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Imprinting-Mutation Mechanisms in Prader-Willi Syndrome

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Summary

Microdeletions of a region termed the "imprinting center" (IC) in chromosome 15q11-q13 have been identified in several families with Prader-Willi syndrome (PWS) or Angelman syndrome who show epigenetic inheritance for this region that is consistent with a mutation in the imprinting process. The IC controls resetting of parental imprints in 15q11-q13 during gametogenesis. We have identified a larger series of cases of familial PWS, including one case with a deletion of only 7.5 kb, that narrows the PWS critical region to <4.3 kb spanning the *SNRPN* gene CpG island and exon 1. Identification of a strong DNase I hypersensitive site, specific for the paternal allele, and six evolutionarily conserved (human-mouse) sequences that are potential transcription-factor binding sites is consistent with this region defining the *SNRPN* gene promoter. These findings suggest that promoter elements at *SNRPN* play a key role in the initiation of imprint switching during spermatogenesis. We also identified three patients with sporadic PWS who have an imprinting mutation (IM) and no detectable mutation in the IC. An inherited 15q11-q13 mutation or a *trans*-factor gene mutation are unlikely; thus, the disease in these patients may arise from a developmental or stochastic failure to switch the maternal-to-paternal imprint during parental spermatogenesis. These studies allow a better understanding of a novel mechanism of human disease, since the epigenetic effect of an IM in the parental germ line determines the phenotypic effect in the patient.

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Introduction

Certain regions of the mammalian genome exhibit gene expression from only the maternally or the paternally inherited allele, a process referred to as genomic imprinting. Imprinted genes are marked differently in the female and the male germ lines, leading to differences in somatic expression and function of the two alleles. Most imprinted genes identified in the mouse and human genomes lie within large clusters (Reik and Maher 1997; Nicholls et al. 1998). Each imprinted gene or region shows several typical features, including monoallelic expression, differential DNA methylation, and asynchronous DNA replication of the maternal and paternal alleles (Nicholls et al. 1998). Despite extensive analysis of imprinted genes, the molecular mechanisms by which parental-specific imprints are established in the germ line and regulated during embryonic and adult development are still poorly understood.

Prader-Willi syndrome (PWS; MIM 176270) and Angelman syndrome (AS; MIM 105830) are distinct developmental and neurobehavioral syndromes that arise from abnormal imprinted-gene expression in humans. Each syndrome occurs at a frequency of ~1/15,000 births (Clayton-Smith and Pembrey 1992; Cassidy 1997). Approximately 70%–80% of patients with PWS or AS have a large 4-Mb deletion in the paternal or the maternal chromosome 15q11-q13 region, respectively (Nicholls et al. 1998). Both syndromes also result from uniparental disomy (UPD), which is of maternal origin in patients with PWS and of paternal origin in patients with AS. Therefore, PWS results from the loss of a gene(s) that is expressed only from the paternally inherited chromosome, and AS results from the loss of maternal-only gene expression. A single gene (*UBE3A*) that displays regional brain-specific imprinting in humans and mice is implicated in AS (Malzac et al. 1998; Nicholls et al. 1998). In contrast, PWS is likely to be a contiguous-gene syndrome (Nicholls et al. 1998), and, consistent with this hypothesis, multiple imprinted and

paternal-only expressed genes have been identified in 15q11-q13 (fig. 1).

An unusual class of patients with PWS and AS have a mutation in the imprinting process. These patients have neither the typical deletion nor UPD, and, although they have biparental inheritance for chromosome 15 DNA markers, they show uniparental DNA-methylation (Glenn et al. 1993; Reis et al. 1994; Sutcliffe et al. 1994; Buiting et al. 1995) and gene-expression (Saitoh et al. 1996) patterns throughout the 1.5–2.0 Mb imprinted domain within 15q11-q13 (fig. 1). Accordingly, in patients with PWS, the paternally inherited chromosome possesses a maternal imprint and vice versa for cases of AS. Furthermore, these findings imply that all genes in the 1.5–2.0-Mb imprinted region are under coordinate regulation (Saitoh et al. 1996). Definition of the molecular pathogenic mechanism in these patients therefore should lead to significant insights into the molecular mechanism of imprinting.

We previously identified inherited microdeletions of 5.5–80 kb in eight families with an AS imprinting mutation (IM) and of 45–200 kb in three families with a PWS IM, defining an element termed the "imprinting center" (IC) (Buiting et al. 1995; Saitoh et al. 1996; Ohta et al. 1999 [in this issue]). On the basis of the inheritance patterns of these microdeletions and the DNA methylation and gene-expression imprint pattern on this chromosome, we suggested that the IC controls imprint switching in the male and female germ line (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996; Nicholls et al. 1998). IC microdeletions can transmit silently for multiple generations, but, when the sex of the transmitting individual changes, the mutation blocks the appropriate resetting of the imprint specific for that individual's germ line. Thus, PWS IC mutations block the normal maternal-to-paternal imprint switch in the male germ line, and AS IC mutations block the paternal-to-maternal imprint switch in the female germ line.

To further investigate the IM molecular mechanism and to define the IC sequence elements that play a role in the imprint switch, we performed molecular genetic studies of a significantly larger series of patients with PWS who have an IM. Two classes of patients with a PWS IM were identified, deletion and nondeletion. By means of deletion studies, we narrowed the IC critical region involved in initiation of the male germ-line switch to <4.3 kb spanning the promoter of the *SNRPN* gene. Therefore, this one gene is intimately involved in the *cis* regulation of all imprinted genes over a 1.5–2-Mb domain within 15q11-q13, during spermatogenesis.

Subjects and Methods

Clinical Reports

Brief reports for patients with novel molecular findings are presented here, for comparison with typical patients with a PWS deletion and UPD, who display neonatal hypotonia with a failure to thrive, hyperphagia and severe obesity, mental retardation with learning disabilities, obsessive-compulsive disorder, hypogonadism, short stature, and small hands and feet (Cassidy 1997). Clinical and cytogenetic studies of two affected sibs in each of the families PWS-J and PWS-T have been reported elsewhere (Ishikawa et al. 1996; Teshima et al. 1996).

Patient PWS-14.—PWS-14 is patient 19 in our previous RFLP analyses (Mascari et al. 1992). At the time she was last seen, patient PWS-14 was 21 years old and had many features of PWS, including short stature, small hands and feet, hypogonadism, truncal obesity (she is on a 600-cal/d diet), behavior problems that included tantrums and stealing of food, and dysmorphic features, including almond-shaped eyes, a narrow bifrontal diameter, and triangular mouth (fig. 2*a*).

Patient PWS-29.—PWS-29 is patient 28 in our previous report of RFLP studies (Mascari et al. 1992). At age 7 years 3 mo (fig. 2*b* and *c*), this African American boy weighed 46.95 kg (>97th percentile) and was 118.1 cm tall (5th percentile). His head circumference was 54 cm (80th percentile). Hand length was 14.6 cm (50th percentile), middle-finger length was 5.4 cm (5th percentile), and ear length was 6 cm (75th percentile). His blood pressure was 110/72 mm Hg. Decreased fetal activity was noted by the mother, as compared with a second pregnancy; otherwise, the pregnancy was nor-

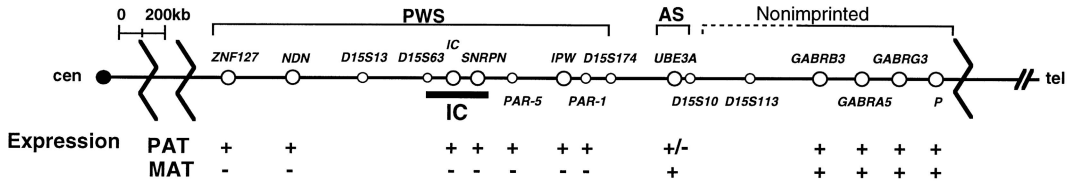

Figure 1 PWS domain in proximal chromosome 15q11-q13. The positions of genes and genetic markers (circles), transcription from paternal ("PAT") or maternal ("MAT") alleles, common cytogenetic deletion breakpoints (zigzag lines), and regions involved in PWS, AS, and the IC are illustrated. A plus sign (+) indicates presence of expression, and a minus sign (−) indicates no expression.

