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Minimal definition of the imprinting center and fixation of a chromosome 15q11-q13 epigenotype by imprinting mutations

(monoallelic expression/DNA methylation/gametogenesis/Prader–Willi syndrome/Angelman syndrome)

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ABSTRACT **Patients with disorders involving imprinted genes such as Angelman syndrome (AS) and Prader–Willi syndrome (PWS) can have a mutation in the imprinting mechanism. Previously, we identified an imprinting center (IC) within chromosome 15q11-q13 and proposed that IC mutations block resetting of the imprint, fixing on that chromosome the parental imprint (epigenotype) on which the mutation arose. We now describe four new microdeletions of the IC, the smallest (6 kb) of which currently defines the minimal region sufficient to confer an AS imprinting mutation. The AS deletions all overlap this minimal region, centromeric to the PWS microdeletions, which include the first exon of the *SNRPN* gene. None of five genes or transcripts in the 1.0 Mb vicinity of the IC (*ZNF127*, *SNRPN*, *PAR-5*, *IPW*, and *PAR-1*), each normally expressed only from the paternal allele, was expressed in cells from PWS imprinting mutation patients. In contrast, AS imprinting mutation patients show biparental expression of *SNRPN* and *IPW* but must lack expression of the putative AS gene 250-1000 kb distal of the IC. These data strongly support a model in which the paternal chromosome of these PWS patients carries an ancestral maternal epigenotype, and the maternal chromosome of these AS patients carries an ancestral paternal epigenotype. The IC therefore functions to reset the maternal and paternal imprints throughout a 2-Mb imprinted domain within human chromosome 15q11-q13 during gametogenesis.**

A new class of genetic disorders has recently been recognized, involving non-Mendelian inheritance in the form of genomic imprinting (1), and include the following: Angelman syndrome (AS), Prader–Willi syndrome (PWS; ref. 2), Beckwith–Wiedemann syndrome, and various pediatric cancers (3), which are the best-known examples to date. AS and PWS arise from various genetic abnormalities in chromosome 15q11-q13, most commonly *de novo*, and involving a differential parental origin of large cytogenetic deletions or uniparental disomy (UPD). These deletions are maternal in origin in AS and paternal in origin in PWS; in contrast, UPD is paternal and maternal in origin in AS and PWS, respectively (2). Thus, the AS gene or genes are active on the maternal chromosome but inactive on the paternal chromosome, while the PWS gene or genes are expressed from the paternal chromosome only (2). About 20% of AS patients show a pattern of inheritance consistent with a mutation in a putative AS structural gene. In contrast, PWS may require at least two genes for the classical clinical phenotype to arise (2). PWS occurs in 1/15,000 births, but the frequency of AS is presently unknown (2).

The 15q11-q13 region has been cloned as a yeast artificial chromosome contig (4). Within the proximal half of this region (see Fig. 1*a*), multiple imprinted genes have been isolated, including *SNRPN* (for review, see ref. 5), *IPW* (6), and *ZNF127* (unpublished data), as well as two uncharacterized transcripts, *PAR-1* and *PAR-5* (8). Since these are expressed from the paternal allele only, each is a candidate to play a role in PWS. Although the maternally expressed AS gene has yet to be isolated, mapping of various deletions has suggested a localization within a 1-Mb domain beginning 200 kb distal of *SNRPN* (ref. 2; K.B., unpublished data). The distal part of 15q11-q13 harbors nonimprinted genes (see Fig. 1*a*; ref. 2).

