nucleotides 82123–82289, AL593857), the second between F2 and Nespas exon V (nucleotides 69172–96104, AL593857) (Fig. 3). Therefore F1 is part of a new alternative noncoding splice variant of Nespas that extends the previously reported length of Nespas (Williamson et al. 2002) by 15.8 kb. We propose that F2 is also part of a Nespas transcript.

Further Transcripts of Nesp

The remaining 10 FANTOM cDNAs matched the sense strand of the genomic sequence and all showed matches to ESTs on the sense strand as shown in Figure 1. Clone A230089C09, designated F3, from a 15-dpc embryonic head library, consisted of exons 1 and 2 of Nesp, which spliced into the region between Nesp and Gnasxl, so identifying two new exons as shown in Figure 1. As expected, this new transcript showed maternal-specific expression because after hybridization with a PCR product of EST AA955518 it was detected exclusively in the MatDp(dist2) embryo (Fig. 2F). The presence of several bands in Figure 2F suggests there are a number of splice variants. One of these may be clone D9300020N02 of 1969 bp, designated F4, also from a 15-dpc embryonic head library, which is an unspliced cDNA that lies 3′ of Nesp and shows homology to one of the new exons identified in F3 (Fig. 1). F4 also showed maternal-specific expression (Fig. 2G) after hybridization with a PCR product of EST AA955518. No amplification was observed in the MatDp(dist2) sample without reverse transcriptase showing the RNA was not contaminated with genomic DNA and therefore providing proof that the amplification seen in the MatDp(dist2) sample with reverse transcriptase was caused by the presence of a real transcript.

New Transcripts of Gnasxl

F5 (clone C130027O20), from a 16-dpc embryonic head library, contains the first exon of Gnasxl and is contiguous with the genomic DNA sequence through the splice

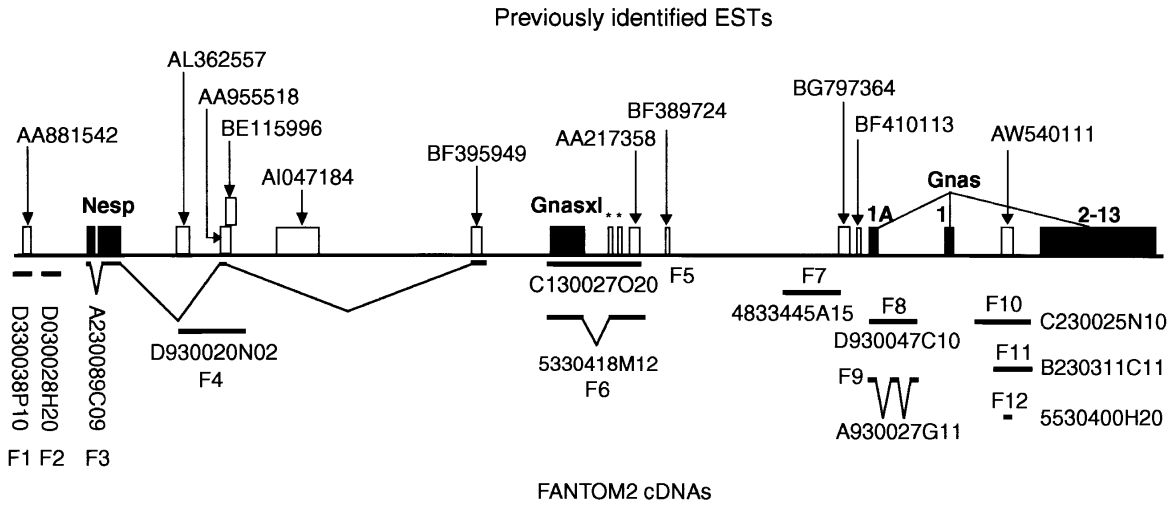

Figure 1 Relative position of FANTOM2 cDNAs. Previously identified exons (solid boxes) and ESTs (open boxes) are shown *above* the line and FANTOM2 clones *below*. Asterisks indicate regions of homology to human A20 and A21 exons. Figure not to scale.