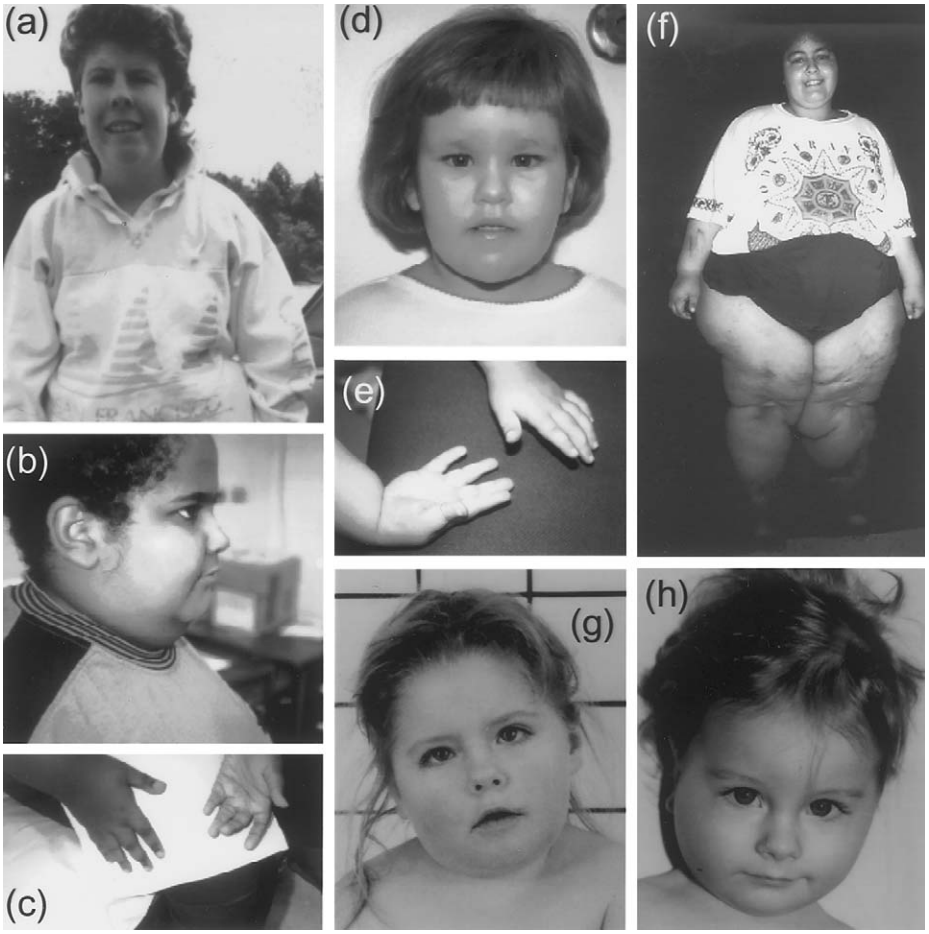

Figure 2 Clinical photographs of PWS patients. *a,* PWS-14. This patient has the smallest known PWS deletion (7.5 kb). *b* and *c,* PWS-29. *d* and *e,* PWS-B. *f,* PWS-G. These three patients with an IM have no 15q11-q13 IC microdeletion or detectable mutation. Note the small hands typical of PWS. *g* and *h,* PWS-P sibs. These sibs have a large IC deletion.

mal. He was born at term by vaginal delivery and weighed 29.23 kg (10th percentile), with a birth length of 48.3 cm (10th percentile). Hypotonia, cryptorchidism, small penis, weak cry, and poor sucking reflex were noted at birth. Feeding difficulties relating to a poor sucking reflex were present during infancy. He had developmental delay, particularly gross-motor and speech delay. Hyperphagia was a recognized problem by age 4 years, and marked obesity developed subsequently. He has a history of sticky saliva and enamel hypoplasia. Behavioral problems were noted by age 4 years and included stubbornness, temper tantrums, and skin picking. The family history was unremarkable. The results of high-resolution chromosome analysis and FISH with the *SNRPN* probe were normal.

Patient PWS-B.—PWS-B is a girl, aged 4 years 2 mo, with features consistent with the diagnosis of PWS (fig. 2*d* and *e*). She initially was seen at age 16 mo with a history of hypotonia during infancy, poor sucking reflex, developmental/speech delay, and small hands and feet. She weighed 2.9 kg at birth, which was 2 wk post term. She was delivered vaginally. Decreased fetal movements were noted; otherwise, the pregnancy was uncomplicated. Levels of organic acids and plasma amino acids in urine were within the normal ranges, and the results of a high-resolution chromosome study were normal (46,XX), including those for FISH analysis with probe A (*D15S11*) and probe B (*GABRB3*) (Oncor). The family history was unremarkable, and the patient has an older sister and a younger brother who are healthy.

At age 20 mo, her height was 74.3 cm (3d percentile), weight was 9.3 kg (5th percentile), head circumference was 47 cm (30th percentile), hand length was 8.6 cm, middle-finger length was 3.6 cm, inner-canthal distance was 3.2 cm, outer-canthal distance was 5.6 cm, ear length was 4.6 cm, and foot length was 10.3 cm. At age 4 years 2 mo (fig. 2*b*), her height was 92.5 cm (5th percentile), weight was 21.3 kg (95th percentile), head

circumference was 49.6 cm (40th percentile), middle-finger length was 4.1 cm (3d percentile), hand length was 10.4 cm (3d percentile), outer-canthal distance was 6.3 cm (3d percentile), inner-canthal distance was 2.5 cm (10th percentile), and ear length was 5.2 cm (25th percentile). She was cooperative and had mild developmental delay. Downturned corners of the mouth, thin upper lip, short upturned nose, tall-appearing forehead, small hands and feet, and truncal obesity (triceps and subscapular skinfold thickness >85th percentile) were noted. She had sticky saliva, strabismus, almond-shaped eyes, small chin, dolichocephalic head shape, narrow bifrontal diameter, clinodactyly of the fifth digits bilaterally, and hypotonia. She had a habit of skin picking and had normal pigmentation. She is described as a happy, affectionate child, and she has had no history of hyperphagia.

Patient PWS-G.—When last seen, PWS-G, a 23-year-old Portuguese woman, was short and morbidly obese and had the gestalt of PWS (fig. 2*f*). Her medical history was significant for neonatal failure to thrive and hypotonia. The family history was nonsignificant, with three older normal sibs; the father is deceased. Height was 136.8cm (<5th percentile for a 10-year-old), weight was 110.7 kg (>95th percentile), and head circumference was 54.2 cm (slightly less than the median). There was mild bitemporal narrowing, with no esotropia or viscous saliva, and hypernasal speech. Obesity was global, with predominance at the chest, abdomen, and upper arms and legs. Breasts were Tanner stage V, with moderate axillary and pubic hair. She had small hands and feet (<5th percentile). There were multiple old and new excoriated skin sores. Neurologically, there were no focal deficits, but there was mild mental retardation and obsessive-compulsive behavior with severe temper tantrums and aggressive food-seeking behavior. She has insulin-dependent diabetes, oligomenorrhea, and hypogonadism. Pigmentation was normal for the family background. The results of high-resolution chromosome analysis were normal.

Family PWS-P.—Two affected sibs are the only children of young, healthy parents of normal intelligence. Fetal movement during both pregnancies was normal. For both sibs, results of a high-resolution cytogenetic study showed normal karyotypes, but FISH studies with *SNRPN* and *GABRB3* probes revealed the lack of a signal for the *SNRPN* probe on one chromosome 15. Patient 1, a girl, was examined at ages 10 mo and 2, 3, and 4 years. At each age, she presented with the age-appropriate spectrum of symptoms usually associated with PWS. She walked and spoke her first words at age 2 years. At age 4 years (fig. 2*g*), her weight was 20 kg (97th percentile), height was 97 cm (10–25 percentile), and head circumference was 50 cm (50th percentile). She was found to have a high forehead with a narrow bifrontal diameter and frontal upsweep of hair, almond-shaped palpebral fissures, convergent squint, downturned corners of the mouth, puffy and delicate hands with slightly tapering fingers, and hypoplasia of the labia minora and clitoris. She was judged to be mildly retarded. She has blue eyes and blond hair, as do her parents, and her skin pigmentation is normal.

Patient 2, a boy, was examined at ages 1 year and 2 years 6 mo. Initially, his history was typical for PWS. On examination at age 2 years 6 mos, he had continued to be hypotonic. In addition, he had normal appetite, poor weight gain, could not sit or walk alone, and spoke only a few words. At the same age, temper tantrums, a habit of picking sores, and thick viscous saliva were present. His facial (fig. 2*h*) and general body appearance resembled that of his sister. He also had hypogenitalism, with small penis, hypoplastic scrotum, and cryptorchidism, and was mildly mentally retarded. Height was 81 cm, and weight was 10 kg (both <3d percentile).

