A new deletion refines the boundaries of the murine Prader–Willi syndrome imprinting center

Amanda J. DuBose¹,Emily Y. Smith¹,Thomas P. Yang²,³,Karen A. Johnstone¹,‡ and James L. Resnick¹,³,*

¹Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, PO Box 100266, Gainesville, FL 32610-0266, USA, ²Department of Biochemistry and Molecular Biology and ³Center for Epigenetics, University of Florida College of Medicine, PO Box 100245, Gainesville, FL, 32610-0245, USA

Received April 10, 2011; Revised May 31, 2011; Accepted June 6, 2011

The human chromosomal 15q11–15q13 region is subject to both maternal and paternal genomic imprinting. Absence of paternal gene expression from this region results in Prader–Willi syndrome (PWS), while absence of maternal gene expression leads to Angelman syndrome. Transcription of paternally expressed genes in the region depends upon an imprinting center termed the PWS-IC. imprinting defects in PWS can be caused by microdeletions and the smallest commonly deleted region indicates that the PWS-IC lies within a region of 4.3 kb. The function and location of the PWS-IC is evolutionarily conserved, but delineation of the PWS-IC in mouse has proven difficult. The first targeted mutation of the PWS-IC, a deletion of 35 kb spanning Snrpn exon 1, exhibited a complete PWS-IC deletion phenotype. Pups inheriting this mutation paternally showed a complete loss of paternal gene expression and died neonatally. A reported deletion of 4.8 kb showed only a reduction in paternal gene expression and incomplete penetrance of neonatal lethality, suggesting that some PWS-IC function had been retained. Here, we report that a 6 kb deletion spanning Snrpn exon 1 exhibits a complete PWS-IC deletion phenotype. Pups inheriting this mutation paternally lack detectable expression of all PWS genes and paternal silencing of Ube3a, exhibit maternal DNA methylation imprints at Ndn and MkRN3 and suffer failure to thrive leading to a fully penetrant neonatal lethality.

INTRODUCTION

A small percentage of mammalian genes are subject to genomic imprinting, an epigenetic mechanism causing unequal expression of parental alleles. Imprinted genes tend to be organized in clusters regulated by one or more imprinting centers (ICs). The IC controls both gene expression and epigenotype within the domain. An imprinted region located at 15q11–q13 is responsible for both Prader–Willi syndrome (PWS) and Angelman syndrome (AS), two neurobehavioral disorders arising from reciprocal patterns of imprinted gene expression (1). Both gene order and allelic patterns of gene expression are conserved at the syntenic region on mouse chromosome 7.

PWS patients lack the paternal-only expression of a number of genes, including NDN, MAGEL2, MKRN3, C15ORF2, SNRPN (a bicistronic transcript of SNURF and SNRPN), UBE3A-AS and several small nucleolar RNAs (snoRNAs) (1). In some regions of the brain, UBE3A expression is restricted to the maternal allele and its function is disrupted in AS patients (2–4). Although most cases of PWS or AS result from a 5–7 mb deletion that removes the entire imprinted domain, some patients harbor microdeletions which disrupt imprinted gene expression (5). The smallest regions of overlap shared by these microdeletions define a bipartite IC comprised of the AS-IC and the PWS-IC (6).

Gene expression patterns in both PWS individuals and mouse mutants support a model in which the PWS-IC functions as a positive regulator of transcription of paternal-only genes at the locus. The AS-IC functions in the maternal germ-line to epigenetically inactivate the PWS-IC so that paternal-only genes are silenced on the future maternal
allele. AS-IC mediated silencing of a large transcript encoding SNURF/SNRPN, several snoRNAs and the UBE3A-antisense transcript (UBE3A-AS) on the maternal allele allows for UBE3A expression by an unknown mechanism (7).

