Recent Assembly of an Imprinted Domain from Non-Imprinted Components

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Genomic imprinting, representing parent-specific expression of alleles at a locus, raises many questions about how—and especially why—epigenetic silencing of mammalian genes evolved. We present the first in-depth study of how a human imprinted domain evolved, analyzing a domain containing several imprinted genes that are involved in human disease. Using comparisons of orthologous genes in humans, marsupials, and the platypus, we discovered that the Prader-Willi/Angelman syndrome region on human Chromosome 15q was assembled only recently (105–180 million years ago). This imprinted domain arose after a region bearing SNRPN (Prader-Willi syndrome) fused with an unlinked region bearing UBE3A (Angelman syndrome) with large stretches of repetitive sequences and retroelements. In their original configurations, SNRPN and UBE3A are expressed from both alleles, implying that acquisition of imprinting occurred after their rearrangement and required the evolution of a control locus. Thus, the evolution of imprinting in viviparous mammals is ongoing.

Introduction

Genomic imprinting refers to the silencing of a gene or region according to its parent of origin. Among vertebrates, imprinting is specific to mammals, with about 83 mammalian genes shown to be imprinted. About half are paternally expressed (that is, the maternally derived allele is suppressed) and half maternally expressed (the paternally derived allele is suppressed) [1]. Many genes subject to imprinting are involved with either developmental disorders or cancer (sometimes both), so understanding how and why genomic imprinting evolved and how it functions is therefore of compelling interest to medicine as well as biology.

Imprinting is an important model system for studying epigenetic regulation—an accelerating field of biology that focuses on how identical DNA sequences are differentially expressed to produce different phenotypes. The molecular mechanism of imprinting resembles X chromosome inactivation in females, another mammal-specific epigenetic phenomenon, suggesting that X inactivation and autosomal imprinting may share a common origin [2,3].

We do not yet understand what selective forces eschewed the benefits of diploidy in favor of parental imprinting, and there are many hypotheses to account for the seemingly perverse evolution of hemizygosity at these loci. Perhaps the most interesting and widely debated is the parental conflict hypothesis [4], now developed into the kinship hypothesis (reviewed [5]), which proposes that imprinting evolved in response to the antagonistic interests of parental genomes.

The origin, as well as the mechanism, of imprinting can be investigated by comparing gene arrangement and expression between divergent species. The observation that genes imprinted in human and mouse (IGF2, M6P/IGF2R) are not imprinted in chicken [6,7] implies that imprinting is specific to mammals. Nonetheless, the gene content and arrangement of human imprinted domains is highly conserved in chicken [8] and other vertebrates. For instance, the content and arrangement of coding genes in the human Beckwith-Wiedemann imprinted cluster (including IGF2, H19, ASCL2, KCNQ1, and CDKN1C) is largely shared with birds and fish, but the non-coding regulatory H19 RNA is missing, along with large stretches of repetitive sequences and retroelements and several sequences thought to exert local control of imprinting [9,10].

The transition of a region from a non-imprinted state in fish and chicken to an imprinted state in placental mammals could therefore be dissected by comparing orthologous regions with the most divergent mammal groups. Marsupials and monotremes diverged from placental mammals 180 and 210 million years ago (MYA), respectively [11], so fill the 310-MY evolutionary void that separates birds and reptiles from humans and mice. This permits the reconstruction of gene content and arrangement over a long evolutionary period and provides informative sequence comparisons with high

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Abbreviations: BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; ICR, imprint control region; kb, kilobase; MYA, million years ago; PWS-AS, Prader-Willi/Angelman syndrome; snoRNA, small nucleolar RNA

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**Synopsis**

Humans and other mammals have two copies of the genome. For most genes, both copies are active. However, some genes are active only when they are inherited from the father, others only when inherited from the mother. These “imprinted” genes are clustered in domains that are controlled coordinately. Only mammals show genomic imprinting. It is not understood how or why genes became imprinted during mammalian evolution. The authors used comparisons between humans and the most distantly related mammals, marsupials and monotremes, to discover how one of these imprinted domains evolved. The authors studied an imprinted domain on human Chromosome 15, mutations which cause Prader-Willi and Angelman syndromes (PWS-AS). They discovered that the PWS and AS genes lie on different chromosomes in kangaroos and platypus and are not imprinted. Other imprinted genes in the domain, including the putative control region, are absent from the genome and derived from copies of genes from yet other chromosomes. The arrangement in kangaroos and platypus is present also in the chicken genome, so it must be ancestral. This study concludes that the PWS-AS imprinted region was assembled relatively recently from non-imprinted components that were moved together or copied from all over the genome.