DNA Extraction, Southern Hybridization, and DNA Methylation

DNA was extracted from blood leukocytes, for DNA-methylation and microsatellite analyses, or from lymphoblast cell lines and was processed for Southern analysis by means of standard procedures (Driscoll et al. 1992; Mascari et al. 1992). The ^{32}P-labeled probes, including subclones of a phage contig spanning the IC and the *SNRPN* gene and CpG island (probes E1–E3, E3–E10, L48.3I, L48.6I, 0.6NE, c4.1, RN4RR, PW71B, 11H11R, and *PAR-5*), were prepared and used for breakpoint, dosage, and DNA-methylation analyses, as described elsewhere (Buiting et al. 1995; Dittrich et al. 1996; Glenn et al. 1996; Saitoh et al. 1996). The 393SS probe was subcloned from phage B8 DNA (Glenn et al. 1996), by *Sca*I digestion and size selection on an agarose gel of a 393-bp sequence upstream of *SNRPN* (GenBank accession number U41384; Huq et al. 1997).

Microsatellite Analysis

PCR studies using 11 short tandem repeats and microsatellites from the 15q11-q14 region (*D15S541, D15S543, D15S11, D15S128, D15S10, D15S210, D15S122, D15S113, D15S165, GABRB3,* and *GABRA5*) were performed for the family of PWS-B, by use of standard protocols (Butler et al. 1996). Primers for polymorphic marker studies were from the Genome Database.

Reverse-Transcription (RT)–PCR Analysis of Gene Expression

mRNAs were extracted from lymphoblastoid cell cultures by use of either RNazol B (Biotecx) or the QuickPrep Micro mRNA purification kit (Pharmacia). Dupli-

cate samples of 5–8 μl mRNA were treated with DNase I, followed by cDNA synthesis using the Superscript-II preamplification system (Gibco BRL) or the r*Tth* RNA PCR kit (Perkin-Elmer), in accordance with the supplier's protocols, with oligo dT as a primer for first-strand cDNA synthesis and one aliquot with (RT+) and one without (RT−) reverse transcriptase. Then, 4 μl of the RT reaction was used for subsequent PCR amplification of both RT+ and RT− cDNAs in a total volume of 25 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP (dATP, dCTP, dGTP, and TTP), 0.35 μM internal control primers (for *FBN*), 0.7 μM specific primers, 2 U *Taq* polymerase (Gibco BRL), by manual hot-start addition of the *Taq* polymerase at 93°C. The cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. The PCR products were separated on a 1% agarose gel in 0.5 × Tris borate–EDTA buffer. Primers were as described for *IPW* (Wevrick et al. 1994), *SNRPN* (RN85 and RN133, or E and L) (Sutcliffe et al. 1994; Glenn et al. 1996), *ZNF127* (DD29 and RN153) (Jong et al., in press), *PAR-1* and *PAR-5* (Sutcliffe et al. 1994), *GAPDH* (GenBank J04038), and fibrillin (*FBN* [GenBank L13923]).

SSCP Analysis

In the families of three PWS patients (PWS-B, PWS-29, and PWS-G) and seven controls (five normal, one with an AS deletion, and one with a PWS deletion), genomic DNA spanning the PWS shortest region of deletion overlap (SRO) was PCR amplified with 11 primer sets, and the PCR products were digested with restriction enzymes (RN528–RN429, *Bst*NI/*Hin*cII or *Bst*NI/*Hae*III; RN428–RN429, *Sau*3AI/*Hin*fI; RN430–RN431, *Bsa*I/*Apo*I or *Hin*fI; RN432–RN433, *Ava*II or *Sca*I; RN434–RN435, uncut; RN434–RN437, *Alu*I or *Sau*3AI; RN436–RN437, *Hin*fI or *Hin*cII; RN438–RN439, *Sau*3AI or *Alu*I; RN440–RN441, uncut; RN495–RN496, *Ava*II/*Pvu*II or *Msp*I; and RN497–RN498, *Ava*II or *Msp*I) and analyzed by nonradioactive SSCP. In brief, the DNA molecules were separated overnight on mutation detection–enhancement gels (FMC Bioproducts) and were visualized by silver staining, in accordance with the manufacturer's conditions. The PCR products containing an abnormal band were cloned into the pCR2.1 vector (Invitrogen) and were subjected to DNA sequence analysis. The primer sequences, map, and PCR conditions are available from the corresponding author.

DNase I Hypersensitivity Studies

Lymphoblastoid cell lines derived from patients with PWS or AS deletions were cultured in standard RPMI medium supplemented with 10% FCS, penicillin, and streptomycin (Life Technologies). Cells (~ 2×10^8) were harvested by centrifugation, washed in PBS, and quantitated with viability >95%, as judged by trypan blue exclusion. The washed pellet was resuspended in lysis buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 15 mM Tris [pH 7.4], 320 mM sucrose, 0.5 mM DTT, 0.2 mM AEBSF [a protease inhibitor; Calbiochem], and 0.5% NP–40), at ~ 4×10^7 cells/ml. The nuclei were washed with cold nuclear buffer (lysis buffer without NP–40), to remove cytoplasmic debris and nucleases. The washed nuclear pellets were resuspended in cold nuclear buffer, at 2.7×10^7 cells/ml, and immediately were subjected to DNase I treatment.

DNase I (Life Technologies) was serially diluted in chilled nuclear buffer (20–12,500 U/ml). Forty microliters of each DNase I dilution, 360 μl of the nuclear suspension, and 4 μl of 10 mM $CaCl_2$ were combined in a 15-ml polypropylene tube, mixed by inversion, and incubated at 22°C for 10 min. One nuclear aliquot was combined with nuclear buffer and $CaCl_2$ but without DNase I, to control for potential endogenous nuclease activity, and another aliquot was combined with nuclear buffer only and then kept on ice, to provide an absolute nuclease-free reference. After incubation, 400 μl stop solution (20 mM EDTA, 1% SDS, and 1 mg proteinase K/ml, at 55°C) was added, mixed gently, and incubated at 50°C–55°C overnight. The samples were extracted with phenol/chloroform and chloroform and were precipitated with 2.5 M NH_4OAc and 2.5 volumes of 95% ethanol at room temperature. DNA precipitates were collected by centrifugation, washed with 70% ethanol, and dissolved in Tris-EDTA. *Bam*HI restriction digests were performed on one-half the recovered material (~25 μg DNA), and the products were prepared for Southern analysis by standard procedures.

Molecular Cloning and DNA Sequencing

Subclones and the DNA sequence for human *SNRPN* are published (Glenn et al. 1996). Genomic bacteriophage λ clones spanning *Snrpn* exons 1–3 were isolated by use of mouse *Snrpn* exons 1–3 cDNA as probe (Gabriel et al. 1998). The DNA sequence was determined from subclones across mouse exon 1 and the 5′ region, by use of an ABI 377 automated DNA sequencer (Applied Biosystems).

Results

Previous analyses of microdeletions in three patients with a PWS IM identified a 25-kb SRO that defines one component of the IC (Buiting et al. 1995). The patients with PWS in these families had the classic clinical phenotype (Saitoh et al. 1997). We therefore sought to iden-

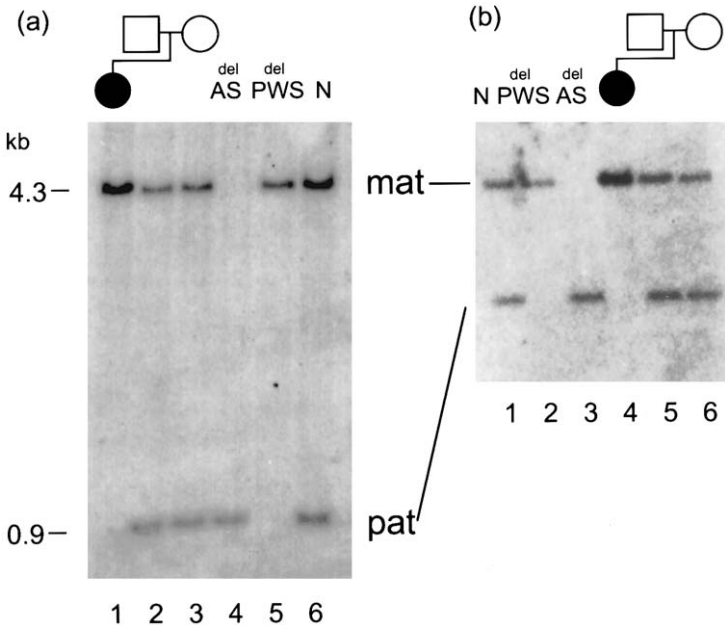

Figure 3 DNA methylation identifies PWS IMs. DNA was digested with *Xba*I/*Not*I, Southern blotted, and probed with the 0.6NE fragment of the *SNRPN* 5′ CpG island. The upper 4.3-kb band represents the methylated allele, which is usually of maternal origin, and the lower 0.9-kb band represents the unmethylated allele, which is of paternal origin. *a,* PWS-14. The *SNRPN* probe is deleted from the paternal allele (lane 1 is overloaded). *b,* PWS-B. Only the methylated band is present, but the patient is not deleted at this locus (lane 4), indicating that the paternal chromosome has a maternal imprint. del = deletion, and N = normal.

tify smaller deletions of the IC in additional patients with PWS, to determine the molecular identity of the critical IC elements.

