The Neuronatin Gene Resides in a “Micro-imprinted” Domain on Human Chromosome 20q11.2

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A small fraction of the genome contains genes that are imprinted and thus expressed exclusively from one parental allele. We report here that the human neuronatin gene (NNAT) on chromosome 20q11.2 is imprinted and transcribed specifically from the paternal allele. The region containing NNAT has multiple CpG islands, and methylation analysis showed that a 1.8-kb CpG island in its promoter region exhibits differential methylation in all tissues examined. This finding is consistent with the island acting as a component of the NNAT imprint control domain. NNAT lies within the singular 8.5-kb intron of the gene encoding bladder cancer-associated protein (BLCAP), which, as we demonstrate, is not imprinted. This study provides the first example, to our knowledge, in humans of an imprinted gene contained within the genomic structure of a nonimprinted gene. Thus, NNAT is in an imprinted “microdomain,” making this locus uniquely suited for the investigation of mechanisms of localized imprint regulation.

Key Words: human chromosome 20, genomic imprinting, neuronatin, NNAT, BLCAP

INTRODUCTION

Genomic imprinting refers to parent-of-origin-specific, monoallelic expression of a gene. Imprinted genes are unique in that although both alleles have identical sequence information and share the same cellular environment, only one of the alleles is expressed. Imprinted genes share several common characteristics including the presence of tandem repeats, differential methylation of CpG islands, boundary elements, nontranslated RNA species, and temporal differences in DNA replication. All of these factors seem to have a mechanistic function in imprint regulation; however, the nature of the primary imprint mark established in the gametes remains elusive [reviewed in 1].

Approximately 45 imprinted genes have been identified, and estimates indicate that as many as 200 may exist in the mammalian genome [2]. Only a handful of these known imprinted loci have been analyzed in any detail, most notably the insulin-like growth factor-2 (Igf2)/H19 domain on mouse chromosome 7 (human chromosome 11p15.5) and the insulin-like growth factor-2 receptor (Igf2r; also known as M6P/IGF2R) locus on mouse chromosome 17 (human chromosome 6q27) [reviewed in 1,3]. However, to better identify and understand the function of the common genetic features found in imprint regulatory domains, it is imperative that additional imprinted loci undergo thorough characterization in many species.

To facilitate interspecies comparison of imprint regulatory regions, we determined the imprint status of the neuronatin gene (NNAT), the human homologue of a gene known to be imprinted in mice [4]. NNAT maps to human chromosome 20q11.2–q12 [5,6] and mouse distal chromosome 2 [4]. The NNAT amino acid sequence is 50% homologous to the proteolipids peroxisomal membrane protein-1 and phospholamban [5] and therefore constitutes a putative member of the proteolipid family. Proteolipids are typically small proteins consisting of one hydrophobic domain and one hydrophilic domain, and they often function as the regulatory subunit of a membrane channel.

NNAT is highly expressed in the central nervous system from mid-gestation through early postnatal development, correlating with the onset and termination of brain development in mice and humans. Its expression seems to be limited, however, to the anterior pituitary in the adult brain [4,7–9]; tissue culture studies indicate that Nnat is also expressed in adult mouse pancreatic β-cells [10,11]. Although computer-simulated virtual northern blot analysis using the SAGE library demonstrated that NNAT expression in glioblastomas is equivalent to that seen in normal brain tissues, it is highly overexpressed in medulloblastomas.
Mouse Nnat is expressed from the paternal allele and lies in a distinct imprinted chromosomal region 30 Mb upstream from the imprinted Gnas cluster [4,12]. Mouse embryos carrying either maternal (no functional copies of Nnat) or paternal (two functional copies of Nnat) duplications of distal chromosome 2 have been studied for phenotypic abnormalities [13]. Although spatial expression of Nnat in mice with a paternal duplication seemed identical to that of wild-type mice by in situ hybridization, there was a notable increase in the depth of the cerebellar folds. Conversely, there was a reduction of cerebellar fold depth in embryos with a maternal duplication that were shown to lack Nnat expression by in situ hybridization [13].

Although mouse Nnat is known to be imprinted, the imprint status of the human homologue has not been determined. We demonstrate here that human NNAT is imprinted and expressed exclusively from the paternal allele during development. Furthermore, we show that this imprinted gene is unusually positioned in that it lies within the singular intron of the gene encoding bladder cancer-associated protein (BLCAP), a gene that is not imprinted.

