

Alternative non-coding splice variants of *Nespas*, an imprinted gene antisense to *Nesp* in the *Gnas* imprinting cluster

Christine M. Williamson,¹ Judith A. Skinner,¹ Gavin Kelsey,² Josephine Peters¹

¹MRC Mammalian Genetics Unit, Harwell, Didcot, Oxfordshire, OX11 0RD, UK

²Laboratory of Developmental Genetics and Imprinting, The Babraham Institute, Cambridge, CB2 4AT, UK

Received: 29 June 2001 / Accepted: 4 October 2001

Abstract. The *Gnas* locus on mouse Chr 2 represents a unique cluster of overlapping imprinted genes. Three of these in the order *Nesp*–*Gnasxl*–*Gnas* are transcribed in the sense direction with *Nesp* having maternal-specific expression, *Gnasxl* having paternal expression, and *Gnas* as being biallelically expressed in most tissues. A fourth imprinted gene, *Nespas*, is paternally expressed, lies antisense to *Nesp*, and expresses an unspliced transcript. Large unspliced antisense transcripts are emerging as a feature of imprinted gene clusters, and such non-coding RNAs may have a *cis*-regulatory function. Here we show that, in addition to an unspliced form of *Nespas*, we can detect five alternatively spliced forms of *Nespas* up to 1.4 kb in length that are non-coding. The splice variants are paternally expressed; they start approximately 2 kb upstream of *Gnasxl* in a region of maternal methylation and end 2.5 kb beyond the ATG of *Nesp*. These variants do not correspond to exons of the human antisense transcript although they start in the same region; the *Nespas* transcript, like its human counterpart, is spliced in various alternative patterns. The identification of a set of small spliced imprinted transcripts in the human and now in the mouse suggests that these antisense transcripts are functionally important.

Introduction

Genomic imprinting is an epigenetic phenomenon affecting a subset of genes that causes silencing of one or other parental copy. To date, 48 imprinted genes have been described, many of which are located in clusters (Beechey and Cattanach, <http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html>), suggesting coordinately controlled regulatory mechanisms. A cluster of imprinted genes has been identified in distal Chromosome (Chr) 2 of the mouse (Peters et al. 1999; Kelsey et al. 1999), a region known from early genetic studies to contain imprinted genes (Cattanach and Kirk 1985; Williamson et al. 1998), as well as the homologous region of the human genome, Chr 20q13.3 (Hayward et al. 1998a, 1998b). Three protein-coding genes have been found in the cluster; these are *Gnas*, which encodes a G-protein α -subunit; *Nesp*, which encodes a neuroendocrine secretory protein (Ischia et al. 1997); and *Gnasxl*, which encodes an extra-large $G\alpha$ isoform (Kehlenbach et al. 1994), but little is known about the function of these latter two proteins. *Gnas* is biallelically expressed in most tissues but shows paternal-specific expression in some (Williamson et al. 1996) and maternal-specific expression in others (Yu et al. 1998). *Nesp* and *Gnasxl* are expressed from opposite parental alleles, with *Nesp* having paternal-specific methylation and maternal expression, and

Gnasxl having maternal-specific methylation and paternal expression (Peters et al. 1999; Kelsey et al. 1999). These genes form a single transcription unit as transcripts of *Nesp* and *Gnasxl* are alternatively spliced onto exon 2 of *Gnas*. Another member of this transcription unit has recently been reported (Liu et al. 2000), and this has maternal-specific methylation and paternal expression. This new transcript is of unknown function and is composed of an alternative first exon of *Gnas*, exon 1a, that splices onto exon 2 of *Gnas*. Thus, the cluster is emerging as a highly complex set of overlapping maternally, paternally, and biallelically expressed transcripts generated by the usage of alternative, unmethylated promoters (Fig. 1).

The cluster was shown to be even more complex by the finding of the antisense gene *Nespas* that is also imprinted. *Nespas* determines a paternally expressed transcript that is transcribed in the antisense direction compared with *Nesp*, and crosses both *Nesp* exons (Wroe et al. 2000). The *Nespas* transcript is weakly expressed, unspliced, and appears to be comprised of an unusually large RNA and/or a collection of differently sized RNAs, as determined by Northern analysis (Wroe et al. 2000). Large (>40 kb) unspliced imprinted transcripts have been described for other antisense genes *Air* (Lyle et al. 2000), *LIT1* (Mitsuya et al. 1999), and *Tsix* (Lee et al. 1999), and there is evidence to suggest they may have a regulatory role. Imprinted antisense transcripts have been described at the human GNAS1 locus that are only about 2 kb in size and are spliced (Hayward and Bonthron 2000). The spliced antisense transcript at GNAS1 extends over more than 30 kb, starting just upstream of XL α s (the homologue of *Gnasxl*) and extending 19 kb upstream of *NESP*. In this report we show that, in addition to the unspliced imprinted antisense transcript in the *Gnas* cluster in the mouse, there are spliced antisense imprinted transcripts with similar features to the antisense transcripts at the human GNAS1 locus.

Materials and methods

The sequence of the spliced *Nespas* cDNA and genomic sequence 5' of *Nesp* have been submitted to the GenBank database under accession numbers AF319945 and AF319946, respectively.