Clinical Analysis of Patients with an IM

Ten patients with PWS, from seven families, were identified for this study. Case reports are presented in Subjects and Methods, and clinical photographs of key patients are shown in figure 2. Two of these patients had been reported previously (Mascari et al. 1992) as having the classic PWS clinical phenotype (PWS-14; fig. 2*a*) or an atypical phenotype (PWS-29; fig. 2*b* and *c*). In retrospect, some of the apparently atypical features of PWS-29, such as the less-typical facies, may be ascribed to the clinical differences found in African American patients with PWS (Hudgins et al. 1998). Whereas one of the affected PWS-T sibs had most of the clinical diagnostic features of PWS, his younger brother, at age 7 years, only had infantile hypotonia, language delay, and above-average weight, without hyperphagia (Teshima et al. 1996). Patients PWS-B (fig. 2*d* and *e*) and PWS-G (fig. 2*f*) are nondeletion and had a typical PWS phenotype.

Biparental Inheritance of 15q11-q13 Markers

The results of RFLP studies were reported previously for PWS-14 and PWS-29 (Mascari et al. 1992), with each patient having biparental inheritance for markers within chromosome 15q11-q13. Likewise, use of microsatellite markers *GABRB3, D15S11, D15S175, D15S659, D15S1365,* and 85CA for patient PWS-G, *GABRB3* for PWS-14, and *D15S128* and *D15S122* for the two affected PWS-P sibs showed biparental inheritance within 15q11-q13 for each patient (data not shown). Microsatellite analysis of PWS-B showed similar results, presented below. The PWS-T sibs showed a deletion at the *SNRPN* locus, by FISH, but not at *D15S11, D15S10,* or *GABRB3,* although microsatellite analysis of parental origin or extent was not performed (Teshima et al. 1996). FISH studies for the PWS-J sibs indicated an *SNRPN* deletion or a reduced signal but no deletion for *D15S11, D15S10,* or *GABRB3* (Ishikawa et al. 1996).

DNA Methylation within 15q11-q13

Probe 0.6NE from *SNRPN* exon 1 detects allele-specific DNA methylation (Sutcliffe et al. 1994; Buiting et al. 1995; Glenn et al. 1996). As shown by the analyses of patients with PWS and AS who have a 15q11-q13 deletion (fig. 3*a,* lanes 4 and 5; fig. 3*b,* lanes 2 and 3), the maternal, methylated allele is detected by a 4.3-kb band, and the paternal, unmethylated allele is detected by a 0.9-kb band, by *Xba*I/*Not*I digestion. The probands PWS-14 (fig. 3*a,* lane 1), PWS-B (fig. 3*b,* lane 4), PWS-

29, and PWS-G and the PWS-T, PWS-J, and PWS-P affected sibs (data not shown) lack the unmethylated 0.9-kb band and have only the methylated 4.3-kb band, which usually is of maternal origin only. A reduced band intensity for patient PWS-14 and for the PWS-T, PWS-J, and PWS-P affected sibs indicates that the *SNRPN* exon 1 region is deleted on the paternal allele. Consistent with this interpretation, the DNA of the fathers of the PWS-T and PWS-P affected sibs was deleted for this probe and had only the 0.9-kb paternal, unmethylated band (data not shown; DNA from the father in family PWS-J was not available). Therefore, these two fathers have inherited the deletion on the maternal chromosome, as have similar cases (Buiting et al. 1995).

Because PWS-14 was deleted for the paternal *SNRPN* exon 1 allele but the father had a normal methylation pattern, the mutation appears to be a de novo event. All other parents in these PWS families also had a normal *SNRPN* methylation pattern. Furthermore, in probands PWS-B, PWS-G, and PWS-29, the 4.3-kb band was of double intensity, compared with normal for a single allele (fig. 3*b;* data not shown). Therefore, these three patients have two copies of the *SNRPN* exon 1 region. Since the methylation pattern was uniparental maternal, the paternal chromosome has a maternal imprint.

The PWS-T (Teshima et al. 1996), PWS-J, and PWS-P affected sibs and patients PWS-14, PWS-B, PWS-29, and PWS-G were also tested for allelic methylation by use of PW71B (Dittrich et al. 1993), as well as *ZNF127* (DN34) for PWS-G (Driscoll et al. 1992). Each patient showed a PWS pattern with only a methylated band, of double intensity, except for the PWS-T sibs, who showed a reduced signal (data not shown). In each case, the parents showed normal biparental methylation patterns, except for the father of family PWS-T, who showed a maternal deletion of PW71 (Teshima et al. 1996). These results indicate that the deletions in neither PWS-14 nor the PWS-P sibs extend across the PW71B (*D15S63*) locus, although the deletion in the PWS-T sibs does, and confirm that DNA methylation throughout the imprinted part of 15q11-q13 is abnormal.

Microdeletions of the IC

Microdeletions of the IC were detected in four of the seven families with a PWS IM that were studied, including all three families with two affected sibs.

Patient PWS-14.—For proband PWS-14, *SNRPN* exons 1–3 cDNA as probe (Glenn et al. 1996) detected the telomeric breakpoint in *Eco*RI-digested DNA (fig. 4*a*). An abnormal 17.5-kb *Eco*RI band in PWS-14 was detected in addition to the normal 14.5-kb exon 1 and 11-kb exon 2 bands, both of which were reduced to 50% intensity, whereas the exon 3 band was of normal intensity and size (fig. 4*a*). In addition, an abnormal 26-kb band and a normal 23-kb *Bam*HI band, which were reduced to 50% intensity, were present in the proband's DNA (data not shown). With this probe, no abnormal band was detected in *Xba*I-digested DNA from PWS-14, although the band for exons 2–3 was of normal intensity, whereas the exon 1 band was of reduced intensity (fig. 4*c*). A proximal probe (L48.3I) from the IC region (Buiting et al. 1995) was intact and unrearranged in PWS-14 (data not shown). Probe 393SS, located 8.1-kb upstream of *SNRPN* exon 1 (fig. 4*c*), detected the centromeric breakpoint in *Xba*I-digested DNA (6.6-kb abnormal band) from proband PWS-14 (fig. 4*b*). The breakpoint fragments were not detectable in the father of PWS-14, by either Southern (fig. 4*a* and *b*) or PCR (data not shown) analysis. Combined with the methylation data (see above), these results indicate that proband PWS-14 has a paternally derived, de novo, 7.5-kb microdeletion of the IC, with breakpoints located ~3 kb proximal and ~4.5 kb distal to *SNRPN* exon 1 (fig. 4*c*).

Family PWS-J.—In DNA from the two affected sibs in family PWS-J (Ishikawa et al. 1996), the c4.1 probe (Buiting et al. 1997) and *SNRPN* exons 4–10 as probe (E4–E10) (Glenn et al. 1996) detected an extra band for both *Eco*RI and *Bam*HI, but the *PAR-5* probe (Sutcliffe et al. 1994) detected an extra band only in *Eco*RI-digested DNA and not in *Bam*HI-digested DNA, defining the telomeric breakpoint of a deletion (fig. 5). The intensity of bands for probes 48.3I, 1.3XE, Y48.5, and RN4RR (Buiting et al. 1995) was reduced to 50% of normal, but the band for the PW71B probe was intact. These results indicate that the distal breakpoint for the PWS-J microdeletion is in the region of *SNRPN* exons 4–10, and the proximal breakpoint is between PW71B and RN4RR (fig. 5).