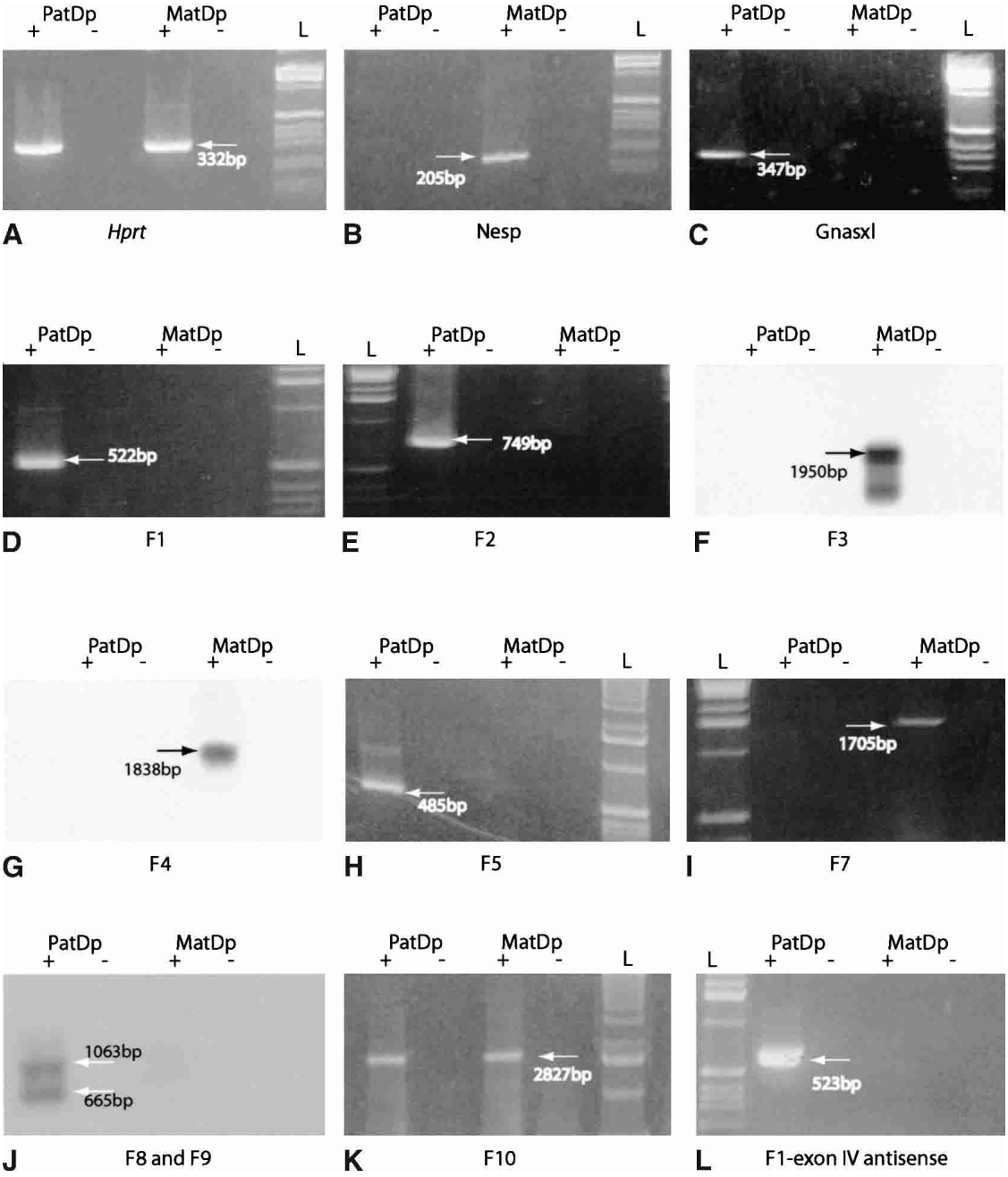

Figure 2 Imprinting status of FANTOM clones F1–F10. Each PCR product is sized with a -kb ladder (Invitrogen). MatDp(dist2) and PatDp(dist2) are abbreviated to MatDp and PatDp, respectively. + and − refers to samples amplified in the presence (+) or absence (−) of reverse transcriptase. Oligo dT-primed cDNA was amplified with: (*A*) *Hprt* primers as an amplification control; (*B*) *Nesp* primers to confirm the MatDp(dist2) cDNA; and (*C*) *Gnasxl* primers to confirm the PatDp(dist2) cDNA. Strand-specific RT-PCR of FANTOM clones 1–10: (*D*) paternal expression of F1; (*E*) paternal expression of F2; (*F*) maternal expression of F3; (*G*) maternal expression of F4; (*H*) paternal expression of F5; (*I*) maternal expression of F7; (*J*) paternal expression of F8 and F9; and (*K*) bi-allelic expression of F10. The PCR products from F3 (*F*), F4 (*G*), F7 (*I*), and F8 (*J*) were blotted and probed as described in Methods. (*L*) F1 is part of Nespas. PCR from exon IV of Nespas to F1 reveals a paternal-specific product. The products in *A–C*, *G*, *H*, and *L* were amplified for 25 cycles. The products in *D–F*, *I* and *J* were amplified for 30 cycles; the product in *K* was amplified for 20 cycles. (L) 1-kb Ladder (Invitrogen).