**RESULTS**

**Imprinting Status of Human NNAT**

The genomic structure of NNAT consists of three exons extending over approximately 2.7 kb (Fig. 1A). At least two alternatively spliced forms of NNAT exist in the human: a 1.2-kb α-form that incorporates all three exons and a 1.1-kb β-form consisting of only the first and third exon. Both of these isoforms are expressed in fetal brain [6]. Sequencing of these three NNAT exons in over 40 individuals failed to demonstrate any polymorphisms, indicating a highly conserved coding region. Indeed, NNAT mRNA remains 98% conserved between mouse and human. However, within intron 1 of NNAT we identified a polymorphic region consisting of a 28-bp stretch of T residues, four of which were polymorphic for a T→A transversion (heterozygosity frequency, 67%; n = 30; bacterial artificial chromosome (BAC) RP11-425M5 positions 131,670–131,679; Figs. 1A and 1B).

To screen for expression, we used RT-PCR on total RNA isolated from adrenal gland, brain, gut, heart, kidney, liver, lung, muscle, placenta, and spleen fetal tissues. These and all other RT reactions included a negative control (data not shown) to ensure that genomic DNA did not contaminate subsequent PCR reactions, which would lead to the amplification of both alleles and thus mask imprinting. Only central nervous system tissue yielded detectable cDNA products, four of which were polymorphic for a T→A transversion (heterozygosity frequency, 67%; n = 30; bacterial artificial chromosome (BAC) RP11-425M5 positions 131,670–131,679; Figs. 1A and 1B).

To determine if NNAT is monoallelically expressed, we analyzed DNA from 11 informative fetal brain samples by RT-PCR followed by nucleotide sequencing. Analysis of the cDNA in all brain samples showed expression of only one allele (Fig. 1B). As the polymorphism used here is in intron 1 of NNAT, the cDNA analyzed represents unspliced, nuclear RNA. Therefore, both the α- and β-isomers of human NNAT are monoallelically expressed from the same allele.

To determine the parental origin for the expressed NNAT allele, we first obtained maternal genotypes using decidua tissue corresponding to the informative fetal brain samples analyzed. For two of these informative samples, the maternal genotypes were homozygous for the A allele (Fig. 1B). Only the T allele was expressed in these two fetal brain tissues, demonstrating that human NNAT is imprinted and expressed specifically from the paternal allele.

**Imprinting Status of Human BLCAP**

We also identified genes adjacent to NNAT, as imprinted genes often occur in clusters. A BLAST search [14] revealed...
that a second gene, **BLCAP**, is on the same BAC clone containing **NNAT** (RP11-425M5; GenBank acc. no. AL109614). Alignment of the **BLCAP** mRNA sequence to BAC RP11-425M5 showed that this gene consists of two exons (Fig. 2A). Exon 1 encompasses approximately 75 bp, whereas the second exon is 1.9 kb and contains most of the 5' untranslated region, the entire protein coding sequence, and the 3' untranslated region. This 2-kb cDNA encodes a putative 87-amino-acid protein. Although the first exon lies about 6 kb downstream of **NNAT**, the second exon is only 1.8 kb upstream of **NNAT**. Thus, **NNAT** sits within intron 1 of **BLCAP**, and the two genes are transcribed in opposite directions (Fig. 2A).

The close proximity of **NNAT** and **BLCAP** indicated that the regulatory mechanisms controlling the imprinting of **NNAT** could also affect **BLCAP** expression. To determine the imprint status of **BLCAP**, we identified an A/G polymorphism in the intron of **BLCAP** 600 bp upstream of the **NNAT** transcription start site (heterozygosity frequency, 25%; n = 20; BAC RP11-425M5 position 133,286; Fig. 2A). As **BLCAP** is transcribed in an orientation opposite to that of **NNAT**, the **BLCAP**-specific RT primers should not reverse transcribe **NNAT**. To further ensure that **NNAT** transcripts would not mask the imprint status of **BLCAP**, we used two separate **BLCAP** RT primers that are approximately 1.1 kb upstream of the **NNAT** transcription start site. We detected **BLCAP** cDNA products in all fetal tissues examined and analyzed them for monoallelic expression. **BLCAP** demonstrated biallelic expression in the fetal brain samples (n = 4) in which **NNAT** was shown to be imprinted (Fig. 2B). Additionally, **BLCAP** was biallelically expressed in fetal adrenal gland (n = 2), heart (n = 3), kidney (n = 2), liver (n = 2), lung (n = 3), and placenta (n = 2) tissues (Fig. 2B).