**Results**

We examined the evolutionary origin of genes in and adjacent to the human PWS-AS imprinted region by isolating, mapping, and assessing transcription of their orthologs in marsupials, platypus, chicken, and fish.

**Isolation of Marsupial and Monotreme Homologs of PWS-AS Genes**

We screened bacterial artificial chromosome (BAC) and cDNA libraries from the model kangaroo **Macropus eugeni** (tammar wallaby) for several human 15q11-q13 genes. cDNA clones containing **GABRB3** and **HERC2** and two BACs containing the AS gene **UBE3A** were confirmed by partial sequencing.

Numerous attempts over several years to isolate other imprinted genes, using PCR amplification, Southern blot analysis, and screening several genomic and cDNA libraries, were consistently unsuccessful. Screening for **MKRN3** identified a hitherto unknown intron-containing source gene **makorin (MKRN1)** [28]. Our conclusion that the marsupial genome lacks **MKRN3** was supported by its absence from the **Monodelphis domestica** (opossum) database. No clones containing tammar **MAGEL2** or **NDN** were ever recovered, and no **MAGEL2** or **NDN** sequences were found in the opossum database.
Similarly, attempts to clone tammar homologs of the imprinted PWS gene SNRPN from a genomic DNA lambda library resulted in the repeated isolation of a non-imprinted paralog SNRPB/B [29], which lies on human Chromosome 20. Subsequently, we discovered a second SNRPN-like transcript encoding a predicted gene product that clusters with the SmN proteins, demonstrating orthology to SNRPN (Figure S1). A single tammar BAC was then obtained containing both SNRPN and SNRPB homologs. Searching the opossum draft assembly for this sequence located a SNRPN ortholog directly adjacent to SNRPB on scaffold 15105. Neither this scaffold, nor any other opossum sequence contained sequences with homology to human SNURF.

We therefore conclude that the marsupial genome contains tandemly arranged SNRPN-SNRPB sequences, and that SNURF is absent.

To determine whether UBE3A is adjacent to CNGA3 also in marsupials, we screened the two tammar UBE3A BACs for CNGA3. We identified CNGA3 in the larger BAC and confirmed its presence by sequencing. We also searched the opossum draft assembly, finding that UBE3A and CNGA3 lie ~60 kb apart in a tail-to-tail arrangement on scaffold 16658, which also contained flanking genes from human Chromosomes 15q and 2 (Table 1).

Mapping PWS-AS Genes in Marsupials

We mapped tammar PWS-AS genes by fluorescence in situ hybridization (FISH) to determine whether their co-location on human 15q is conserved in marsupials. GABRB3 and HERC2 (lambda clones), as well as the BAC containing UBE3A and CNGA3 co-localized in the middle of tammar Chromosome 5p (Figure 2). This was unexpected because tammar 5p has homology to the short arm of the human X chromosome (reviewed [30]). This location was also different to the localization on tammar 1q of a lambda clone originally thought to contain SNRPN [31], but now identified as SNRPB. We mapped the tammar BAC containing both SNRPN and SNRPB unequivocally to the middle of tammar Chromosome 1q (Figure 2).

Thus, the arrangement of marsupial and monotreme orthologs of PWS-AS genes differs from that in humans and...
mice. **UBE3A** and flanking genes do not co-localize with **SNRPN**, but instead share synteny with genes on human Chromosomes Xp and 2. To determine which arrangement is ancestral, we extended our comparison to other vertebrates, making use of information in public databases.

**Other Vertebrate Genomes Reveal the Ancestral Arrangement of PWS-AS Genes**

We searched the UCSC database (http://www.genome.ucsc.edu) for dog, chicken, and fish homologs of genes in and near the human PWS-AS region, including **UBE3A**, **GABRB3**, and **HERC2** (tammar PWS-AS orthologs), as well as **CNGA3** and genes adjacent to this region in tammar (lying on Xp in human). Orthologs of these genes all lay on scaffolds mapping to chicken Chromosome 1 (Table 1). A block of nine human 15q11-q13 genes (from **UBE3A** to the centromeric **TUBGCP5**) was found sandwiched between large blocks of human Xp and Chromosome 2 genes on chicken Chromosome 1. **CNGA3**, flanking a block of human Chromosome 2 genes, lies only 2 kb from **UBE3A**. In zebrafish, although the synteny groups are duplicated and somewhat broken up, **UBE3A** and **CNGA3** lie on Chromosome 6 along with several other genes from the human X, 15, and 2 blocks and are separate from genes that flank this region in human, which lie on Chromosomes 7 and 20.