Mice and RNA. Mice with maternal [MatDp(dist2)] and paternal [PatDp(dist2)] duplication for the distal Chr 2 imprinting region were generated by standard methods: intercrossing reciprocal translocation heterozygotes (Searle and Beechey 1978). The reciprocal translocation T(2;8)26H with a breakpoint in Chr 2 in band H1 and a breakpoint in Chr 8 in band A4 was used as described previously (Williamson et al. 1996). The duplication embryos were identified at 15.5dpc by typing for the marker *D2Mit226* (Williamson et al., 1995). Poly(A)⁺ RNA was extracted from embryos using the FastTrack 2.0 mRNA isolation kit (Invitrogen) and treated with RNase-free DNase I as described previously (Wroe et al. 2000). The RNA was subsequently used for expression analysis.

All animal studies were carried out under the guidance issued by the

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers AF319945 and AF319946.

Correspondence to: J. Peters; E-mail: j.peters@har.mrc.ac.uk

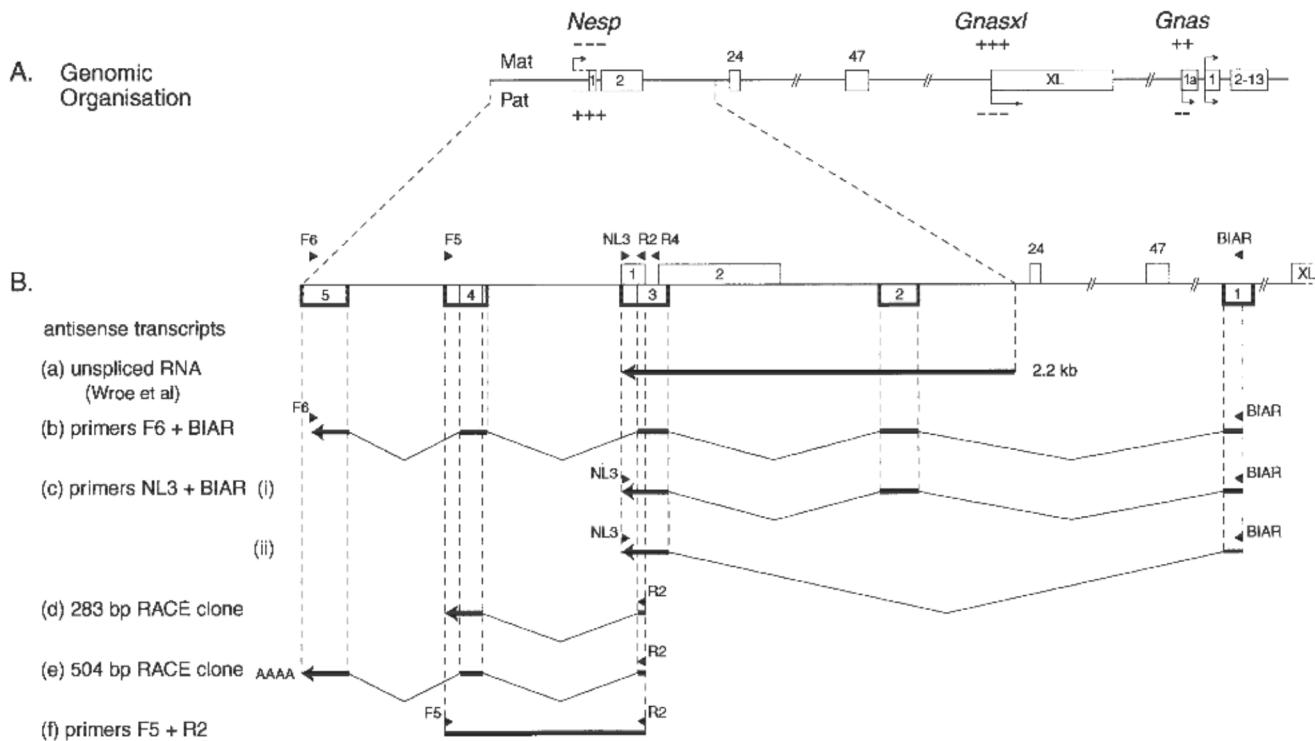


Fig. 1. (A) Genomic organization of cluster. The methylation status of CpG regions are shown as --- (unmethylated) or +++ (methylated) either above or below the line to refer to the maternal or paternal chromosome, respectively. The arrows refer to the direction of transcription. (B) Summary of antisense transcripts. Boxes below the line represent the exons identified in *Nespas*, and boxes above the line refer to previously identified

exons and two ESTs. 24 represents EST AV247408, and 47 represents EST AI047184. Arrowheads refer to specific primers, and the arrows refer to direction of transcription of the antisense transcripts. The genomic region between the dotted lines, from primer F6 to the region between exon 2 of *Nespas* and EST 24, is to scale; approximately, 1 mm represents 34 bp. The poly(A) tail is represented by AAAA.

Medical Research Council in *Responsibility in the Use of Animals for Medical Research* (July 1993) and Home Office Project Licence Number 30/1518.