Conservation of gene order and imprinting patterns suggests that mouse mutants can provide faithful models of imprinting mechanisms at the PWS/AS locus. The smallest region of overlap of microdeletions defining the human PWS-IC currently stands at 4.3 kb including the SNRPN promoter and exon 1, and includes a differentially DNA methylated region (DMR) characterized by DNA hypermethylation of the maternal allele (8). A differentially methylated enhancer associated with an evolutionarily conserved sequence located just outside of the minimal PWS-IC in the first intron of SNRPN, termed the conserved activator sequence (CAS), may also contribute to PWS-IC function (9).

The murine PWS-IC remains poorly characterized. A 35 kb deletion removing the first six exons of Snrpn and 16 kb of 5’ flanking sequence exhibits a complete PWS-IC imprinting defect, indicating that the entire murine PWS-IC is contained within this deletion. Paternal inheritance of this deletion is characterized by a highly penetrant neonatal lethality and absent expression of paternal-only genes (10). To date, smaller deletions within the boundaries of the 35 kb deletion have not yielded a similar complete PWS-IC phenotype. Paternal transmission of a 0.9 kb deletion removing Snrpn exon1 led to normal expression of paternal-only genes and appropriate DNA methylation at the remaining portion of the Snrpn DMR (11). A 4.8 kb deletion, revealed to be 5.07 kb by complete DNA sequencing of the region, that extended further into the DMR yielded partial neonatal lethality with residual expression of the paternal-only genes (7).

RESULTS

Generation of a 6 kb deletion at the PWS-IC

The imprinting defects characteristic of the PWS-ICIC allele suggest that the entire PWS-IC is located within a 6 kb region centered around Snrpn exon (12). An ES cell clone containing a loxP site at −3.7 kb, with reference to the 5’ end of Snrpn exon 1, and a floxed PGK-neo cassette at +2.3 kb was generated by gene targeting (Fig. 1C). Following transfection of a Cre-expressing plasmid, G418-sensitive clones lacking the PGK-neo cassette but retaining the floxed Snrpn allele were identified by polymerase chain reaction (PCR) and southern blot (Fig. 1D). A clone with the 6 kb region flanked by loxP sites was successfully transmitted to the germline. These mice were mated with the germline Cre-expressing line, 129-Alpen1(cre)Nagy/J (13), to create the 6 kb deletion allele termed PWS-ICΔ6kb (Fig. 1E and F).

Paternal inheritance of the 6 kb PWS-IC deletion leads to reduced birth weight and survival

Paternal inheritance of a 35 kb deletion removing the entire murine PWS-IC is associated with low birth weight, failure to thrive, fully penetrant neonatal lethality and loss of expression of paternal-only genes from the region. At birth, mice inheriting the PWS-ICΔ6kb allele paternally (PWS-IC+/Δ6kb) are smaller than wild-type littermates and often lack milk spots. PWS-IC+/Δ6kb neonates weigh significantly less than their wild-type littermates and rarely survive beyond postnatal day (P) 2, although we did observe one pup surviving to P7 (Fig. 2). Similar to the previously reported PWS-ICΔ5kb mutation, we observed no overt phenotypes following maternal inheritance of the PWS-ICΔ6kb allele.
PWS gene expression is undetectable in PWS-IC\textsuperscript{+}/\Delta6kb pups

We investigated the molecular consequences of paternal inheritance of the 6 kb PWS-IC deletion by analyzing gene expression in newborn brain. Paternally expressed genes at the PWS/AS locus are arranged in two clusters. The centromeric cluster of genes comprising \textit{Snrpn}, several snoRNAs and \textit{Ube3a-as} are likely to be processed from a common primary transcript (14–18). \textit{Snrpn} RNA and the snoRNAs Snord115 and Snord116 were not detected in the newborn PWS-IC\textsuperscript{+}/\Delta6kb brain (Fig. 3B). A low level of Snord116 RNA was detected in the 35 kb PWS-IC deletion control. As previously noted, this low level of expression may be a consequence of continued maintenance of the mouse line or result from the backcross onto the C57BL/6 background (19).