donor site of the exon and through the region that contains sequences homologous to exons A20 and A21, identified in human, as shown in Figure 1. F5 was detected in the PatDp(dist2) embryo but not in the MatDp(dist2) embryo (Fig. 2H), showing the transcript is paternally expressed on the sense strand and therefore represents an alternative form of the Gnasxl transcript. Clone 5330418M12, designated F6, from a pituitary library, is a spliced form of F5 in which Gnasxl splices into the putative mouse A20 exon. Human transcripts in which XLαs, the homolog of Gnasxl, splices

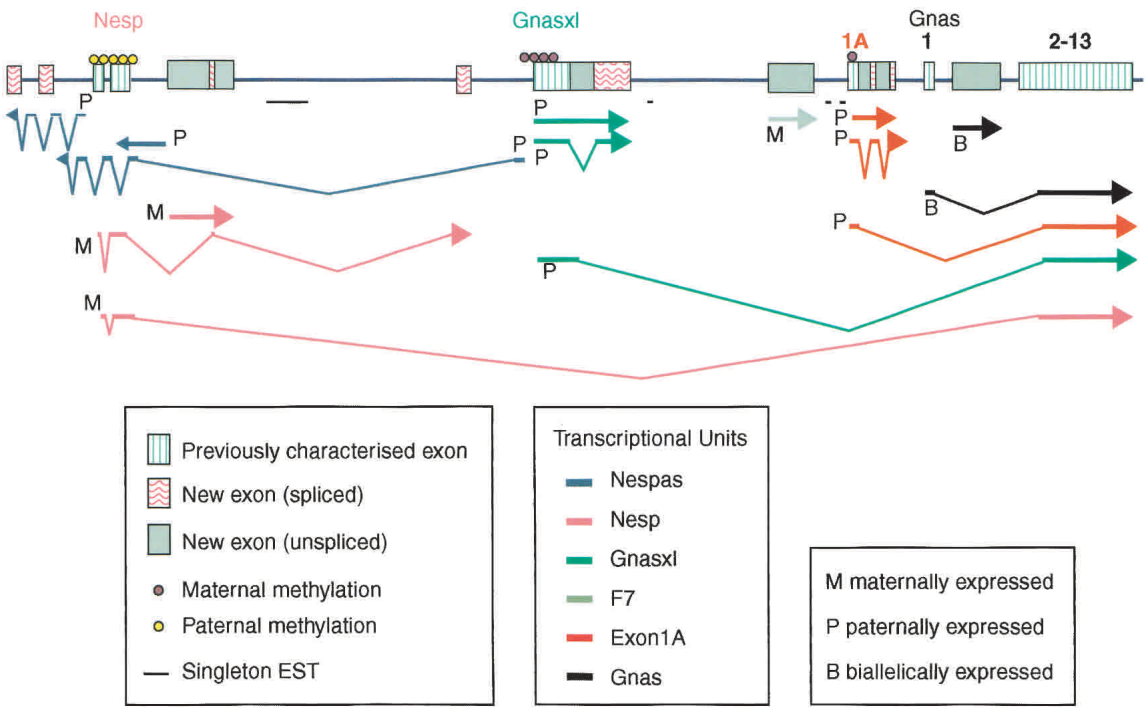

Figure 3 Transcript map of the *Gnas* complex imprinted locus. Six transcriptional units were defined from this study; these are Nespas, Nesp, Gnasxl, F7, exon 1A, and Gnas. Each transcriptional unit is associated with a differentially methylated promoter, and consists of several splice variants. F7 is the exception, with unexpected maternal expression indicating a link to the Nesp promoter. The arrows show direction of transcription. Figure is not drawn to scale.

into A20 or A21 had been identified previously (Hayward et al. 1998). F6 may be expressed prenatally and this may account for our inability to detect this spliced form in our expression analysis of whole 15.5-dpc embryos (Fig. 2H).