A new class of familial PWS and AS patients with a mutation in the imprinting process has recently been recognized (8–13), offering insights into the imprinting mechanism. These patients have neither the typical deletion nor UPD, but they show biparental inheritance with uniparental DNA methylation throughout 15q11-q13. We have previously identified 45- to 200-kb inherited microdeletions in two AS and three PWS imprinting mutation families, defining a genetic element we term an imprinting center (IC; ref. 13). The deletion *per se* does not cause the disease phenotype, because the microdeletions are also present in a phenotypically normal parent and ancestors (8, 13). Based on the pattern of inheritance, we postulated that the IC is involved in resetting of the imprint during gametogenesis (13). In this model, mutation of the IC would result in fixation of an ancestral epigenotype, with silent transmission through the same sex but advent of the imprinted phenotype after inheritance through the opposite sex (13). To substantiate this hypothesis, we have now examined allelic expression of five imprinted genes from 15q11-q13 in PWS and AS imprinting mutation families. In addition, we have examined the extent of microdeletions in four new AS imprinting mutations to define the minimal region sufficient to cause an imprinting mutation.

MATERIALS AND METHODS

Patients. One PWS and five AS families were diagnosed with imprinting mutations on the basis of clinical features (PWS-U,

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Abbreviations: AS, Angelman syndrome; IC, imprinting center; PWS, Prader–Willi syndrome; RT, reverse transcription; UPD, uniparental disomy; VNTR, variable number of tandem repeats.

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ref. 13; AS-C, AS-J, and AS-H, ref. 14; and AS-SCH and AS-R, unpublished data) and uniparental DNA methylation within 15q11-q13, but biparental inheritance and no typical deletion nor UPD (refs. 9, 13, and 15; AS-SCH and AS-R, unpublished data).

Southern Hybridization. DNA extraction and Southern hybridizations were performed by standard procedures (5, 13, 16) using ^{32}P-labeled probes from a 150-kb phage contig spanning PW71 (*D15S63*)-*SNRPN* (13). If repetitive sequences were present in the probe, preassociation with human placental DNA was performed (13). DNA from peripheral blood was used for DNA methylation studies (5, 7, 12, 13, 16).

RNA Extraction and Reverse Transcription (RT)-PCR. Total RNA was extracted from lymphoblast cell lines using RNAzolB (Cinna/Biotecx Laboratories, Friendswood, TX), 5 μg was reverse-transcribed with SuperscriptII (GIBCO/BRL) or rTth RNA PCR kit (Perkin–Elmer) using oligo dT as a primer (5), and one-tenth or one-twentieth of the RT reaction was used for subsequent PCR amplifications. Primers and conditions for PCR were as described: 60A and 60B for *IPW* (6); RN85 and RN133 for *SNRPN* (5); *PAR-1* and *PAR-5* as described (8); and DD29 and RN153 for *ZNF127* (unpublished data). Primers from the *ERG* (17) or *PDHA1* (*E1α*) (18) genes were used as positive controls.

Expressed Polymorphisms. RT-PCR products or genomic DNA from *IPW* (using primers 60C and 60D) were digested with *Hph*I (6). For the *SNRPN* exon 2-expressed polymorphism (19, 20), 200 ng of total RNA was RT-PCR-amplified with primers RN133 (exon 0) and RN85 (exon 3) (5). Exon 2 and flanking intron genomic DNA sequences were amplified with DD38 (5′-CTACTCTTTGAAGCTTCTGC-3′) and DD41 (5′-CCCTCAGCCTTATCATACAG-3′) (5). RT-PCR and genomic PCR products were digested with *Bst*UI, and the products were electrophoresed on an agarose gel and stained with EtBr.

RESULTS

Identification of New Microdeletions and Minimal Definition of the IC. The use of probes from the IC region (13) between PW71 (*D15S63*) and *SNRPN*, for quantitative dosage Southern and breakpoint analyses, led to the identification of microdeletions in four new AS imprinting mutation families (summarized in Fig. 1*b*).