Attempts to retrieve **SNURF-SNRPN** and **MKRN3** sequence from the chicken and zebrafish databases yielded only the ancestral **SNRPB** and **MKRN1**, suggesting that non-mammal vertebrates lack **SNURF-SNRPN** and **MKRN3**. No sequences orthologous to **MAGEL2** and **NDN** were found in chicken and fish genomes (Table S2).

The finding that human 15q11-q13 genes lie between blocks of human Xp and Chromosome 2 genes in chicken and fish, as well as marsupials and monotremes, and that **MKRN3**, **MAGEL2**, **NDN**, and **SNURF** are absent, implies that this is an ancestral vertebrate arrangement. Its occurrence also in marsupials and monotremes implies that the ancestral arrangement was retained in marsupials and monotremes, but the region was rearranged and augmented more recently in placental mammals.

**Are SNRPN and UBE3A Imprinted in Marsupials and Monotremes?**

Of the greatest interest was to determine whether marsupial and monotreme **SNRPN** and **UBE3A** are imprinted...
like their human orthologs, even though they do not share the gene arrangement of the placental PWS-AS region.

Expressed polymorphisms in these two genes in marsupials were therefore sought by screening DNA from 60 tammar wallabies. Sequence analysis revealed a SNRPN polymorphism in one heterozygote and a UBE3A polymorphism in two. Brain RNA (the tissue showing imprinting of both UBE3A and SNRPN in placental mammals) was extracted from these animals and a fragment amplified from the resulting cDNA template using gene-specific primers that spanned introns. Sequencing the PCR products revealed expression at similar levels of both parental alleles for both genes (Figure 3).

We also investigated the expression of monotreme UBE3A (but not SNRPN, since it appears to be absent from the monotremé genome). DNA from five platypuses was screened, and a UBE3A polymorphism detected in one heterozygous animal, from which brain tissue was isolated and RNA extracted. Amplification and sequencing from brain cDNA revealed expression of both alleles (Figure 3). Because the alleles differed by a base pair insertion, this result was confirmed by primer extension assays (Figure S2).

Expression studies therefore show that UBE3A (the Angelman syndrome gene) is biallelically expressed in marsupial and monotreme brain. The Prader-Willi gene SNRPN, represented by a tandem duplication of the non-imprinted gene SNRPB in marsupials, also does not appear to be imprinted.

Discussion

Our results demonstrate that the PWS-AS imprinted region of placental mammals was assembled from a variety of disparate genomic elements and suggest that imprinting was acquired relatively recently in the placental lineage. These conclusions follow from differences in arrangement and expression of SNRPN and UBE3A in the three major mammal groups, placentals, marsupials, and monotremes.

SNRPN and UBE3A lie together in all placental mammals investigated, including the fully sequenced human, mouse, and dog. In the basal placental clades, Afrotheria and Xenarthra, sequence assembly is not sufficiently advanced to ascertain gene arrangement, but chromosome painting experiments reveal that human Chromosome 15q is represented as a single block in elephant and armadillo [32,33]. In contrast, we demonstrate that the two primary PWS-AS loci SNRPN and UBE3A lie on different chromosomes in marsupials and monotremes, and that other PWS-AS loci have no orthologs in marsupials or monotremes. Since this gene arrangement is shared by birds and fish, it must be ancestral.

This implies that a major rearrangement occurred in the placental lineage to unite UBE3A and SNRPN. The evolutionary breakpoints lie between SLC9A7 and TUBGCP5 and between UBE3A and CNGA3 in the ancestral sequence. Fission between UBE3A and CNGA3 was evidently preceded by
some 20 [28], and the intronless MAGEL2 and NDN arose by retrotransposition from genes on different sites on the X. Since no snoRNA arrays lie near the ancestral UBE3A or SNRPN, these must also have been seeded from other locations. Thus, the expanded and fused region was evidently a target for insertions and rearrangements. It seems to have remained unstable, since the mouse and human regions differ by several retrotranspositions [27], and the region contains breakpoint hotspots that are the source of many PWS and AS deletions [34].