A New Maternal Transcript

Clone 4833445A15, F7, from a newborn head library, maps between Gnasxl and exon 1A and lies 2138 bp from the 5′ of exon 1A and is expressed in MatDp(dist2) but not PatDp(dist2) (Fig 2L). This is the first maternally expressed transcript to be identified between Gnasxl and exon 1A.

Further Transcripts of Gnas Exon 1A

Clone D930047C10, F8, from a 15-dpc embryonic head library, starts at exon 1A, is contiguous with the genomic sequence and extends 1080 bp as far as nucleotide 1535 (accession no. AF152375). F8 showed paternal specific expression (Fig. 2J). Therefore F8 appears to represent an unspliced form of the paternally expressed exon 1a transcript on the sense strand. Clone A930027G11, F9, from a retina library, is a spliced form of F8. Using the primers for F8 a smaller product of ~665 bp was amplified in PatDp(dist2) but not MatDp(dist2) (Fig. 2J) and this would be consistent with the existence of a splice form as shown for F9 in Figure 1.

New Gnas Transcripts

Clone C230025N10, designated F10, from a newborn cerebellum library, lies in the intron between exon 1 and 2 of *Gnas* but does not extend into either exon (Fig. 1). The transcript was detected in both MatDp(dist2) and PatDp(dist2) thereby showing that this transcript is bi-allelically expressed (Fig. 2K). Two other FANTOM cDNAs, F11 and F12, (clone numbers B230311C11 from a *Chrysolina quadrigemina* library and 5530400H20, from a library of 10-day-old head, respectively) represented shortened forms of F10 and are predicted to be bi-allelically expressed. F10 is consistent with bi-allelic expression of *Gnas* itself (exons 1–13).

DISCUSSION

Analysis of the FANTOM full-length cDNA collection has allowed a comprehensive transcript summary of the *Gnas* complex imprinted locus. The *Gnas* locus was known to be transcriptionally rich, with multiple ESTs mapped previously to the genomic BAC (Fig. 1). Thirteen full-length FANTOM cDNA clones, representing six transcriptional units (TU), describe novel splice variants of Nespas, Nesp, Gnasxl, exon 1A, and Gnas, as well as a completely novel, maternally expressed transcript 5′ of exon 1A, designated F7. We predict that each alternatively spliced transcript will be associated with the differentially methylated promoters described previously (Kelsey et al. 1999; Peters et al. 1999; Liu et al. 2000) for each transcriptional unit (Fig. 3). Although the origin of F7 remains a conundrum, the maternal expression of this transcript predicts an association with the Nesp promoter. We observed a common pattern of alternate splicing. In addition to the previously described transcripts in which the 5′ exons of Nesp, Gnasxl, and exon 1A were spliced onto exon 2 of Gnas, each 5′ exon was spliced onto an alternate 3′ UTR independent of *Gnas*. Additionally, each of these alternate 3′ UTRs were expressed as spliced or unspliced variants. The alternatively spliced messages from each transcriptional unit were mono-

allelically expressed from the same allele. This emerging pattern of transcription may offer insights into the control or processing of imprinted transcripts, though the functional significance of this observation is as yet unclear.

The start site of Nespas was known to lie ~2 kb upstream of Gnasxl (Li et al. 2000) in a maternally methylated region and the transcripts extend for more than 15 kb of genomic sequence (Williamson et al. 2002). Nespas has now been extended farther by almost 16 kb upstream of Nesp, so that it now covers >30 kb. It may be of significance that the antisense transcripts at human *GNAS* also cover >30 kb of genomic sequence, starting just upstream of XLαs (homologous with Gnasxl) and extending 19 kb upstream of NESP (Hayward and Bonthron 2000). Furthermore, Nespas may have a function in heart, for it was shown previously to be expressed strongly there (Wroe et al. 2000), and the F1 clone that extends Nespas came from an embryonic heart library. Nespas, like a number of other imprinted antisense genes, such as *Air* (Wutz et al. 1997; Lyle et al. 2000), is noncoding, paternally expressed, and extends across a large genomic region. Antisense transcription appears to be a common feature of imprinted regions and there is evidence that it has a role in regulating expression from its sense counterpart (Wutz et al. 1997; Lee 2000; Sleutels et al. 2002).

F10, F11, and F12, like *Gnas*, were bi-allelically expressed, however, we cannot exclude the possibility that these clones are tissue-specifically imprinted. *Gnas* itself is bi-allelic in most tissues but is maternally expressed in certain cell types in mouse (Yu et al. 1998) and human (Hayward et al. 1998).