\textit{Ube3a} expression is increased in PWS-IC\textsuperscript{+}/\Delta6kb mice

\textit{Ube3a} is normally paternally silenced by a mechanism speculated to depend upon \textit{Ube3a-as}, a transcript antisense to \textit{Ube3a}. Consistent with this model, paternal inheritance of the 35 kb deletion of the PWS-IC results in increased paternal expression of \textit{Ube3a} (20). To determine the imprinted status of \textit{Ube3a} in PWS-IC\textsuperscript{+}/\Delta6kb mice, we took advantage of the B6.CAST.e7 strain in which the PWS/AS region is congenic...
for Mus musculus castaneus on a C57BL/6 background. DNA sequencing of a region containing a single nucleotide polymorphism demonstrated biparental expression of Ube3a in PWS-IC+/Δ6kb mice, while the wild-type littermates exhibited predominantly maternal expression (Fig. 3C). Thus, similar to the 35 kb deletion, the 6 kb PWS-IC deletion allele lacks elements necessary for silencing paternal Ube3a.

DNA methylation analysis of Mkrn3 and Ndn in PWS-IC+/Δ6kb mice

The paternally expressed genes Ndn and Mkrn3 both contain DMRs characterized by DNA hypermethylation of the normally silent maternal allele. Disruption of the PWS-IC leads to paternal hypermethylation in both individuals with PWS and mice lacking PWS-IC function (21–23). We used DNA methylation sensitive restriction endonucleases to investigate the role of the 6 kb PWS-IC deletion on DNA methylation status of these two loci. Paternal inheritance of the 6 kb PWS-IC deletion resulted in increased DNA methylation at both the Mkrn3 and Ndn loci, although to varying extent (Fig. 4). Increased DNA methylation appeared more extensive and reproducible at Ndn than at Mkrn3. The same DNA preparations were analyzed at both loci, suggesting that DNA methylation defects in the absence of the PWS-IC may be more penetrant at the Ndn locus. Partial hypermethylation of these paternal alleles was evident following paternal inheritance of both the 4.8 and 35 kb PWS-IC deletions (11, 22).

DISCUSSION

ICs are DNA sequences that regulate both epigenotype and allelic gene expression (12). At the PWS/AS locus, overlapping microdeletions define a bipartite IC controlling both paternal- and maternal-only gene expression. A previously described 4.8 kb deletion at the murine PWS-IC exhibited a partial imprinting phenotype. Here, we have extended this deletion 1 kb further upstream of Snrpn exon 1. Paternal transmission results in fully penetrant neonatal lethality, undetectable expression of paternal-only genes and increased DNA methylation at Mkrn3 and Ndn. These traits are identical to a 35 kb PWS-IC deletion and indicate that all PWS-IC elements are located within the 6 kb deletion boundaries.

This 6 kb PWS-IC deletes most of the DMR, the Snrpn promoter region and the murine CAS. Both the promoter and the CAS are associated with paternal-specific DNase I hypersensitive (DH) sites at the human SNRPN gene in lymphoblasts and have been proposed to be involved in PWS-IC function (9). However, the human CAS is located in SNRPN intron 1, just outside of the minimally defined human PWS-IC. The inclusion of the transcription activating CAS in the 6 kb murine PWS-IC is consistent with the postulated function of the PWS-IC as a positive regulator of paternally expressed genes in the PWS/AS domain (7). The partial imprinting defect characteristic of the previously described murine 4.8 kb PWS-IC mutation indicates that this deletion contains some but not all elements required for a fully functional PWS-IC. Both the murine Surpn promoter region and the CAS element are included in the 4.8 kb deletion, suggesting DNA sequence elements associated with these regions may contribute to PWS-IC activity. However, the partial imprinting defect of the 4.8 kb deletion indicates that one or more elements outside of this deletion are additionally required for full PWS-IC activity. Because the 4.8 kb deletion and the 6 kb deletion described here share the same 3′ boundary, we postulate that functional elements that confer full PWS-IC activity must be present within this differential 1.0 kb interval. A DH site has been detected within this 1.0 kb interval (S. Rodriguez-Jato, J.R. Khadake, T.P. Yang, unpublished data). We are currently refining its location and determining its parent of origin. If present only on the paternal allele, this would be consistent with the hypothesis that the PWS-IC is comprised of multiple functional elements that contribute to PWS-IC activity by creating a chromatin...
Snrpn, the snoRNAs and Ube3a-as are all processed from a common transcript. While the majority of transcription initiates within the PWS-IC, some transcripts initiate at several alternative upstream exons and splice into Snrpn exon 2, or less commonly, further downstream (24). Although the splice acceptor site at Snrpn exon 2 is intact, these transcripts are not detected in mice with a paternal 6 kb PWS-IC deletion, indicating that these upstream transcription initiation sites are themselves subject to regulation by the PWS-IC.