AS Family SCH. Probe 71.19.12HR, a 450-bp *Hin*dIII/*Rsa*I fragment mapping 12 kb proximal to probe PW71B (B. Dittrich and B.H., unpublished data), detects, in the patient and his mother, an abnormal 8.5-kb *Hin*dIII fragment (Fig. 2*a*) or 9.5-kb *Xba*I fragment (data not shown). This defines the centromeric breakpoint of this deletion. Probes PW71B, Y48.5, YL46T, and L48.6I showed reduced dosage, whereas probe L48.3IP, a 750-bp fragment from L48.3I, detects the 8.5-kb *Hin*dIII junction fragment (Fig. 2*a*) and thus defines the telomeric breakpoint. Based on these data, the deletion is estimated to span 80 kb (Fig. 1*b*). For prenatal diagnosis, probes 71.19.12HR and L48.3IP did not detect a 8.5-kb *Hin*dIII junction fragment in DNA from chorionic villus sampling, excluding a deletion (Fig. 2*a*). Therefore, the pregnancy was continued, and an apparently normal boy was subsequently born.

DNA Methylation Studies in the AS-SCH Family. Probe PW71B detects allele-specific DNA methylation patterns (10, 12). As expected, the AS proband in the AS-SCH family lacks the maternal 6.6-kb band for PW71B (Fig. 3*a*). However, the normal mother also has an abnormal methylation pattern due to a deletion at this locus (see above), having a maternal band but lacking the paternal 4.7-kb band (Fig. 3*a*). These results indicate that the patient's deletion is maternal in origin and that the mother inherited the deletion from her father. The maternal grandfather has a normal methylation pattern (Fig. 3*a*) and no reduced dosage with PW71B, indicating that the deletion occurred *de novo* in either the grandfather's germ line or early in embryogenesis of the patient's mother.