Family PWS-P.—For family PWS-P, quantitative Southern blot analysis with probe kb17 showed that both sibs are deleted for the exon 1 region of *SNRPN*. Further analysis showed that the deletion included *IPW* and *PAR-1* distally (data not shown). Upstream of *SNRPN,* several probes (RN28*5*/*IC3,* a PCR probe for the AS SRO including exon IC3, and Y48.5) (Buiting et al. 1995; Dittrich et al. 1996) showed 50% intensity, compared with that of normal controls. Probe kb64, a genomic clone that maps ~1 kb distal to *D15S128* and spans exon IC2 of the *IC* transcript (Dittrich et al. 1996), detected an abnormal fragment in *Bam*HI-digested DNA and in *Kpn*I-digested DNA of the patients and the father (*Bam*HI, normal band 20 kb and abnormal band 12 kb; *Kpn*I, normal band ~25 kb and abnormal band 20 kb). These results indicate that the deletion in PWS-P spans >180 kb downstream of the *SNRPN* gene and that the proximal breakpoint lies between IC2 (kb64) and Y48.5 (fig. 5).

Family PWS-T.—In family PWS-T (Teshima et al. 1996), the two affected sibs and the father had reduced

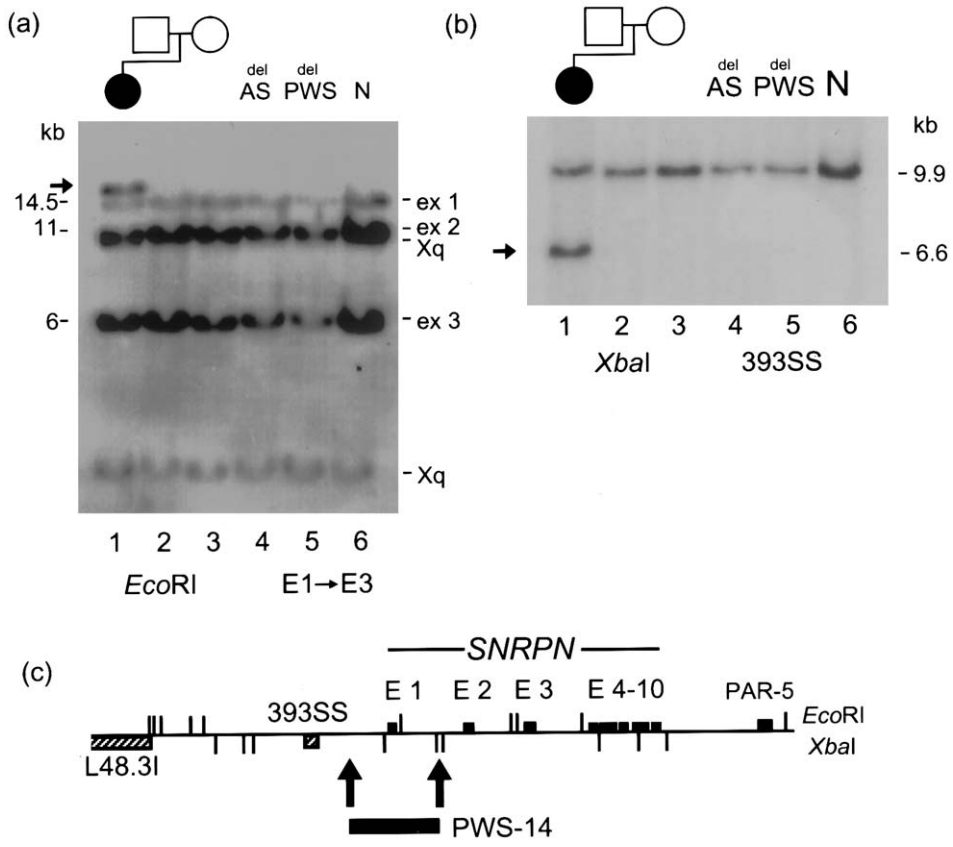

Figure 4 Microdeletion of 7.5 kb in patient PWS-14. *a*, Distal breakpoint. *SNRPN* exons 1–3 cDNA as probe detected the distal breakpoint in *Eco*RI-digested DNA. *b*, Proximal breakpoint. The 393SS probe detected the proximal breakpoint in *Xba*I-digested DNA. *c*, Map of the IC microdeletion. The deletion is shown as a blackened bar. Arrows indicate breakpoint fragments. E = exon.

intensity of the bands for probes from YAC end 11H11R (Mutirangura et al. 1993) to *PAR-1* (Sutcliffe et al. 1994) (figs. 1 and 5), although FISH showed that the deletion did not extend to *D15S11*, *D15S10*, or *GABRB3* (Teshima et al. 1996). In addition, PW71 was deleted by DNA-methylation analysis (Teshima et al. 1996), and *D15S13* (189-1) was shown to be intact by quantitative Southern blot analysis (data not shown). Therefore, the patients and the father have a relatively large deletion (figs. 1 and 5).

The extent of all four new microdeletions and of the three described elsewhere (Buiting et al. 1995) are summarized in figure 5. The PWS-O distal breakpoint is located ~600 bp distal to *SNRPN* exon 1; therefore, the PWS SRO has been reduced in this study, from ~25 kb (Buiting et al. 1995) to 4.3 kb spanning exon 1 of *SNRPN* (fig. 5).

Imprinted-Gene Expression within 15q11-q13

Analyses of imprinted-gene expression were performed for the patient with the smallest microdeletion, of only 7.5 kb (PWS-14; fig. 6*a*), and for one patient with a PWS nondeletion IM (PWS-B; fig. 6*b*). Several imprinted genes that are only paternally expressed were studied, including the *SNRPN*, *IPW*, and *ZNF127* genes, as well as the *PAR-1* and *PAR-5* expressed-sequence tags (ESTs) (fig. 1). These studies showed that none of these five genes or transcripts was expressed in the two patients with a PWS IM or in the controls with PWS deletions or UPD, whereas the control genes *GAPDH* and *FBN* were expressed (fig. 6*a* and *b*). Likewise, *SNRPN* was not expressed in blood or lymphoblast cells from PWS-G (data not shown). All genes and transcripts were expressed in cell lines from the mother of PWS-14, the father of PWS-B, a normal control, and a control with an AS deletion (fig. 6*a* and *b*).

DNase I Hypersensitivity of the SNRPN *Gene and the IC*

We demonstrated above that the PWS component of the IC spans 4.3 kb of exon 1 and the CpG island of the *SNRPN* gene (fig. 5). Since the IC acts in *cis*, we considered that the first exon of the *SNRPN* gene, which encodes the translation start of an independent protein

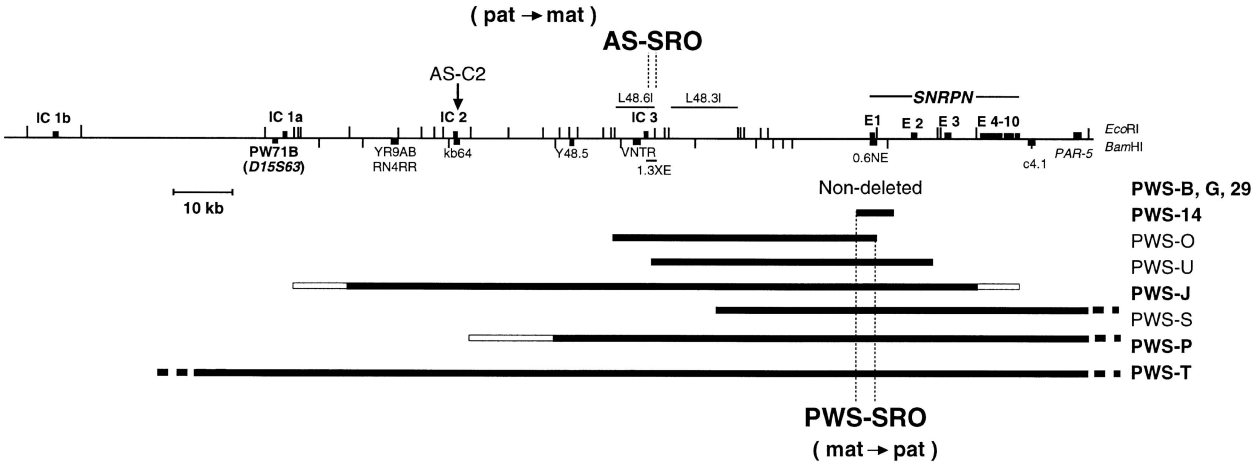

Figure 5 Summary map of PWS microdeletions, with SRO. The extent of each deletion is shown by a blackened bar, with new cases in boldface (PWS-B, PWS-G, and PWS-29 are nondeleted); records for families PWS-O, PWS-U, and PWS-S and for the AS SRO are published elsewhere (Buiting et al. 1995; Saitoh et al. 1996). A point mutation in family AS-C2 occurs in the splice donor site of an *IC* transcript (Dittrich et al. 1996). The microdeletions in families with PWS or AS affect the maternal-to-paternal ("mat→pat") or the paternal-to-maternal ("pat→mat") switch in the male or female germ line, respectively. Exons of the *IC* transcript and *SNRPN* (*upper blackened boxes*) and of the probes (*lower blackened boxes* or *horizontal lines* [for L48.6I and L48.3I]) are shown.