3' RACE. 3' rapid amplification of cDNA ends was performed with RACE (Gibco BRL) according to the instructions provided by the manufacturer. The 3' end of *Nespas* was amplified with gene-specific primer R2 (5' AACCACTGCGAACTTCTAA) and AUAP primer (Gibco BRL). PCR products were subcloned by using the TA cloning kit (Invitrogen). Colonies were replica plated onto colony/plaque screen (NEN Research Products) and hybridized with an appropriate probe as described (Wroe et al. 2000). PCR products for labeling were gel purified with the QIAquick gel extraction kit (Qiagen). DNA was extracted from positive colonies with the Qiagen plasmid mini kit, and cycle sequencing was performed as described below.

5' RACE. 5' rapid amplification of cDNA ends was performed with SMART RACE cDNA amplification kit (Clontech). The 5' end was amplified with gene-specific primer BICF (5' GCCCACCCACTCCAGGC-CCTGG) and UPM primer (Clontech). PCR products were cloned as described for 3' RACE, and clones were identified by probing with gel-purified PCR product from cDNA amplified with primers BICF and BIAR (Fig. 2A).

RT-PCR. Reverse transcription was performed with mouse murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, Md.). All primers were supplied by SigmaGenosys (Cambridge, UK), and their sequences were as follows: F6 (5' TTCTGTACTAGTCATAGGAT-GTGG 3'), F5 (5' CTATATTAACAAGGCAAAGGCAA 3'), NL3 (5' AGTGGAGGCACCTCTCGGA 3'), R4 (5' AGAACGCTGGGCTCTGA-AGAC 3'), BIAR (5' CGTATGGCGGCCAGGCCTAG 3'), BICF (5' GC-CCACCACCTCCAGGCCCTGG 3'), BIBR (5' CGGTGGAAACGC-CCAAGAGG 3'), BICR (5' GATATGGGTGGGAGGTCCAA 3'), NXLR2 (5' CGGCACACTACAGCCCCTTG 3').

Sequence analysis. Cycle sequencing was performed with ABI Big DyeTM terminator mix (Perkin Elmer), and products were electrophoresed on an ABI 377 automated sequencer. Genomic sequence 5' of *Nesp* was obtained from clone MPMGc121H20234Q2 (129/Ola mouse cosmid library number 121, RZPD Berlin) by cycle sequencing with primer F6tr (5'-GGGTGCTGGTAAATGCCCGTTGATGG-3'). Sequences were analyzed for similarity by using the BLAST programs at <http://www.ncbi.nlm.nih.gov/blast>, and ORF finder was used to look for coding potential.

Results

The extent of *Nespas* at the 5' end. The 5' ends of imprinted genes tend to be associated with differentially methylated regions. We therefore expected the 5' end of the paternally expressed *Nespas*, identified by Wroe et al. (2000), to be associated with a maternally methylated region 3' of *Nesp*. Indeed, at the time one major site of maternal-specific methylation had been identified at the *Gnasxl* locus and provided a good candidate region for the start of *Nespas* (Fig. 1A). A putative start site had been identified in the homologous region in human (Hayward and Bontron 2000) and by analogy would be expected to lie between primers BIBR and BICR in the mouse at nucleotide 13136, as shown in Fig. 2A. The analysis performed by Li et al. (2000) in the mouse suggested the start site was at either nucleotide 13082 (120 bp from the start of their primer) or between 13121 and 13151 (within 15 bp of the human start site). We performed 5' RACE using primer BICF (Fig. 2A) and identified a start site at 13094 from two separate clones, and so it is different from that identified by Li et al. (2000). We also used an RT-PCR approach in which several primers were designed from the mouse genomic sequence (AJ251761) between primer BICF and *Gnasxl* (Fig. 2A); primer BICR was 7 bp upstream of the

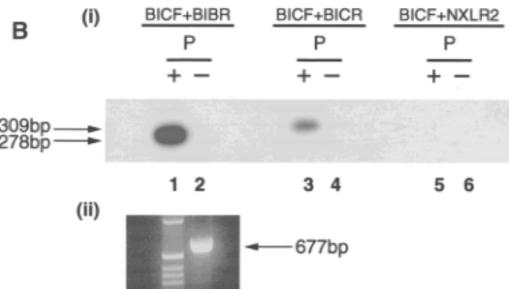
A

Fig. 2. Analysis of the 5' end of the paternally expressed *Nespas*. (A) Sequence showing the approximate positions of primers used to amplify the 5' end of *Nespas*. The direction of transcription of *Nespas* is from right to left. Bold bases: * proposed human start site at 13136 (Hayward and Bontron 2000); ** a proposed mouse start site by RACE (this study). Double line below sequence represents possible positions of start site from Li et al. (2000). Nucleotide numbers refer to the annotated sequence (AJ251761). (B) (i) RT-PCR analysis of PatDp.dist2 (P) cDNA with