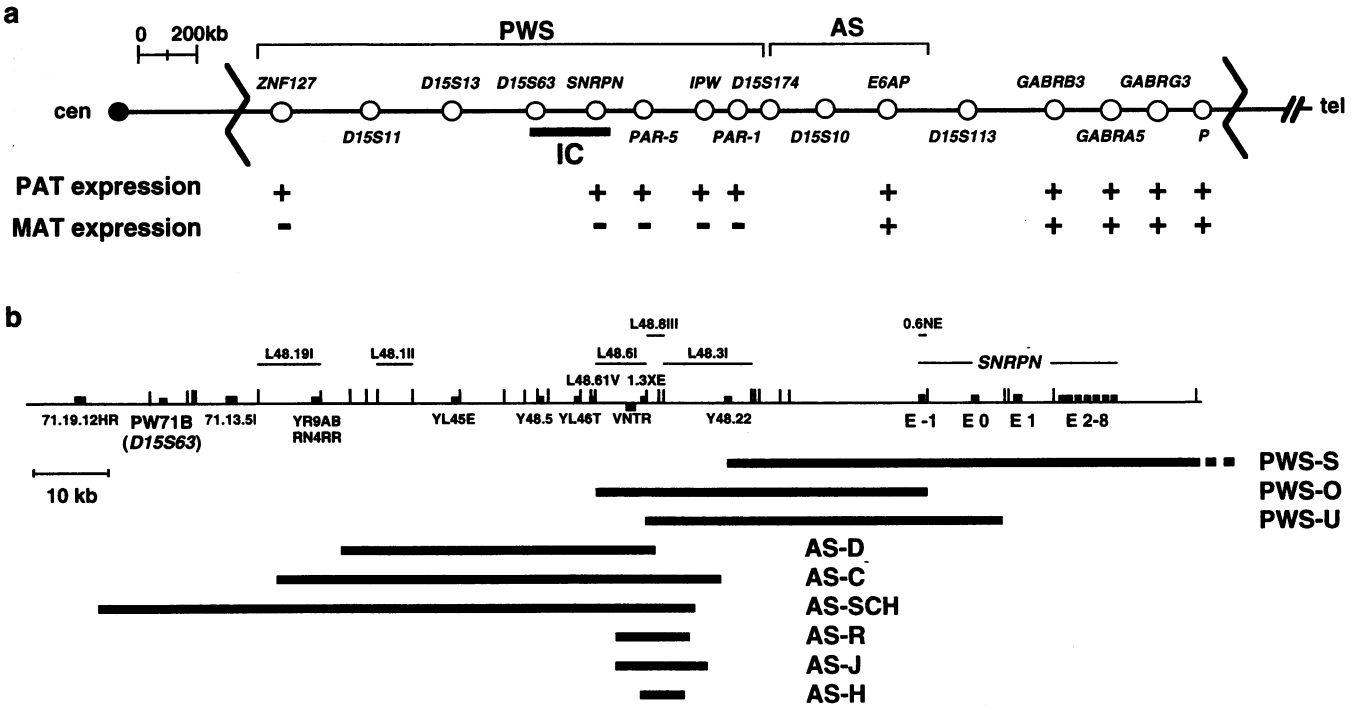

FIG. 1. Microdeletions in the 15q11-q13 IC. (*a*) Genetic map of chromosome 15q11-q13, illustrating the position of genes and genetic markers (circles), transcription from paternal (PAT) or maternal (MAT) alleles, common cytogenetic deletion breakpoints (zigzag lines), and regions involved in PWS, AS, and the IC. (*b*) Map showing microdeletions within the 15q11-q13 IC. The extent of deletions in AS families H, J, R, and SCH are based on data in Fig. 2. The *Eco*RI restriction map (vertical lines), probe location (bars), and extent of deletions in AS families C, D, and PWS families O, S, and U are from ref. 12.

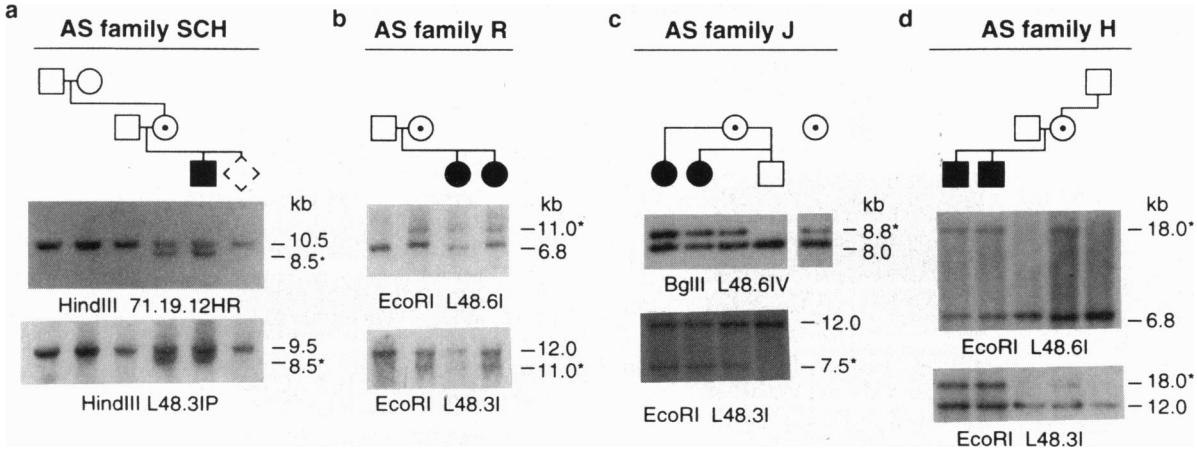

FIG. 2. Identification of 15q11-q13 IC microdeletions. The proximal (*Upper*) and distal (*Lower*) breakpoints are shown for the following families: (*a*) AS-SCH; (*b*) AS-R; (*c*) AS-J; and (*d*) AS-H. The restriction enzyme and probe used is listed. The mothers in the AS-J and the AS-H families demonstrate mosaicism, both by qualitative and quantitative (data not shown) analysis. Each experiment was performed three times. *, Breakpoint fragment.