We conclude from our expression studies that UBE3A and SNRPN are biallelically expressed in marsupial and monotreme brain. Because imprinted expression of UBE3A is found within neurons, but not glial cells of the brain [35], it is possible that we could not detect some level of allelic attenuation for UBE3A in marsupials and monotremes. However, considering that the brain of AS patients produces only about 10% UBE3A expression [22], we consider that even semi-quantitative methods such as direct sequencing should have detected this level of imprinting in marsupial and monotreme brain. Our finding that SNRPN and UBE3A are not imprinted in their original locations is consistent with the hypothesis that rearrangement was required for the establishment of imprinting.

Since UBE3A and SNRPN appear not to be imprinted in marsupials and monotremes, we would not expect to find sequences that control imprinting of these genes, so the absence of SNURF and the ICR from the marsupial genome is particularly telling. SNURF lies in the ICR (as defined by the region of shortest deletion in human PWS patients) and the UBE3A anti-sense transcript originates either at its 5’ end or at alternative upstream exons [36]. We propose that coordinate regulation of the PWS-AS domain required introduction of these sequences into the unstable fused region.

The time at which chromosome rearrangement occurred and imprinting was acquired can be deduced from the mammalian phylogeny (Figure 4). Gene arrangement in the PWS-AS domain is conserved at least between dog, mouse, and human, and the region was syntenic even in the most distantly related placentals, implying that it predated the placental radiation 105 MYA. The ancestral arrangement in chicken and fish is shared by marsupials and monotremes, which diverged from placentals 180 and 210 MYA, respectively. Major rearrangements therefore occurred after placentals and marsupials diverged 180 MYA, but before the placental radiation 105 MYA. Our demonstration that SNRPN and UBE3A are also biallelically expressed in marsupials shows that imprinting evolved considerably later (180–80 MYA) in this region than, for instance, in the IGF2 region. This is consistent with the view that imprinting evolved after the evolution of viviparity in therian mammals, but shows, also, that at least some loci evolved imprinting considerably after this time. The recent findings that the imprinted gene Nsat is eutherian-specific [37], and DLK1 is not imprinted in the opossum [38], also suggest that novel genes could be imprinted more recently. Thus, imprinting has been acquired at different times in different domains, after the evolution of viviparity, and suggests that viviparity is a necessary but not a sufficient condition for the evolution of imprinting.

Is there any significance in our finding that the PWS-AS
imprinted domain once shared synteny with the genome region that became part of the placental X and was recruited into the X inactivation system in placental mammals? There has been continuing speculation that genomic imprinting and X chromosome inactivation are related by descent [2,3]. One possibility is that the entire region was added to the ancestral therian X and recruited to the X inactivation system, and then the PWS-AS region was subsequently relocated to an autosome, carrying elements that control cis-silencing. This could also explain the acquisition by this human Chromosome 15 region of MAGE (cancer-testis antigen) genes, nearly all of which accumulated on the X chromosome in placentals [39].

In conclusion, we show that the Prader-Willi/Angelman imprinted domain on human Chromosome 15q11–13 was assembled relatively recently from unlinked and non-imprinted components in a mammalian ancestor. Two non-imprinted regions fused 105–180 MYA, and several retroposed genes and snoRNAs from different regions were independently inserted. We propose that genomic rearrangement early in the eutherian lineage was required for the acquisition of imprinting at this locus.

Materials and Methods

Tissue samples. Tammar wallaby tissue samples were sourced at the Research School of Biological Sciences, Australian National University, Canberra, and the Department of Zoology, University of Melbourne, according to the Animal Experimentation Ethics Committee. Platypus samples were supplied courtesy of Dr. F. Grützner, Australian National University, Canberra, Australia.