most 5' maternally methylated *Hpa*II site (Kelsey et al. 1999). RT-PCR analysis was done on DNase I-treated poly(A)⁺ from 15.5 dpc embryos with paternal duplication for the distal Chr 2 imprinting region [PatDp(dist2)]. Amplification was observed with primers BICF and BIBR [Fig. 2B (i), lane 1] and a weaker product was obtained with primers BICF and BICR (lane 3). No product was detected with BICF and NXLR2 after blot hybridization (lane 5) even though the 677-bp genomic region was amplifiable with BICF and NXLR2 as shown in Fig. 2B (ii) (lane 2). Strand-specific reverse transcription with primer BICF and subsequent amplification with primers BICF and BIBR showed the paternal transcript was expressed from the antisense strand (data not shown). The amplification observed with BICF and BIBR indicates there is a start site upstream of BIBR, possibly within BICR. This would be consistent if the position identified by Li et al. (2000) is between 13121 and 13151 and could account for the weak amplification with BICF and BICR. In conclusion, using 15.5 dpc embryos, we have identified at least two start sites for *Nespas*, at least one of which is different from the site identified by Li et al. (2000).

Identification of spliced Nespas transcripts. The extent of the paternally expressed antisense transcript was investigated further by RT-PCR. Primer BIAR (Figs. 1B and 2A), which is part of the paternally expressed antisense transcript (see above), and primer F6, identified in a 3' RACE clone (see below), amplified a band of 1.2 kb in PatDp(dist2) embryos (Fig. 3A, lane 1), but not in MatDp(dist2) embryos (Fig. 3A, lane 3). Comparison of the cDNA with genomic sequence [AJ251480 and AJ245856 (Wroe et al. 2000), and AJ251761 (Hayward and Bontron 2000)] revealed that the cDNA [Fig. 1B(b)] was spliced and that the splicing followed the GT/AG rule in an antisense orientation with respect to *Nesp*. Five exons were identified: exons 1 and 2 lie between *Nesp* and *Gnasxl*; exon 3 spans the 5' end of *Nesp* exon 2, the 95-bp intron of *Nesp*, and the 3' end of *Nesp* exon 1; and exons 4 and 5 lie upstream of *Nesp*. The cDNA sequence and splice junctions are shown in Fig. 3B.

Another RT-PCR showed alternative transcripts extending from BIAR to NL3 [Fig. 1B, c (i) and (ii)], with and without exon 2, respectively], and these are expressed in the PatDp(dist2) (Fig. 3A, lane 5) and not in the MatDp(dist2) (Fig. 3A, lane 7). Thus, the



primer pairs BICF + BIBR, BICF + BICR, and BICF + NXLR2. The presence (+) and absence (-) of reverse transcriptase is shown. The PCR products were weak and thus were probed with a 217-bp, gel-purified PCR product from cDNA amplified with primers BICF and BIAR. Amplification was observed with BICF + BIBR and BICF + BICR, but not with BICF + NXLR2. (ii) PCR amplification of PAC 583L07 (RPCI21 Mouse PAC Library, HGMP Resource Centre, Hinxton, UK) with primers BICF and NXLR2 (lane 2), 1 kb ladder (lane 1).

transcripts in Fig. 1B [b, c (i) and (ii)] represent three alternative splice forms of *Nespas*.

Two further splice variants were found in experiments to characterize the 3' end of the *Nespas* transcript. 3' RACE was performed with DNase I-treated poly(A)⁺ RNA from 15.5 dpc PatDp(dist2) embryos, and two RACE clones of 283 and 504 bp [Fig. 1B, (d) and (e), respectively] were identified that extended from primer R2 in *Nesp* exon 1, and both revealed splicing in an antisense orientation. The donor site of the 283-bp transcript in exon 3 was identical to the site identified in the RT-PCR product [Fig. 1B (b)]; however, an alternative acceptor site was used 18 bp within exon 4, and the transcript extended through a donor site in exon 4. The transcript represented by the 504-bp clone showed a similar splicing pattern to that represented by the 283-bp clone except that in exon 4 an alternative donor was used which spliced into exon 5 in the same way as the transcript shown in Fig. 1B (b).

RT-PCR analysis using transcript-specific forward primers F5 and F6 (shown in Fig. 1B) and reverse primer R2 confirmed that the two RACE clones were alternative transcripts of the exclusively paternally expressed *Nespas* transcript. Spliced RT-PCR products of the expected size, 275 bp (Fig. 3C, lane 1) and 442 bp (lane 5), were detected in cDNA derived from 15.5 dpc PatDp(dist2) embryos, but not in the cDNA of age-matched MatDp(dist2) embryos (lanes 3 and 7). Interestingly, no amplification was detected with primers F5 and R2 in the MatDp(dist2) (Fig. 3C, lane 3), thus suggesting that the maternally expressed *Nesp* transcript probably starts downstream of primer F5 on the sense strand. The two RACE clones [Fig. 1B, (d) and (e)] represent another two alternatively spliced isoforms of *Nespas*, and thus five alternative forms have been identified in total.