AS Family R. Use of probe L48.6I (Fig. 1*b*) detects a novel variable number of tandem repeats (VNTR) polymorphism in *Sac*I-digested DNA, with three different alleles identified in the AS-R family. The two AS children inherited different paternal alleles and lack a maternal allele (Fig. 3*b*), indicating a maternal deletion of the VNTR. Probes proximal to L48.6I show normal dosage (data not shown), whereas with L48.6I an 11.0-kb *Eco*RI junction fragment in both children and the mother was identified (Fig. 2*b*). While probe L48.8III shows reduced dosage in *Eco*RI-digested DNA, probe L48.3I detects the distal 11.0-kb deletion junction fragment (Fig. 2*b*). Based on a fine restriction map of the region, the microdeletion spans 10 kb (Fig. 1*b*).

AS Family J. Probe L48.6IV detects the centromeric breakpoint in *Bgl*II-digested DNA from the two affected half-sibs and mother in this family (Fig. 2*c*), while probe L48.3I detects the telomeric breakpoint in *Eco*RI (Fig. 2*c*), *Xba*I, or *Bam*HI (data not shown) digests. Interestingly, the mother shows a reduction in intensity of the breakpoint fragments compared with that in the patients (Fig. 2*c*), indicating that she is mosaic for deletion and normal cell lines. A breakpoint fragment was not detected in a normal brother. Although the father was unavailable, one affected sib was informative for the IC-VNTR polymorphism using the 1.3XE probe, in *Bgl*II digests, and did not inherit a maternal allele (data not shown). Based on the data, the extent of the deletion is 13 kb (Fig. 1*b*).

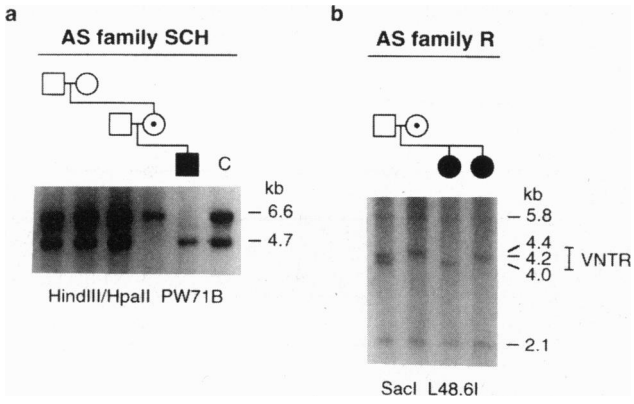

FIG. 3. Inheritance of microdeletions. (*a*) DNA methylation at PW71 in the AS-SCH family. The proband has DNA methylation typical for an AS patient (12). The mother shows a PWS pattern of DNA methylation (12), but is clinically normal, and this pattern results from deletion at this locus. (*b*) Analysis of a VNTR polymorphism within the IC in the AS-R family. The father is heterozygous, the mother is homozygous for a third, larger allele, and the two affected daughters each inherited a different paternal allele only. The restriction enzymes and probes used are listed in *a* and *b*.

AS Family H. Probe L48.6I detects the centromeric breakpoint and probe L48.3I detects the telomeric breakpoint in *Eco*RI (Fig. 2*d*) or *Bam*HI-digested DNA from the two affected sibs and mother in this family. The mother shows a reduced intensity for breakpoint fragments, compared with patients (Fig. 2*d*), indicating that she is mosaic for the deletion. By Southern and PCR analyses of the deletion junction, the deletion was not present in the maternal grandparents, the two maternal brothers, or the sister. Probe 1.3XE on *Eco*RI (and *Bgl*II or *Xba*I)-digested DNA also detects the centromeric breakpoint, in addition to showing a maternal deletion for the IC-VNTR polymorphism. The affected sibs inherited a 7.0-kb VNTR band from the father, who was homozygous, and the 18.0-kb breakpoint band from the mother, who had a 6.9-kb VNTR band (data not shown). Fine mapping of the deletion junction allowed precise determination of the size of the deletion as only 6 kb (Fig. 1*b*).