It is difficult to speculate how comprehensive a transcript map the FANTOM2 clones provide. We found no cDNA matches in 122 kb of BAC sequence upstream of the most 5′ clone, F1. There is some mouse EST evidence of transcription in this region; however, it is not known whether these ESTs are part of the *Gnas*-imprinted complex. No cDNA matches were found in the 3.9-kb region between exon 13 of *Gnas* and the 3′ end of the BAC sequence. This is consistent with human studies, where the next gene 3′ of Gnas is *TH1*, which lies >70 kb downstream of GNAS1 and is outside the imprinted region (Bonthron et al. 2000).

Analysis of the FANTOM2 data set has revealed a further level of complexity of the single *Gnas* locus and indicates the massive increase in coverage and diversity found in the mouse transcriptome. Complex regions of transcription like the *Gnas* locus provoke our definitions of a gene beyond simple exon sharing. The FANTOM2 consortium coined the phrase "Transcriptional Unit" to define a functional clustering of transcripts. This single gene locus contains at least six imprinted TU, each containing several splice variants. Genetic studies have provided strong evidence that the complex phenotypes associated with the region may be attributable to functional differences between these TU. These data indicate the depth of the mouse transcriptome is more than the number of genes in the genome; diversity of transcripts from a single gene locus is the variable that underlies dynamic gene networks.

METHODS

FANTOM2 cDNA Clone Analysis

New clones were identified as part of the FANTOM2 annotation process (FANTOM2 Main Paper) and were mapped to BAC sequence of the region (accession no. AL593857, clone number RP23–439H2).

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 standard methods of intercrossing reciprocal translocation heterozygotes (Searle and Beechey 1978). The reciprocal translocation T(2;8)26H was used as described previously (Williamson et al. 1996). MatDp(dist2), PatDp(dist2), and wild-type embryos were identified at 15.5 dpc by typing for the marker *D2Mit226* (Williamson et al. 1995). The MatDp(dist2) and PatDp(dist2) embryos were used to establish the imprinting status of the FANTOM2 clones mapping to the *Gnas* cluster.

Strand-Specific RT-PCR

Poly(A)$^+$ RNA was extracted from MatDp(dist2) and PatDp(dist2) embryos using the FastTrack 2.0 mRNA isolation kit (Invitrogen) and the RNA was treated with RNase-free DNase I using the Message Clean kit (GenHunter Corporation). Primers to amplify cDNAs expressed from the *Gnas* cluster were designed from the BAC sequence (accession no. AL593857, clone number RP23–439H2) and were supplied by SigmaGenosys (Cambridge, UK). The relative positions of the primers are shown in Figure 1, and their sequence is provided in a Supplementary Table 2.

Strand-specific RT-PCR was performed as described by Wroe et al. (2000). Each RNA sample was reverse transcribed in the presence and absence of reverse transcriptase. Strand-specific primers and 1 µg of oligo(dA)$_{80}$ [Genosys] 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 (Promega), to trap any oligo(dT) that might have co-purified with the poly(A)$^+$ RNA (Nguyen et al. 1995). 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, consisting of 20–30 cycles (as described in the legend of Fig. 2) of 1 min each at 94°C, 55°C, and 72°C by using Thermoprime Plus DNA polymerase (Advanced Biotechnologies). Extensor Long Master Mix (Advanced Biotechnologies) was used to amplify FANTOM cDNAs 3, 8, and 9. cDNA (200ng) and each primer (300 nM) were used in a 50-µL reaction and amplified using the following conditions: 94°C for 10 sec, 62°C for 30 sec, and 68°C for 2 min for 10 cycles followed by 20 cycles at 94°C for 20 sec, 60°C for 30 sec, and 68°C for 2 min.

Southern Hybridization

DNA was transferred onto charged nylon membranes (Hybond N$^+$; Amersham Pharmacia) by alkaline transfer. PCR products were radiolabeled with 25 µCi of [$-^{32}$P]dCTP (NEN) using Megaprime (Amersham Pharmacia). The Southern filters were hybridized using Church and Gilbert hybridization buffers (Church and Gilbert 1984). The products of F3 (f) and F4 (g) were probed with PCR amplified BAC DNA (clone number RP23–439H2) using forward and reverse primers for EST AA955518, and the PCR products of F8 and F9 (j) were probed with the PCR amplified BAC DNA using primers FAN 8F and FAN 8R.

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