3' end of spliced Nespas. The 3' end of the transcript represented by the 504-bp clone had a poly(A) signal with the sequence AT-TAAA (Fig. 3B, nucleotides 1222–1227), a common natural variant of the conventional sequence AATAAA. The poly(A) addition site in this clone was at nucleotide 1262 (Fig. 3B) and thus represents the 3' end of the *Nespas* transcript. The splice variants of *Nespas* would, therefore, appear to be up to 1.4 kb in length. One hundred and ten base pairs of sequence at the 3' end of the 504 bp clone (Fig. 3B nucleotides 1153–1262) were not present in the available mouse genomic sequence (AJ251761) and represented a

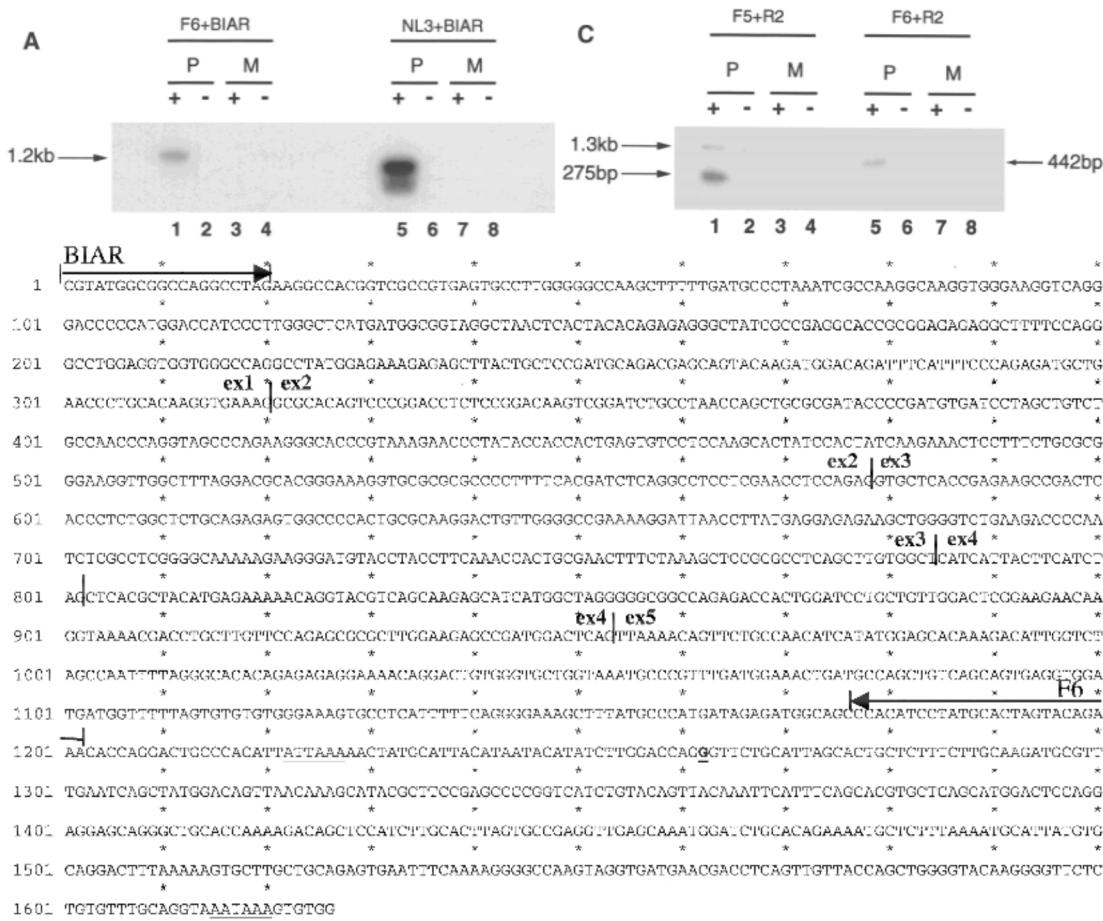


Fig. 3. (A) Paternal-specific expression of spliced antisense transcripts. Expression analysis was done by RT-PCR on MatDp(dist2) (M) and Pat-Dp(dist2) (P) poly(A)⁺ RNA from 15.5-dpc embryos derived from the reciprocal translocation T26H by using primers F6 + B IAR and NL3 + BIAR. After 25 cycles, the PCR products were weak and thus were probed with a 217-bp, gel-purified PCR product from cDNA amplified with primers BICF and BIAR. The presence (+) and absence (-) of reverse transcriptase are shown. (B) Sequence of the spliced antisense transcript extending from primer BIAR to the second poly(A) signal of *Nespas*. The precise end of the transcript (AF319945) as identified by 3' RACE is the G at nucleotide 1262, which is shown in bold and underlined. The sequence

between nucleotides 1263 and 1626 represents genomic sequence and shows the relative position of a potential poly(A) signal AATAAA; both poly(A) signals are underlined. Primers BIAR and F6 are shown by arrows on the sequence. The splice junctions are indicated by vertical lines, and an alternative junction is also shown. (C) Paternal-specific expression of alternatively spliced antisense transcripts with primer pairs F5 + R2 and F6 + R2. After 25 cycles, the PCR products were weak and thus were probed with a 275-bp, gel-purified PCR product from cDNA amplified with primers F5 and R2. Primers F5 and R2 amplified a 1.3-kb unspliced product in PatDp(dist2).