**Methylation Analysis of CpG Islands**

Imprinted genes are normally associated with CpG islands that are within or near these genes, and differential methylation of these islands is important in the maintenance of their imprint status [1,15]. Analysis of the **NNAT**/**BLCAP** genomic domain with the computer program Webgene (http://www.itba.mi.cnr.it/webgene/) demonstrated the presence of four CpG islands in this region (Fig. 3A). The promoter regions of both genes contained CpG islands greater than 1 kb, and a CpG island less than 500 bp was detected within the coding sequence of each gene. Sequencing of bisulfite-treated DNA from fetal brain, kidney, liver, and pancreas demonstrated that the promoter CpG island for **NNAT** (region 3, positions 132,019–133,799; BAC RP11-425M5) contained a 1:1 distribution of methylated to unmethylated alleles at all CpG dinucleotides examined, consistent with the results expected for differential methylation (Fig. 3B). The two smaller CpG islands (regions 2 and 4, positions 130,973–131,529 and 134,849–135,251, respectively; BAC RP11-425M5) also presented a similar pattern of differential methylation in these four tissues. In contrast, all CpG dinucleotides examined in the **BLCAP** promoter CpG island (region 1, positions 125,283–126,670; BAC RP11-425M5) were unmethylated in all tissues examined (Fig. 3B).
which was not imprinted in all tissues examined. Of the four CpG islands identified within the NNAT/BLCAP genomic domain, the 1.8-kb NNAT promoter CpG island showed consistent differential methylation in all tissues investigated, whereas the 1.3-kb BLCAP promoter CpG island remained

**DISCUSSION**

We have demonstrated here that NNAT is imprinted in the human central nervous system during development. This gene lies within the 8.5-kb intron of a second gene, BLCAP,
unmethylated on both parental alleles, concordant with the respective imprint status of each gene.

This atypical genomic structure of an imprinted gene within the intron of a nonimprinted gene has been documented before in mice but not in humans. The mouse imprinted gene U2af1-rs1 sits within the 26-kb intron of the biallelically expressed Murr1 on proximal chromosome 11 [16]. Nonetheless, there are several differences between the mouse U2af1-rs1 and human NNAT domains. U2af1-rs1 lies within an intron three times the size of the BLCAP intron in which NNAT is situated, and whereas NNAT contains three introns, U2af1-rs1 is intronless. Furthermore, no human homologue of mouse U2af1-rs1 has been identified.

In contrast, the NNAT/BLCAP genomic structure and imprint status seem to be conserved between humans and mice (GenBank acc. no. AB041829). Therefore, NNAT is the first example, to our knowledge, of an imprinted gene that lies within the intron of a separate, biallelically expressed gene in both humans and mice. Although BLCAP homologues are present in species such as zebrafish and Drosophila melanogaster, NNAT homologues are not found outside the mammalian lineage. These findings indicate that the NNAT/BLCAP structure originated either through a retrotransposition event early in mammalian evolution or through a gain-of-function event within the intron of BLCAP that resulted in the formation of NNAT.

BLCAP was initially identified in a mRNA differential display between invasive and noninvasive human bladder transitional cell carcinomas [17]. The downregulation of BLCAP in invasive transitional cell carcinomas indicates its possible function as a tumor suppressor. The finding that BLCAP is not imprinted is intriguing, given its close proximity to NNAT. Studies of the IGF2/H19 domain have shown that imprinting control mechanisms are capable of exerting influence over distances of at least 100 kb [reviewed in 1]. Therefore, mechanisms must exist within the NNAT/BLCAP domain that allow these two genes to be regulated differently despite their propinquity.

For example, differential imprint regulation of NNAT and BLCAP may exist because chromatin boundary elements such as those established by CTCF prevent the imprint of NNAT from spreading to nearby genes [18–21]. CTCF-binding sites were found in both the IGF2/H19 and DLK1/GTL2 imprinted domains; however, consensus CTCF-binding sites are not present in the NNAT/BLCAP domain. The lack of CTCF-binding sites does not preclude the possibility that insulators other than CTCF may lie between NNAT and BLCAP.

The NNAT/BLCAP domain also lacks other characteristics common to imprinted genes. Imprinted genes are often associated with tandem repeat sequences [1]; however, tandem repeats were not detected within this domain (tandem repeat finder, http://c3.biomath.mssm.edu/trl/basic.submit.html; nucleic acid dot plot, http://arbl.cvmbs.colostate.edu/molkit/dnadot/). Additionally, antisense RNAs often occur near imprinted genes. BLAST searches of RP11-425M5 did identify two overlapping, unspliced expressed sequence tags (ESTs) between exon 1 of NNAT and exon 2 of BLCAP; however, both 5’ and 3’ rapid amplification of cDNA ends (RACE) failed to detect a cDNA product in fetal brain (data not shown). This indicates that these ESTs are either unspliced BLCAP RNA or genomic DNA contamination in the EST database. Finally, imprinted genes tend to occur in clusters, yet additional BLAST searches of overlapping BACs using both the nonredundant and EST database showed no other genes within a 280-kb region.