(T. A. Gray, S. Saitoh, and R. D. Nicholls, unpublished data), is an unlikely candidate for the IC element and that the *cis*-acting IC factor most likely corresponds to a DNA element within the 5′ part of the *SNRPN* gene. To assess possible promoter function, we performed a DNase I hypersensitive site (DHS) analysis of the *SNRPN* 5′ region, since sites exhibiting a high degree of DNase I hypersensitivity generally represent binding sites for DNA sequence–specific binding factors (Gross and Garrard 1988).

Two cell lines were used to assess the DNase I accessibility of the chromatin at the 5′ end of *SNRPN* and to discern any parent-of-origin specificity. These lines were derived from patients with PWS (PWS-109) or AS (AS-139) who have a 4-Mb deletion of paternal or maternal origin and in whom the remaining allele, therefore, has been maternally or paternally derived, respectively. Chromatin exposed to DNase I will be cleaved, and the position of the site of cleavage can be estimated by measurement of the length of the generated fragment relative to a landmark restriction site. DNA from DNase I–treated nuclei was cut with *Bam*HI, Southern blotted, and probed with a 2.2-kb *Eco*RI/*Bam*HI fragment in the first intron of *SNRPN* (Glenn et al. 1996). A major DHS ~3.1 kb from the *Bam*HI intronic site was identified only in the AS-139 cell line (fig. 7). This maps the exposed chromatin to a region spanning ~100–300 bp upstream of the *SNRPN* transcription start site. The maternally inherited *SNRPN* 5′ region in PWS-109 was devoid of DHS, indicating a closed, inaccessible chromatin conformation. The major DHS was verified in a second experiment using *Eco*RI-digested DNA and a probe including exon 1 and 500 bp of *SNRPN* intron 1 (data not shown). A second paternal-specific DHS was identified within intron 1, but it appeared as a dispersed signal (fig. 7), since it lies within the region spanned by the probe, and is best detected with other restriction digests and probes (C. C. Glenn, R. D. Nicholls, and T. P. Yang, unpublished data). These findings unequivocally demonstrate that the *SNRPN* chromatin is in an open configuration exclusively on the paternally derived allele. The paternal-specific DHS identified here and results from standard transfection assays (Huq et al. 1997) are consistent with this region corresponding to a functional promoter of the *SNRPN* gene.

Evolutionary Footprints in the IC

To further define genetic elements within the 4.3-kb PWS SRO, we performed a phylogenetic analysis of human *SNRPN* and mouse *Snrpn* exon 1 and the 5′ putative promoter regions. A phylogenetic footprint is defined by at least six contiguous bases of absolute conservation in orthologous genes of evolutionarily distant species (Gumucio et al. 1992). Our sequence comparison identified six phylogenetic footprints of 7–10 nucleotides in the human *SNRPN* and mouse *Snrpn* promoter regions, corresponding to the 4.3-kb human PWS SRO in the IC (fig. 8). There was also a 10-bp block of T_4A_6, but previous studies of the γ-globin gene promoter have shown that no specific *trans*-acting factors bind simple-sequence tracts of this nature (Gumucio et al.

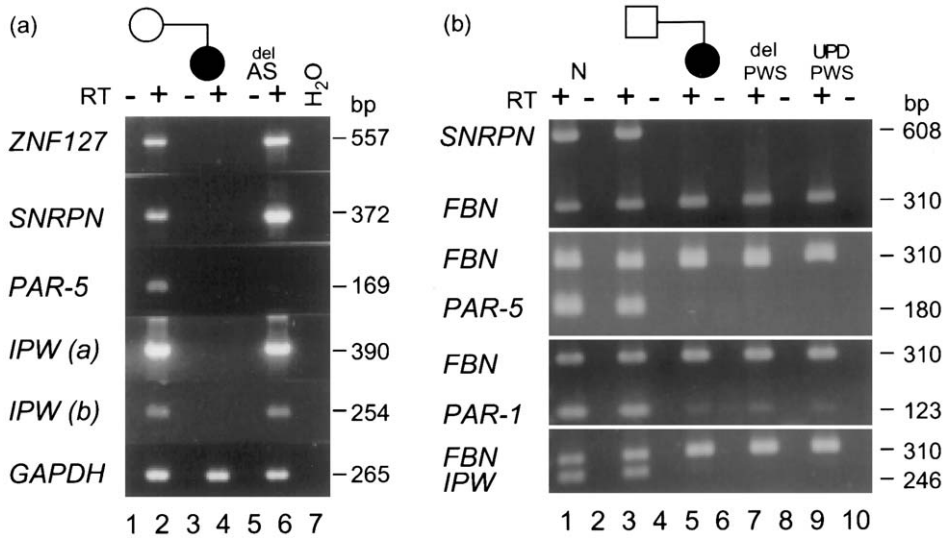

Figure 6 Silencing of paternal-only gene expression in PWS IMs. Paternally expressed genes and transcripts across a 1.0–1.5-Mb domain (*ZNF127, SNRPN, PAR-5, IPW,* and *PAR-1*) are not expressed in (*a*) PWS-14, a patient with PWS who has a 7.5-kb microdeletion, or in (*b*) PWS-B, a patient with PWS who does not have an IC mutation. RT-PCR expression analysis of lymphoblast cell lines of the patients and controls are shown. RT-PCR analysis of the control *GAPDH* (*a*) and *FBN* (*b*) genes shows expression in all the RT+ samples.

1992). Since these are the only conserved nucleotides in the 4.3-kb PWS-SRO region, these sites represent candidate binding elements for the factor(s) involved in somatic expression and/or the maternal-to-paternal imprint switch in the male germ line.

Patients with a PWS Nondeletion IM

Three patients with an IM (PWS-B, PWS-G, and PWS-29) did not show a microdeletion in a 150-kb IC region flanking the promoter of the *SNRPN* gene (fig. 5), on extensive analysis. For the family of PWS-B, the proband, two unaffected sibs, the parents, and the paternal grandparents were typed by DNA microsatellite markers within 15q11-q14 (fig. 9). The younger brother, who is unaffected, has the same nonrecombinant paternal haplotype as the proband (fig. 9). This result indicates either a de novo mutation or the lack of a chromosome 15q11-q13 mutation. It should be noted that the paternal haplotype for PWS-B was inherited from the paternal grandmother (fig. 9).

Mutation analysis was performed for the three patients with nondeletions, by use of 11 PCR primer sets across the 4.3-kb PWS SRO. To be a mutation-causing PWS, a sequence change must either occur de novo or be paternally inherited. No mutations were found in these patients, although three polymorphisms were detected (table 1). For example, SSCP analysis of DNA from the family of PWS-B identified a paternally inherited sequence change, by use of *Ava*II/*Pvu*II-digested PCR product from primers RN495 and RN496, and DNA sequence analysis identified a single-nucleotide change (G→C) at position −64 of the *SNRPN* promoter region (table 1 and fig. 8). This sequence change was classified as a polymorphism, since a normal sib has the same paternally inherited and nonrecombinant haplotype as PWS-B (fig. 9). A maternally inherited—and, hence, polymorphic—sequence change was identified in the 4.3-kb PWS SRO in patient PWS-29 DNA as a single-base change (G→T) at position +94 in *SNRPN* intron 1 (table 1). An A→G change at position +64 in *SNRPN* intron 1 in PWS-G DNA (table 1), which had not been maternally inherited (the father and unaffected sibs were not available for study), was classified as a polymorphism, because it is not evolutionarily conserved in mouse DNA, as was also found for the other two polymorphisms (fig. 8).