new sequence that did not show any matches to ESTs in the database. This sequence was subsequently shown to be contiguous with the adjoining genomic sequence obtained from cosmid MPMGC121H20234Q2 (RZPD). The poly(A) addition site of *Nespas*, identified in the 504-bp clone, thus lies approximately 2.5 kb upstream of the *Nesp* ATG start codon as shown in Fig. 3D. Analysis of the extra genomic sequence (Fig. 3B, nucleotides 1263–1626) revealed a putative poly(A) signal, AATAAA, 64 bp downstream of the first one in *Nespas*, but this has not yet been shown to be utilized. The 3' end of the 283-bp clone appeared to have no poly(A) signal but contained an A-rich stretch that was also found on the complementary strand in the genomic sequence (AJ245401, nucleotides 210–221). The stretch of 12 As was probably present within a larger antisense RNA and had provided a priming site for the oligo dT. It is possible that this RNA could represent a non-polyadenylated RNA that has co-purified with the poly-T-selected RNA.

Polyadenylated unspliced *Nespas* transcript. In the RT-PCR analysis shown in Fig. 3C, a larger 1.3-kb product was also am-

plified in cDNA derived from poly(A)⁺ RNA with primers F5 and R2 (Fig. 3C, lane 1) which on sequencing was shown to be an unspliced form (Fig. 1B f). Similar results were obtained with primer F5 and reverse primers R4 and NL3r (data not shown); primer R4 was designed to the 95-bp intron of *Nesp* and is specific for *Nespas*, and NL3r is the reverse complement of primer NL3. The unspliced product is assumed to be part of the polyadenylated antisense transcript (Wrode et al. 2000) and provides another 1.15 kb of sequence 3' so that the paternally expressed polyadenylated unspliced transcript is at least 3.35 kb in size. The poly(A) signal of the unspliced form is as yet undetermined.

Sequence analysis reveals no coding potential. The mouse *Nespas* cDNA was analyzed for similarity to the human genomic sequence (AL132655, AJ251760) by using the BLAST program. Although extensive sequence conservation across the *Nesp*–*Gnasxl* interval had been demonstrated previously (Hayward and Bonthron 2000), the splice donor and acceptor sites of *Nespas* were not conserved in human except for the donor site of exon 1 and the acceptor site of exon 3 (data not shown). Regions of homology were detected in

exons 1, 3, 4, and 5; the exon 1 homology has been described (Hayward and Bontron 2000). The two regions of homology in exon 3 showed 78% homology over 101 bp (nucleotides 584–684, AF319945, Fig. 3B), 100% over 23 bp (nucleotides 750–772) and coincide with *Nesp* exons on the sense strand. The match in *Nespas* exon 4 was 85% over 49 bp (nucleotides 842–890), and the matches in exon 5 were 79% over 273 bp (nucleotides 1041–1309; Fig. 3B), 86% over 200 bp (nucleotides 1428–1626; Fig. 3B), and were to regions that encompassed the *Nespas* poly(A) sites. The *Nespas* spliced cDNA showed no significant similarities to expressed sequence tags in the databases other than the homology to exon 1 of the human antisense and the mouse IMAGE clone 932324, which represents an unspliced form of *Nespas* (Wroe et al. 2000). Thus, exons 2–5 of *Nespas* do not show any homology with exons II–V of the spliced antisense identified in human.

The largest spliced *Nespas* cDNA was analyzed for open reading frames (ORFs) using the ORF finder program. The largest ORF, starting with a potential initiator methionine in exon 1 (AJ251761, nucleotide 13312), predicted a polypeptide of 103 amino acids; this was dissimilar to the 97-amino acid ORF predicted for the human GNAS1 antisense (Hayward and Bontron 2000). The 103-amino acid ORF is unlikely to code for a protein because the ATG did not conform to the Kozak consensus and the translated sequence was not conserved in human. We conclude from this comparative analysis that the spliced forms of *Nespas* are non-coding.

Discussion

There are a growing number of examples of imprinted genes producing non-coding RNAs that act in *cis* and have a regulatory function. Probably the most intensively studied is H19 (Brannan et al. 1990), which is only 90 kb from the reciprocally imprinted gene *Igf2*, which encodes a potent fetal growth factor. A similar arrangement was found for the reciprocally imprinted pair of genes, *Dlk1* and *Glt2*, whereby *Dlk1* is protein encoding and *Glt2* makes a non-coding RNA (Schmidt et al. 2000). An increasing number of antisense genes producing non-coding RNAs that are oppositely imprinted to their sense counterparts are being identified. These include *Air*, a gene that is antisense to *Igf2r*, the insulin-like growth factor type-2 receptor (Wutz et al. 1997, 2001; Lyle et al. 2000); *LIT1/Lit1*, which is antisense to KvLQT1, a gene associated with Beckwith-Wiedemann syndrome (Mitsuya et al. 1999); *Tsix*, which is antisense to *Xist*, which is necessary for X inactivation (Lee 2000), and *Nespas/GNAS1* antisense (Wroe et al. 2000; Hayward and Bontron 2000). One common feature of *Air*, *LIT1*, and *Tsix* is that they make very long unspliced transcripts, but *Tsix* has also been shown recently to produce complex alternative splicing (Sado et al. 2001). There is evidence in the mouse, but not yet in humans, for an unspliced *Nespas* transcript. It is contiguous with genomic DNA for at least 3.35 kb (this paper), has been estimated to be 11.2 kb (Li et al. 2000), and may include ESTs AV247408 and AI047184, which appear to represent sequences transcribed in an antisense orientation; the complete size remains to be determined.