Nucleic acid extraction, amplification, and sequencing. Total genomic DNA was extracted from tail clippings of 20 pouch young (py) tammar wallabies (collected June 2005), from the brain of three py tammars (collected November 2003), and from ear punches of 30 adult tammars (collected March 2005) using the DNeasy Tissue Kit (Qiagen, Valencia, California, United States) or the protocol outlined in [40]. Total genomic DNA was extracted from two male and one female platypus brain samples (collected September 2004). High-copy number plasmids were extracted using either the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, Wisconsin, United States) or the protocol outlined in [40]. Total genomic DNA was extracted from two male and one female platypus brain samples (collected September 2004). High-copy number plasmids were extracted using either the Wizard Plus SV Miniprep DNA Purification System (Promega). Total RNA was extracted from the brains of polymorphic platypus and tammar wallabies using the RNeasy Mini Kit (Qiagen) according

Figure 4. Assembly of the PWS-AS Imprinted Region in Placental Mammals during Vertebrate Evolution

Relationships between fish, birds/reptiles, and the three mammal groups are presented as a phylogeny (top). In the ancestral arrangement, shared by marsupials, monotremes, birds, and fish, the block of imprinted human 15q genes (pink) is flanked by human X (green) and human 2 (purple) blocks. These three blocks were separated by at least two fissions and were rearranged next to an unlinked block of genes (on chick Chromosome 10, pale blue) to make up the present regions of human Chromosome 15q (and with two more inversions, of mouse Chromosome 7). SNRPB (brown) is present on a different chromosome in fish, birds, and marsupials, but its duplicate SNRPN (orange) is transposed next to UBE3A in placentals. Other human 15q genes absent from non-placental vertebrates (orange) were independently added to the imprinted region in the placental lineage.

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Evolution of the PWS-AS Imprinted Domain

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Table 2. Description of Probes Used and DNA Libraries Screened

| Probe Description                  | Probe Source                  | DNA Library                               | Library Source                     |
|-----------------------------------|-------------------------------|-------------------------------------------|------------------------------------|
| Full-length cDNA of human SNRPW   | R. D. Nicholls                 | j. ZAP II phage M. eugenii cDNA library   | Clontech Laboratories Incorporated, Palo Alto, California, United States |
| 3.0-kb fragment from human GABRB3 cDNA | R. D. Nicholls                 | j. EMBL3 phage library (average insert size:15–20 kb) containing M. eugenii genomic sequence | Steven Wilcox [45] |
| 1.1-kb product from human HERC2 cDNA | R. D. Nicholls                 |                                           |                                    |
| 213-bp product from exon 7 of CNGA3 in platypus | Amplified using primers: CNGA3F1 (GAGGACGGGAGTCACTCC) CNGA3R1 (GTCAGGGTGAGCCAGGA) | Me_Kba BAC library containing M. eugenii genomic sequence in 11-fold coverage | Arizona Genomics Institute, Tuscon, Arizona, United States |
| 1,712-bp product encompassing exon 10 and surrounding intronic sequence of UBE3A in tammar | Amplified using primers: UBE3aEx10UTR (GCGCCCACCAAGACCTTA) | BAC library containing M. eugenii genomic sequence in 2.2-fold coverage | Victorian Institute of Animal Science, Victoria, Australia |
| 112-bp product encompassing last exon and 3' UTR of SNRPN in tammar | Amplified using primers: SnrpnEx10UTR (AAATCAATTTAAAAGGAGCACACT) | Oa_Bb BAC library containing Omithorhyncus anatius genomic sequence in 11-fold coverage | Clemson University Genomics Institute, Clemson, South Carolina, United States |
| 280-bp cDNA probe from exons 10–13 of UBE3A in tammar | Amplified using primers: UBE3A1F (TACTTATCCAGCAGCAAGA)UBE3A1R (GCAAGATGAGATGTAGGTAAC) |                                           |                                    |
| 268-bp product from exon 14 of UBE3A in platypus | Amplified using primers: PlatyUBE3aF (TTTTCGGAGAGGTTTTCATATGGTG)PlatyUBE3aR (TATGTCCTGTGCAGGCTTGAG) |                                           |                                    |
| Overgo probe from exon 2 of SNRPB in platypus |                                           |                                           |                                    |

Table 3. PCR Products Used in Expression Studies

| Product Description                  | Primers Used                  |
|-------------------------------------|-------------------------------|
| 247-bp product from exons 9–10 and 3' UTR of SNRPN in tammar wallaby | SNRPN exon9 F (CCTCTCCCTCTGAAATGAAGCCC)SNrpnpEx10UTRR (AAATCAATTTAAAAGGAGCACACT) |
| 517-bp product from exon 5 and exon 6 of UBE3A in tammar wallaby | UBE3A exon6 F (CACAACGACTCATCTATAGACCCG)UBE3A exonR (GCAGCCTTCTTTCTTACCT) |
| 508-bp product from exon 14 and 3' UTR of UBE3A in platypus | UBE3UR_F4 (TTTCCACATCTCCTAGGTTC)UBE3UR_R8 (ACACAGCTTCAAACAGCT) |

Probes were hybridized to various DNA libraries (Table 2) and exposed to autoradiography film at −70 °C until sufficiently exposed.