Loss of Expression of Imprinted Genes in PWS Imprinting Mutation Patients. We examined expression of the three known paternal-only imprinted genes within 15q11-q13, *SNRPN* (5), *IPW* (6), and *ZNF127* (unpublished data) and two uncharacterized transcripts, *PAR-1* and *PAR-5* (8), in two affected sibs of the PWS-U family (13). None of these five genes or transcripts was expressed in the two PWS imprinting mutation patients, whereas the control *ERG* gene was expressed (Fig. 4a). However, all five genes were expressed in the normal mother and the phenotypically normal father (Fig. 4*a*), who has the same deletion on his maternal chromosome (13).

Biparental Expression of Imprinted Genes in AS Imprinting Mutation Patients. We also used expressed polymorphisms to examine allele-specific transcription of the *SNRPN* (*Bst*UI polymorphism in exon 2; refs. 19 and 20) and *IPW* (*Hph*I polymorphism in exon 2; ref. 6) genes in AS imprinting mutation families. Both affected sibs in the AS-H family were heterozygous for polymorphisms in *SNRPN* and *IPW* (Fig. 4*b*, DNA lanes) and RNA from cells of both individuals showed heterozygous expression (Fig. 4*b*). This included expression of the normally silent maternal (*Upper*) allele for *SNRPN* and maternal (*Lower*) *IPW* allele in the AS-H family, as well as the paternal allele for each gene. Likewise, the AS-C proband showed biparental expression of both paternal and maternal alleles for *SNRPN*, while the AS patients in the AS-J family were heterozygous (DNA lanes) for at least one of the two

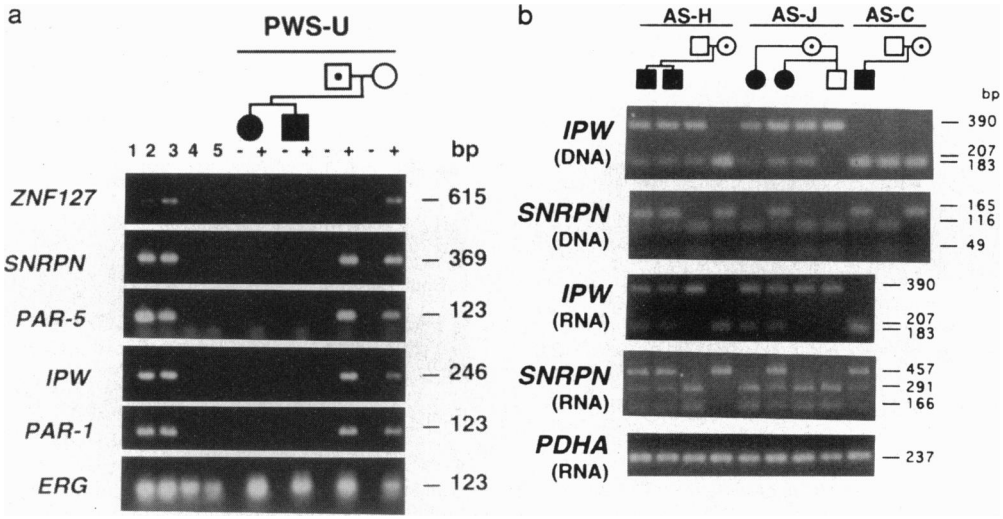

FIG. 4. Expression analysis in imprinting mutation patients. (*a*) Lack of expression of paternally expressed genes in patients from the PWS-U family. RT-PCR expression analysis of lymphoblast cell lines from members of the PWS-U family (−, RNA without prior reverse transcription; and +, reverse-transcribed RNA), as well as AS (lane 2, deletion; and lane 3, UPD) and PWS (lane 4, deletion; and lane 5, UPD) controls, is shown for the *ZNF127*, *SNRPN*, *PAR-5*, *IPW*, and *PAR-1* genes or transcripts and the control *ERG* gene. Lane 1 represents a H_2O control. (*b*) Biparental expression of paternally expressed genes in AS imprinting mutation patients from families AS-C, -H, and -J. DNA analysis by PCR and RNA analysis by RT-PCR, followed by restriction enzyme digestion with *Bst*UI for *SNRPN* or *Hph*I for *IPW*, is shown for each family. RT-PCR of the control *PDHA1* (*E1α*) gene is shown (*Bottom*).