Our further analysis of *Nespas* led to the identification of at least five paternally expressed splice variants that show exon skipping, exon truncation, and inclusion of sequences that could be described as intronic. A similar heterogeneous population of paternally expressed, spliced antisense transcripts, also with up to five exons, has been identified in humans. As in the mouse, the spliced antisense transcripts extend from a maternally methylated region just upstream of XLαs and span NESP (Hayward and Bontron 2000). There are notable regions of sequence homology in exon 1 and at the 3' end encompassing the *Nespas* poly(A) sites, thus suggesting the presence of functional constraints. However, none of the splice sites in the human were conserved except for the

first donor splice site (Li et al. 2000), which we now show is used in the mouse. Only two of the splice sites of the mouse *Nespas* are conserved in human. Overall there is little sequence conservation, and in neither species do the spliced transcripts encode a common protein product. However, the finding of a set of small spliced antisense transcripts in the region of *NESP* in human (Hayward and Bontron 2000) and now in the mouse suggests these antisense transcripts are functionally important.

Like *Nespas*, the transcriptional start sites of other noncoding transcripts *Air* (Lyle et al. 2000), *LIT1* (Mitsuya et al. 1999), and *Tsix* (Lee et al. 1999) lie in differentially methylated regions. Deletion of the CpG island at the start of *Air* resulted in loss of expression of the imprinted antisense transcript and deregulation of imprinting of its sense counterpart *Igf2r* (Wutz et al. 1997, 2001). Similarly deletion of *Tsix* deregulates *Xist* expression (Lee 2000), and loss of *LIT1* derepresses several imprinted transcripts (Horike et al. 2000). Thus, this emerging class of untranslated RNAs appears to be important as regulators of regional gene expression. The untranslated RNA itself may be the regulator and act by occluding the promoter of the sense transcript or by causing localized heterochromatinization of the sense allele (Reik and Constancia 1997). However, the antisense transcript itself may not be needed for regulation, but competition between the sense and antisense promoters for shared enhancers, for example, could be the regulatory mechanism. Another possibility is that transcription in the antisense direction somehow prevents transcription from the sense allele. The start site of the *Nespas* transcripts lies approximately 2 kb upstream of *Gnasxl*; the region between the start site and *Gnasxl* is maternally methylated and thus probably represents a regulator for the paternal-specific expression of both genes. The reciprocal imprinting of *Nesp* and *Nespas* in heart enabled us to predict that *Nespas* has a function in repressing the expression of *Nesp* in *cis* on the paternal chromosome (Wroe et al. 2000). However, this cannot be the only function of *Nespas* because non-complementary expression of *Nesp* and *Nespas* has been identified at mid-gestation (Ball et al. 2001) and in adult interspecific mice (Li et al. 2000). An alternative model is that these antisense transcripts have no function and arise as a consequence of an open chromatin structure in the paternally nonmethylated region. Whether the unspliced and alternatively spliced transcripts of *Nespas* are tissue-specific and have distinct functions during mouse development remains to be investigated. Gene knockout studies will be required to evaluate the role of *Nespas*, and one approach will be to delete the promoter region. If the deletion results in loss of imprinting of the *Nesp* gene, further studies will be required to investigate whether the *Nesp* silencing is mediated by the *Nespas* RNA or control elements in the promoter region. Analysis of their role will provide new insights into RNA-based regulatory mechanisms.

Acknowledgment. We are indebted to C.V. Beechey and E. Prescott for mouse resources, A.M. Mallon for informatics advice, A. Ford for Fig. 1, S. Thomas and K. Glover for labeled gel photos. Sequences were generated at the Sequencing, microArray and Genotyping Facility, MRC Harwell. G. Kelsey is a Senior Fellow of the MRC.

References

- Ball ST, Williamson CM, Hayes C, Hacker T, Peters J (2001) The spatial and temporal expression pattern of *Nesp* and its antisense *Nespas*, in mid-gestation mouse embryos. *Mech Dev* 100, 79–81
- Brannan CI, Dees EC, Ingram RS, Tilghman SM (1990) The product of the H19 gene may function as an RNA. *Mol Cell Biol* 10, 28–36
- Cattanach BM, Kirk M (1985) Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 315, 496–498
- Hayward BE, Bontron DT (2000) An imprinted antisense transcript at the human GNAS1 locus. *Hum Mol Genet* 9, 835–841
- Hayward BE, Kamiya M, Strain L, Moran V, Campbell R et al. (1998a) The human GNAS1 gene is imprinted and encodes distinct paternally