**FISH.** Mitotic metaphase chromosomes were prepared from tammar wallaby fibroblast cell lines grown from ear punctures of adult male wallabies. FISH was carried out as previously described [31]. Probes were fluorescence-labeled by the BioNick Labeling System (Invitrogen) in accordance with the manufacturer's instructions. Probes were detected using fluorescein isothiocyanate (FITC)- conjugated avidin and biotin-conjugated anti-avidin antibody (Vector Laboratories, Burlingame, California, United States). Chromosomes and cell nuclei were counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in 2X SSC for 1 min and mounted. Fluorescence signals were visualized and captured using a Zeiss (Oberkochen, Germany) Axioplan epifluorescence microscope equipped with a CCD (charge-coupled device) camera (RT-Spot, Diagnostic Instruments, Sterling Heights, Michigan, United States). Images were manipulated using IPLab imaging software on an Apple Macintosh computer (Cupertino, California, United States). Gray scale images were captured with source images superimposed into color images.

**Expression analysis.** Tammar py heterozygous for an expressed polymorphism ranged in age from 104 d to 116 d. PCR products used in expression studies were amplified using primers shown in Table 3. PCR products were purified prior to sequencing using the QIAquick gel extraction kit (Qiagen). Single nucleotide primer extension (SNUPE) assays were undertaken on adult platypus samples using the MassARRAY analyzer system (Sequenom, San Diego, California, United States) by the staff at the Australian Genome Research Facility.

**Bioinformatic analysis.** Human protein sequence from genes of interest (Table S1) were extracted and interrogated against the mouse (assembly: March 2005), dog (assembly: May 2005), opossum (assembly: October 2004), chicken (assembly: February 2004), and zebrafish (June 2004) databases deposited on the UCSC genome browser (http://www.genome.ucsc.edu) using the BLAT algorithm with default settings [42] (Table S2). In an attempt to identify highly diverged homologs of proteins SVRF, SNRP, MAGEL2, and NDN the more sensitive tBLASTn algorithm [43] was used on databases accessible through the Ensembl website (http://www.ensembl.org). No significant alignments in addition to those found using BLAT were produced. Sequence from the draft platypus genome assembly (version 0.0), related to SNRPB, was extracted from the Washington University Genome Sequencing Center (http://genome.wustl.edu/tools/blast) using tBLASTn.

Members of the Sm family of proteins (Table S3) were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw) with
default parameters. Phylogenetic analysis was performed with the maximum parsimony method using PAUP* version 4.0 b 10 [44]. Gaps were treated as missing data. Most-parisonymous trees were searched using a heuristic strategy, starting trees were obtained via stepwise addition for tree-bisection-reconnection (TBR) branch swapping, one tree was held at each step during stepwise addition, and a maximum of 1,000 best trees were saved in each replicate. 1,000 replications were performed for the bootstrap analysis. A pictorial consensus tree was created using the Phylodendron tree printer (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

Supporting Information

Figure S1. Relationships between Vertebrate Sm Proteins Encoded by the Genes SNRPN (SmN) and SNRPB/B (SmB) Generated Using Maximum Parsimony Analysis

Marsupial (opossum and tammar) SmN sequences are sister to placental SmN and distant from vertebrate SmB. Support for tree topology is indicated by bootstrap values (1,000 replicates).

Figure S2. Primer Extension Assay

Relative allelic concentrations of PCR products amplified from the UBE3A gene of platypus brain genomic DNA and cDNA for the three individuals sampled. Standard deviation indicated by error bars.

Table S1. Human Protein Sequences Used in Study

Table S2. Location of Putative Orthologs Aligned to Human Proteins of Interest in Mouse, Dog, Opossum, Tammar Wallaby, Platypus, Chicken, and Zebrafish

Percent identity between query and target sequence is given, along with the coverage of this alignment relative to query sequence length. An asterisk (*) denotes that no significant alignment was found. % Id, percent identity; % cov, percent coverage.

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