Discussion

In this study, we performed molecular genetic studies of seven families, involving 10 patients with PWS and a mutation in the imprinting process, to better define the sequence elements that play a role in the maternal-to-paternal imprint switch in the male germ line. We identified two subclasses of patients with a PWS IM, deletion and nondeletion. Previously, microdeletions had been characterized completely in only three cases of PWS (Buiting et al. 1995), defining a 25-kb PWS SRO. Two additional cases were identified by FISH and molecular analyses, but deletion extents were not determined (Sut-

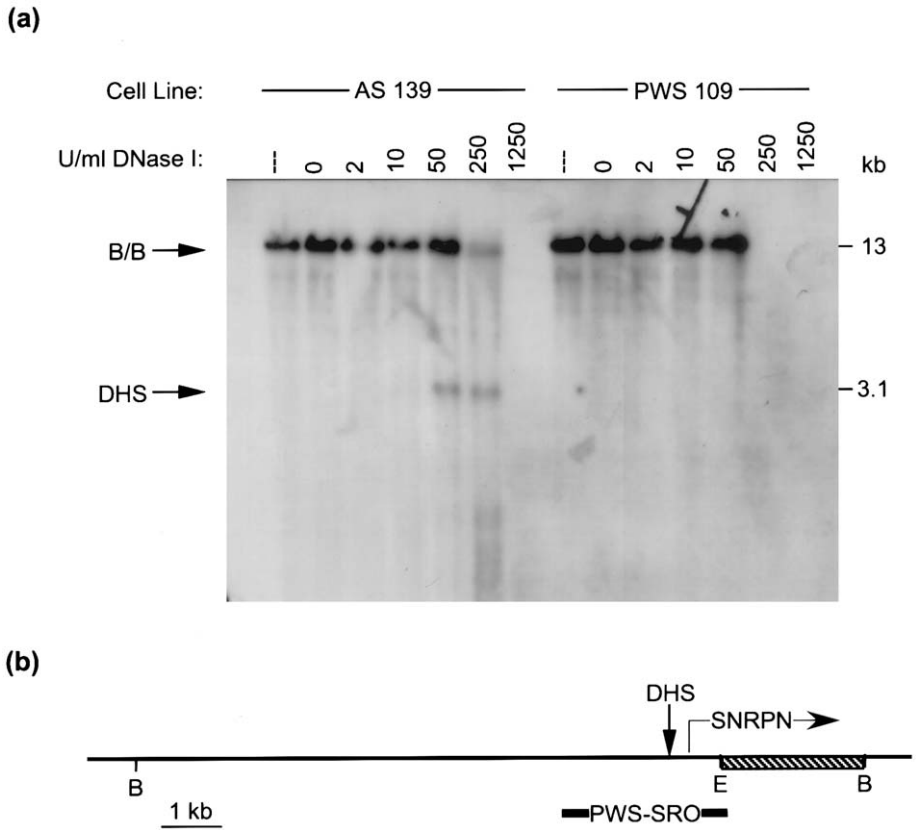

Figure 7 A paternal-specific DHS in the PWS SRO. *a,* Nuclei from lymphoblastoid cell lines with a maternally derived (AS-139) or paternally derived (PWS-109) 15q11-q13 deletion were treated with varying concentrations of DNase I. The resulting genomic DNAs were cut with *Bam*HI ("B"), Southern blotted, and probed with an *Eco*RI ("E")/*Bam*HI fragment from intron 1 of *SNRPN*. In AS-139, a major subfragment was generated in nuclei exposed to 50 and 250 U DNase I/ml, indicating the presence of a DHS ~3.1 kb upstream of the distal *Bam*HI site. No specific DHSs were seen in PWS-109. *b,* Schematic diagram depicting the relative positions of the PWS SRO (*blackened bar*), the probe fragment (*hatched bar*), the *SNRPN* exon 1 transcription start (*thin arrow*), and the major DHS (*thick arrow*).

cliffe et al. 1994; Butler et al. 1996). Our new analyses have identified, in one patient with PWS, a very small deletion of only 7.5 kb that narrows the critical region for the IC element involved in the male germ-line switch to only 4.3 kb (the PWS SRO). This region represents the promoter for the *SNRPN* gene, implying that this one gene plays a critical role in the germ-line *cis* regulation of all imprinted genes over a 1.5–2-Mb domain within 15q11-q13.

Epigenetic Effects of PWS IMs

Microdeletion.—Four of these IM cases (in families PWS-T, PWS-P, and PWS-J and patient PWS-14) demonstrated a microdeletion. For the first two cases, the PWS patients inherited an IM from their father, who had inherited the mutation from his mother. These familial inheritance patterns are consistent with our previous model, in which a mutation in the PWS-SRO component of the IC in the germ line of an ancestral female would lead to fixation of the maternal imprint in that chromosome (Buiting et al. 1995). In PWS-14, the IC mutation was de novo on the paternally inherited chromosome, but the grandparental origin of this mutation and of that in PWS-J should be grandmaternal, since a maternal epigenotype was fixed. Since the mutated chromosome exhibits a normal maternal imprint, it can transmit silently through females. However, it is blocked from switching to a paternal imprint in the germ line of a male, and such males have a 50% risk of having a child who inherits only maternally imprinted chromosomes 15, leading to PWS.

The abnormal imprint, or epigenotype, in such cases of PWS includes all normal paternally expressed genes in the 1.5–2-Mb imprinted domain, from *ZNF127* through *IPW* (fig. 1), based on the loss of paternal-only gene expression in PWS microdeletions ranging in size from 7.5 kb (PWS-14) to larger PWS deletion IMs (Saitoh et al. 1996). DNA-methylation changes in cases of deletions and nondeletions are also consistent with this conclusion. Since all paternal-only gene expression in

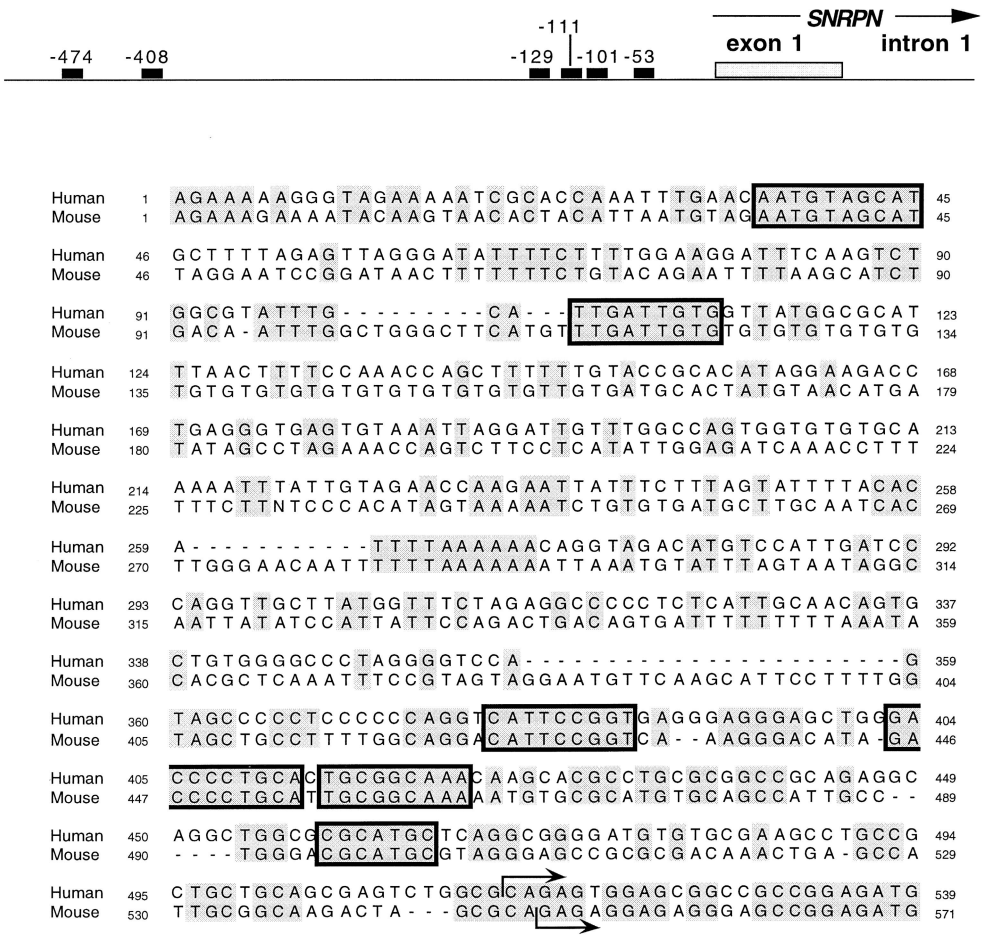

Figure 8 Phylogenetic footprints within the PWS SRO. The transcription start site for human *SNRPN* and mouse *Snrpn* genes are indicated by arrows, and mouse and human promoter sequences are aligned 5′ of these start sites (determined by use of the Clustal algorithm, with modifications to maximize parsimony). Six putative evolutionarily conserved footprints, which are likely to represent TF binding sites, are identified (*blackened boxes* [*top*] and *boxed sequences* [*bottom*]). One or more of these TF binding sites are also candidates for the IC imprint-switch element.