- and biallelically expressed G proteins. Proc Natl Acad Sci USA 95, 10038–10043
- Hayward BE, Moran V, Strain L, Bonthron DT (1998b) Bidirectional imprinting of a single gene: GNAS1 encodes maternally, paternally and biallelically derived proteins. Proc Natl Acad Sci USA 95, 15475–15480
- Horike S, Mitsuya K, Meguro M, Kotobuki N, Kashiwagi A et al. (2000) Targeted disruption of the human LIT1 locus defines a putative imprinting control element playing an essential role in Beckwith-Wiedemann syndrome. Hum Mol Genet 9, 2075–2083
- Ischia R, Lovisetti-Scamihorn P, Hogue-Angeletti R, Wolkersdorfer M, Winkler H et al. (1997) Molecular cloning and characterisation of NESP55, a novel chromogranin-like precursor of a peptide with 5-HT_{1B} receptor antagonist activity. J Biol Chem 272, 11657–11662
- Kehlenbach RH, Matthey J, Huttner WB (1994) XLαs is a new type of G protein. Nature 372, 804–808
- Kelsey G, Bodle D, Miller HJ, Beechey CV, Coombes C et al (1999) Identification of imprinted loci by methylation-sensitive representational difference analysis: application to mouse distal chromosome 2. Genomics 62, 129–138
- Lee JT (2000) Disruption of imprinted X inactivation by parent-of-origin effects at *Tsix*. Cell 103, 17–27
- Lee JT, Davidow LS, Warshawsky D (1999) *Tsix*, a gene antisense to Xist at the X-inactivation centre. Nat Genet 21, 400–404
- Li T, Vu TH, Zeng ZL, Nguyen BT, Hayward BE et al. (2000) Tissue-specific expression of antisense and sense transcripts at the imprinted *Gnas* locus. Genomics 69, 295–304
- Liu J, Yu S, Litman D, Chen W, Weinstein LS (2000) Identification of a methylation imprint mark within the mouse *Gnas* locus. Mol Cell Biol 20, 5808–5817
- Lyle R, Watanabe D, te Vruchte D, Lerchner W, Smrzka OW et al. (2000) The imprinted antisense RNA at the *Igf2r* locus overlaps but does not imprint the flanking *Mas* gene. Nat Genet 25, 19–21
- Mitsuya K, Meguro M, Lee MP, Katoh M, Schulz TC et al. (1999) LIT1, an imprinted antisense RNA in the human KvLQT1 locus identified by screening for differentially expressed transcripts using monochromosomal hybrids. Hum Mol Genet 8, 1209–1217
- Peters J, Wroe SF, Wells CA, Miller HJ, Bodle D et al. (1999) A cluster of oppositely imprinted transcripts at the *Gnas* locus in the distal imprinting region of mouse chromosome 2. Proc Natl Acad Sci USA 96, 3830–3835
- Reik W, Constancia M (1997) Genomic imprinting. Making sense or antisense? Nature 389, 669–671
- Sado T, Wang Z, Sasaki H, Li E (2001) Regulation of imprinted X-chromosome inactivation in mice by *Tsix*. Development 128, 1275–1286
- Schmidt JV, Matteson PG, Jones BK, Guan XJ, Tilghman SM (2000) The *Dlk1* and *Gtl2* genes are linked and reciprocally imprinted. Genes Dev 14, 1997–2002
- Searle AG, Beechey CV (1978) Complementation studies with mouse chromosomes. Cytogenet Cell Genet 20, 282–303
- Williamson CM, Miller HJ, Beechey CV, Peters J (1995) Microsatellite marker D2Mit226 for the identification of duplication/deficient mice for the distal region of Chr 2. Mouse Genome 93, 860
- Williamson CM, Schofield J, Dutton ER, Seymour A, Beechey CV et al. (1996) Glomerular-specific imprinting of the mouse *Gsα* gene: How does this relate to hormone resistance in Albright hereditary osteodystrophy? Genomics 36, 280–287
- Williamson CM, Beechey CV, Papworth D, Wroe SF, Wells CA et al. (1998) Imprinting of distal mouse chromosome 2 is associated with phenotypic anomalies in utero. Genet Res 72, 255–265
- Wroe SF, Kelsey G, Skinner JA, Bodle D, Ball ST et al. (2000) An imprinted transcript, antisense to *Nesp*, adds complexity to the cluster of imprinted genes at the mouse *Gnas* locus. Proc Natl Acad Sci USA 97, 3342–3346
- Wutz A, Smrzka OW, Scheifer N, Schellander K, Wagner EF et al. (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. Nature 389, 745–749
- Wutz A, Theussi HC, Dausman J, Jaenisch R, Barlow DP et al. (2001) Non-imprinted *Igf2r* expression decreases growth and rescues the *Tme* mutation in mice. Development 128, 1881–1887
- Yu S, Yu D, Lee E, Eckhaus M, Lee R et al. (1998) Variable and tissue-specific hormone resistance in heterotrimeric Gs protein alpha-subunit (*Gsalpha*) knockout mice is due to tissue-specific imprinting of the *Gsα* gene. Proc Natl Acad Sci USA 95, 8715–8720

Copyright of Mammalian Genome is the property of Kluwer Academic Publishing / Academic and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.