We have provided evidence for differential methylation of the NNAT promoter CpG island, however, which is consistent with findings for many other imprinted genes [1]. In contrast, the BLCAP promoter CpG island is unmethylated. This principal difference in CpG island methylation correlates with the imprint status of these two genes and is concordant with the proposed mechanistic involvement of differential methylation in the regulation of imprinting [1]. Mouse Nnat also shows differential methylation in the promoter region, with the maternal allele being fully methylated and the paternal allele being unmethylated [13]. Thus, we predict that the large CpG island in the NNAT/Nnat promoter region may be crucial in regulating the imprint status of this gene. The smaller CpG islands located in the coding regions of BLCAP and NNAT also seem to be differentially methylated, although not at a ratio of 1:1. Therefore, the involvement of these CpG islands in regulating NNAT imprinting is less certain.

Recent studies of IGF2 and IGF2R indicate that imprinting evolved approximately 150 million years ago in an ancestor common to marsupials and eutherian mammals [22,23]. The most actively debated theory for why imprinting evolved concludes that imprinted genes result as a consequence of a parental conflict to control the extraction of resources from the mother by her offspring [24]. This hypothesis predicts that paternally expressed genes would promote offspring growth, whereas maternally expressed genes would reduce their growth.

Nonetheless, embryos with maternal or paternal duplications of Nnat show no substantial size difference from that of their wild-type littermates; the only observed morphological changes are alterations in the depth of cerebellar folds [13]. This region of the brain is involved in the coordination and control of voluntary movement. Thus, mice with Nnat mutations may show abnormalities in basic behavioral activities such as walking or eating. As female mice with null mutations for the imprinted genes mesoderm-specific transcript (Mest) and Peg3 show severe nurturing deficiencies [25,26], it will also be important to determine if Nnat inactivation affects behavior. Furthermore, it will be important to determine if imprinting of NNAT and other genes involved in neuronal function occurred later in mammalian evolution than the imprinting of IGF2 and IGF2R, genes with a considerable effect on body size during development.

The human imprinted gene NNAT lies within a small intron of BLCAP, a nonimprinted gene. This finding indicates that all of the control elements necessary for initiating and maintaining the NNAT imprint could exist within the 8.5-kb intron of BLCAP. Consequently, this “micro-imprinted” domain is uniquely suited for investigating the control elements required for localized regulation of genomic imprinting in the human genome.
PCR amplification and DNA sequencing. Human fetal tissues were obtained from the National Institutes of Health-supported Birth Defects Research Laboratory at the University of Washington. DNA was isolated from these tissues with the Dneasy kit (Qiagen, Valencia, CA). NNAT intronic polymorphisms were amplified using the forward primer 5′-TTCTTGTCTGGAAAGGCGG-3′ and the reverse primer 5′-CTCATGCTGCGTCCATTAGGC-3′. Cycling conditions were identical to those used for NNAT except that the annealing temperature was increased to 62°C. The PCR products were then sequenced as described above with the primer 5′-CTGAGGCAAGACCTTGCCAACAG-3′.

Genomic imprinting analysis. RNA Stat-60 (Tel-Test, Friendswood, TX) was used according to the manufacturer’s protocol to isolate total RNA from fetal tissue samples. The isolated RNA (2 μg) was treated with DNaseI (Life Technologies, Grand Island, NY) and then divided into two samples for reverse transcription in either the presence or the absence of reverse transcriptase. The NNAT RT primer was 5′-ACCTGTGGCCAGATGCTCGT-3′, and the RT primer for BLCAP was either 5′-GGCATCGTACAGACCTGACCT-3′ or 5′-TGCGGAGGACACAGACG-3′. Reverse transcription used Superscript II (Life Technologies). The resulting cDNAs were amplified by PCR and sequenced as described above. All DNA amplifications and imprint status determinations were made two or more times.

DNA methylation analysis using bisulfite sequencing. Genomic DNA samples were treated with sodium bisulfite using the CpGenome DNA modification kit (Intergen, New York, NY). Bisulfite-treated DNA was amplified using two rounds of nested PCR (30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s), purified, and sequenced as described above. Primer sets were selected to amplify DNA fragments from the four identified CpG islands present in the BLCAP/NNAT domain; these primer sequences are available on request from the authors. A 300-ng amount of bisulfite-treated DNA was empirically determined to avoid stochastic PCR amplification bias due to a limiting number of starting DNA molecules, and this amount was therefore used as template in the first round of PCR amplification. The conversion of all non-CpG dinucleotide cytosines served as a positive control to ensure that the initial DNA template underwent complete denaturation. All bisulfite-treated DNA amplification reactions were duplicated.

RACE analysis. For RACE, we used the Marathon-Ready fetal brain cdNA kit according to the manufacturer’s directions (Clontech, Palo Alto, CA). For 5′-RACE we used the primer 5′-TCGAGGAGACACTGCTGAGG-3′, and for 3′-RACE we used the primer 5′-CAGGTCTCTGCGTCTGAGG-3′.

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