MATERIALS AND METHODS

Gene targeting

A targeting vector was constructed from a phage library of fragments generated from BAC 397F16 (Research Genetics). Homology arms consisted of a 3′ 3.5 kb EcoRI and a 5′ 8.6 kb HindIII/EcoRI fragment. Oligonucleotides containing a loxp site were inserted into the HpaI site of the 8.6 kb HindIII/ EcoRI fragment, splitting it into a 2.6 kb 5′ homologous arm and a 6 kb PWS-IC region. A PGK-neo gene flanked by loxp sites was placed between the 3′ homology arm and the PWS-IC region, and a PGK-tk cassette was placed between the 3′ arm and the pBluescript KS + vector backbone. Homologous recombination was performed in CJ.7 cells (129S1/Sv strain) as previously described (12). Clones with the appropriate homologous recombination were identified by Southern blot. Homologous recombination at the 5′ end was identified by Spel digest and at the 3′ end by HpaI/EcoRI digest.

The floxed neomycin cassette was removed by transient transfection of the ES cells with a Cre expressing plasmid, pCAGcre (25). Colonies sensitive to G418 were analyzed by PCR and southern blot. An ES cell line with a floxed 6 kb region of the PWS-IC was selected for injection into C57BL/6 blastocysts and chimeric mice identified. The region of the PWS-IC was selected for injection into C57BL/6 blastocysts and chimeric mice identified. The ES clones with homologous recombination were outside of the exposed to Kodak XAR film. The probes used for identifying a positively charged nylon membrane (Hybond, GE Healthcare) digested with restriction endonucleases as indicated, electrophoresed through 0.8% TAE agarose gels and transferred to a positively charged nylon membrane (Hybond, GE Healthcare). Membranes were hybridized to 32P-labeled probe and exposed to Kodak XAR film. The probes used for identifying ES clones with homologous recombination were outside of the targeting vector’s arms of homology. At the 5′ end, a 800 bp EcoRI fragment was used while at the 3′ end, a 358 bp EcoRI fragment was used, both from a phage library of BAC 397F16 (Research Genetics) fragments.

Gene expression analysis

Analysis of paternal-only gene expression by either RT–PCR or northern blot was as previously described (12). Ube3a allelic expression was determined in newborn brains obtained from matings of B6.CAST/c7 females with PWS-IC+/−Dkk male. B6.CAST/c7 are C57BL/6 congenic for the Mus castaneous PWS/AS region (26). RT–PCR for Ube3a was performed across a polymorphism between Mus castaneous and Mus musculus domesticus using primers Ube3a 5′-CACATATGA TGAAGCTACGA-3′ and Ube3a 6R, 5′-CAGACTCCCTT CATATTCC-3′ (20). The RT–PCR product was sequenced by the UF Center for Epigenetics.

ACKNOWLEDGEMENTS

The authors thank Ryan Fiske of the UF Mouse Models Core.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the Foundation for Prader-Willi Research and the National Institutes of Health (R01 HD 037872). E.Y.S. was supported by a UF Alumni Fellowship.

REFERENCES