genes, and both patients showed heterozygous expression (Fig. 4*b*). In contrast, expression of *SNRPN* was monoallelic for the AS-H mother, and, likewise, expression for *IPW* was monoallelic for the AS-H father and AS-J mother, when informative (Fig. 4*b*).

DISCUSSION

We have identified four new microdeletions in AS imprinting mutation families, in addition to the two AS and three PWS microdeletions previously characterized (13). The new AS microdeletions are as small as 6 kb (AS-H), and all six share a common deleted region of just 2 kb (Fig. 1*b*). This defines a minimal 2- to 6-kb region in which deficiency produces a typical AS phenotype, as well as uniparental DNA methylation and aberrant expression within 15q11-q13. Since one (PWS-S) of three PWS microdeletions does not overlap this region (13), it is likely that the IC has a bipartite structure. The deletion of the centromeric part of the IC (AS microdeletions) appears to prevent the paternal → maternal imprint switch, whereas deletion of the telomeric part of the IC (PWS microdeletions) appears to prevent the maternal → paternal imprint switch. Identification of smaller deletions in additional PWS imprinting mutation families will be critical in defining the latter element.

In two families (AS-J, AS-H), the mother of AS imprinting mutation patients was shown to be mosaic for the microdeletion. These results indicate an early somatic origin for the microdeletions. The mutations apparently fix the grandpaternal epigenotype into the chromosome, before resetting the new imprint in the mother's germ line. This strikingly illustrates the transgenerational effect of imprinting mutations.

In the two affected sibs in the PWS-U family, we have shown that paternally expressed *SNRPN*, *IPW*, *ZNF127*, *PAR-1*, and *PAR-5* transcripts are repressed. Repression of *SNRPN* gene expression, also seen in the PWS-O family (8), is most simply explained by deletion of the promoter and first exon of this gene (5). Recently, Sutcliffe *et al.* (8) demonstrated that neither *PAR-1* nor *PAR-5* transcripts were expressed in affected individuals of the PWS-O family. The *IPW* gene, which does not encode a protein product (6), and *PAR-1* (8) map 180 kb distal to *SNRPN* (6, 15) while the *ZNF127* gene, which encodes a novel zinc finger polypeptide (unpublished data), maps about 1 Mb proximal to *SNRPN* (4) (Fig. 1*a*). Therefore, PWS imprinting mutations abolish gene transcription of paternal origin over a 1.0–1.5 Mb domain within 15q11-q13.

We (13) have previously suggested that the IC functions in resetting the imprint in 15q11-q13 and that IC mutations block this resetting. Our new data confirm a prediction of this model that gene transcription in imprinting mutation patients is altered as a consequence of failure to reset the imprint. Normal individuals have a paternal (active *ZNF127*, *SNRPN*, *PAR-5*, *IPW*, and *PAR-1*) and a maternal (inactive state for these genes) imprint, or epigenotype (Fig. 5). An IC microdeletion arising on an ancestral maternal chromosome "fixes" the maternal epigenotype into that chromosome. Following paternal transmission, PWS results as individuals inheriting this chromosome are effectively homozygous for a maternal epig-

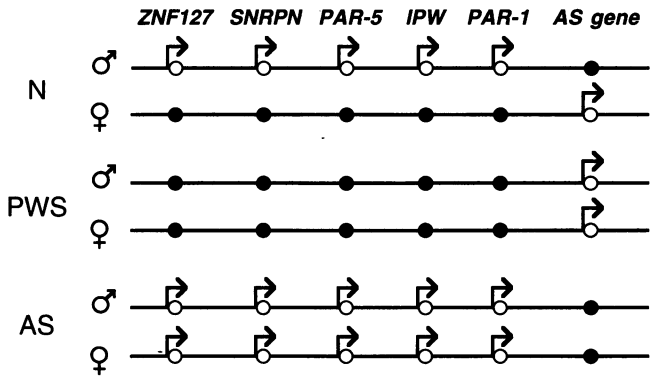

FIG. 5. Model for fixation of an ancestral epigenotype by imprinting mutations in the IC. In normal individuals, five genes within 15q11-q13 are expressed from the paternal allele only (active gene, open circles with arrow; and inactive gene, closed circles), while the putative AS gene is expected to be expressed from the maternal allele only. In PWS imprinting mutation patients, the paternally inherited chromosome has a maternal epigenotype based on DNA methylation and gene expression analyses, whereas the maternally inherited chromosome in AS imprinting mutation patients has a paternal epigenotype. The direction of transcription (arrows) is arbitrary.

enotype and do not express the paternal-only transcribed genes (Fig. 5).

Our finding of biparental expression of *SNRPN* and *IPW* in AS imprinting mutation patients from three families with different microdeletions strongly supports the "epigenotype fixation" hypothesis. In contrast, this result makes it unlikely that the IC directly affects transcriptional control of genes, such as by enhancer or locus control region activity. An IC microdeletion arising on an ancestral paternal chromosome fixes the paternal epigenotype, which, following maternal transmission, leads to effective homozygosity for a paternal epigenotype; genes normally transcribed from the paternal allele only are therefore biparentally transcribed (Fig. 5). The putative AS gene, normally expressed from the maternal allele only, is predicted to be silenced as a consequence of the paternal epigenotype (Fig. 5). Since the AS gene lies 0.2–1.0 Mb distal to *SNRPN* (Fig. 1*a*), this would extend the domain containing imprinted genes whose imprints are reset by the IC to 1.5–2.5 Mb.

These results provide conclusive evidence that large chromosomal domains contain coordinately regulated imprinted genes. This was first suggested as imprinted genes cluster within specific chromosome regions, such as *H19*, *Igf2*, *Ins-2*, *Mash-2*, and $p57^{KIP2}$ (for review, see refs. 3, 21, and 22); *Igf2r* and *Mas* (23); and *SNRPN*, *IPW*, *ZNF127*, and *PAR-5* (refs. 5, 6, 8; unpublished data). Chromosome regions containing imprinted genes also show asynchronous replication timing over domains up to several megabases in size (2, 24). Our finding of coordinate regulation of imprinted genes by a 15q11-q13 IC over a 2-Mb chromosomal domain now provides a molecular basis for the clustering of imprinted genes in large domains.

In Wilms tumor, other pediatric or adult cancers, and Beckwith–Weidemann syndrome, "loss of imprinting" in chromosome 11p15 is characterized by conversion to uniparental DNA methylation at *IGF2* and *H19*, loss of expression of the maternally expressed *H19* gene, and biparental expression of the closely linked *IGF2* gene (3, 25). These data are consistent with a model in which the maternal chromosome gains a fixed paternal epigenotype, as proposed for PWS and AS. Similarly, maternal inheritance of a targeted deletion of the *H19* gene leads to biparental expression of *Igf2* (21), which may be explained by the *H19-Igf2* enhancer local competition model or if the *H19* gene itself is an IC for this locus (21). Therefore, loss of imprinting patients may have mutations within an IC, which may be the *H19* gene or an independent genetic element within 11p15 (25). The latter hypothesis may also account for maternal translocations seen in Beckwith–Weidemann syndrome (7). Further analysis of the sequence elements present within the 15q11-q13 IC and the study of the function of such elements, also addressed through analysis of mouse models, will provide the keys to understanding how genomic imprinting is reset during gametogenesis and how imprinting mutations give rise to abnormal phenotypes.

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