15q11-q13 is lost, these results clearly explain why patients with a PWS IM have the classic phenotype for the disorder (Saitoh et al. 1997; this study). These conclusions have been confirmed by an equivalent deletion in a mouse model of a PWS IM, with loss of paternal gene expression over several megabases (Yang et al. 1998), as in humans.

Nondeletion.—Several sporadic cases (PWS-B, PWS-29, and PWS-G) had neither a detectable deletion of the IC region nor a mutation in the PWS SRO. Similar findings were recently found for approximately one-half the patients with a PWS or AS IM (Bürger et al. 1997; Buiting et al. 1998). As with deletion cases, patients with PWS nondeletions have not switched the imprint throughout the imprinted 2-Mb domain in 15q11-q13, as was demonstrated here for PWS-B, a patient with a nondeletion, by gene-expression and DNA-methylation studies. Therefore, we consider a prefertilization imprint-switch failure to be most likely, since the IC has not been shown to have a function in somatic cells, although a postzygotic event cannot be excluded (Bürger et al. 1997; Buiting et al. 1998). In one family (that of PWS-B), a normal sib shares the same paternal haplotype as the patient, which is similar to that in two other families (Buiting et al. 1998) and indicates either a de novo mutation in the father's germ line or the lack of a mutation in 15q11-q13. Whereas a mutation in a gene encoding a *trans*-acting factor could occur, this might be expected to affect all germ cells and, hence, all offspring, which is inconsistent with our observations, unless each such mutation was de novo (the effect also would have to be specific for 15q11-q13). Since all patients with a PWS or AS nondeletion IM are sporadic (Bürger et al. 1997; Buiting et al. 1998; this report), we

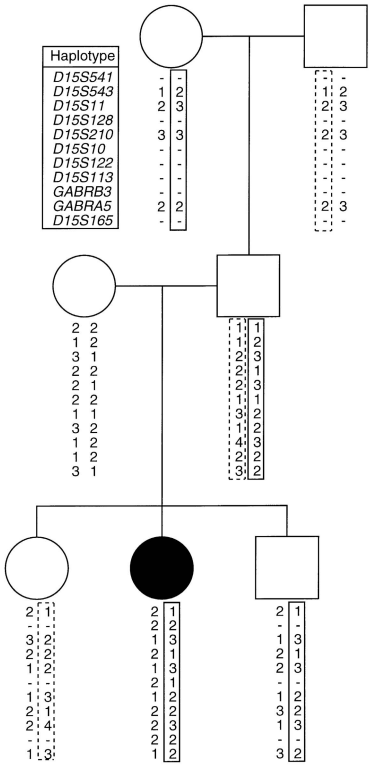

Figure 9 Microsatellite haplotype analysis of the family of PWS-B. Haplotypes were generated by use of the marker loci shown. One normal sib has the same paternal haplotype (*boxed*) as patient PWS-B, and this haplotype was derived from the paternal grandmother.

favor the former hypothesis—namely, that there is no mutation, with a developmental or stochastic failure to switch the imprint in the male or female germ line, respectively. Developmental delay can lead to defects in sex determination (Mittwoch 1992), and germ-cell delay can occur as a consequence of skewed X inactivation (Lau et al. 1997). Whereas this hypothesis may be difficult to prove, since neither the parent nor the child actually has a mutation, animal models may provide support for these ideas.

SNRPN *Promoter Contains the IC Critical Elements for Imprint Switching in Males*

All seven PWS microdeletions fully characterized here and previously (Buiting et al. 1995) overlap and narrow the PWS SRO of the IC, to 4.3 kb (fig. 5). There are two high-copy *Alu* repeat sequences at the proximal (centromeric) end of the PWS SRO, reducing to only 3.7 kb the amount of unique sequence in the PWS SRO. Indeed, the distance from the distal point of the PWS SRO (just inside *SNRPN* intron 1) to the first 5′ *Alu* is only 2.03 kb; therefore, the latter segment is most likely to contain the IC critical element(s).

The critical PWS-SRO region corresponds to the *SNRPN* transcription promoter, on the basis of four lines of evidence. First, this region contains the *SNRPN* transcription start site in a 5′ differentially methylated CpG island (Sutcliffe et al. 1994; Glenn et al. 1996). Second, our DNase I hypersensitivity studies indicated paternal-specific open chromatin in this region, which is typical of binding sites for DNA sequence–specific transcription factors (TFs) (Gross and Garrard 1988). This correlates with the paternal-only transcription of *SNRPN*. Preliminary studies of the remaining 120 kb of the IC failed to identify similar sites, at least in lymphoblastoid cell lines. Third, our phylogenetic footprint analyses of the PWS SRO from human *SNRPN* and the equivalent region of mouse *Snrpn* identified six conserved elements, which are likely to be recognition sites for positive or negative TFs involved in somatic cell gene expression of *SNRPN*. Since *SNRPN* expression is upregulated in the postnatal brain (Schmauss et al. 1992; Grimaldi et al. 1993), there may be both silencing elements in nonexpressing tissues and activating elements in expressing tissues. Furthermore, one or more of these conserved-sequence elements may also be involved in the imprint-switch process. Finally, transfection studies of fragments from the proximal part of the *SNRPN* 5′ region (−207 to +53 bp) have established this region as having promoter function (Huq et al. 1997). However, the latter studies (Huq et al. 1997) likely define only a "minimal" promoter, since the constructs used lack two phylogenetically conserved elements (−408 and −474 bp, relative to *SNRPN;* fig. 8) upstream of the transcription start site.

Therefore, the *SNRPN* promoter appears to contain the critical IC elements for the imprint switch during spermatogenesis. The imprint switch may involve a two-step process of initial erasure of all previous imprints, followed by an active resetting, or a single switch wherein a parental imprint is imposed specific for the sex of that individual. The IC represents the first step in imprint switching, since all known imprinted genes within 15q11-q13 and mouse models do not switch their gametic and somatic methylation or expression patterns unless the IC signal is received (Glenn et al. 1996; Saitoh

Table 1

PWS-SRO Polymorphisms in Patients with a PWS Nondeletion IM

	Nucleotide			
Family	Patient	Mother	Father	Position[a]
PWS-B	G→C	G	G→C	−64
PWS-G	A→G	A	…	+64
PWS-29	G→T	G→T	G	+94

Note.—Changes in sequence from the cloned *SNRPN* locus (Glenn et al. 1996) are indicated by use of an arrow.

[a] Relative to the *SNRPN* transcription-initiation site at +1.

et al. 1996; Nicholls et al. 1998; Yang et al. 1998; Jong et al., in press; this study). Therefore, the IC signal must be bidirectionally transmitted to each imprinted gene within 15q11-q13 (fig. 1), which then switches its own imprint. At present, we can only recognize mutations in PWS and AS in the first step of the imprint-switch process. It will be important in future work to identify mutations in each step of the imprint-switch process during gametogenesis, by means of patient analyses or, more likely, by involving targeted mutagenesis and transgenesis in the mouse.

Models for Imprint Switching during Spermatogenesis

On the basis of our findings, non–mutually exclusive models for imprint switching during gametogenesis can be proposed. In the first model, imprint switching in female and male germ cells may involve a form of promoter or enhancer competition. Expression in the oocyte of a novel, noncoding, paternal-only *IC* transcript may preclude transcription from the *SNRPN* promoter, allowing silencing to be established at the latter site (Dittrich et al. 1996; Buiting et al. 1998; Lyko et al. 1998; Nicholls et al. 1998). This may be accomplished because the *IC* transcript splices to *SNRPN* exon 2 (Dittrich et al. 1996), and, consistent with this role, it was mutated in all patients with an AS IM who were examined (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996; C. Farber, B. Dittrich, K. Buiting, and B. Horsthemke, unpublished data). Silencing then may spread, from the 5′ end of the *SNRPN* gene, throughout 15q11-q13 by an unknown mechanism, although this could involve chromatin factors involved in silencing in other organisms (Paro and Harte 1996), which would establish the maternal imprint characteristic of the oocyte and would complete the switch from a previous paternal imprint. In contrast, an active *SNRPN* promoter would prevent expression of the *IC* transcript during spermatogenesis. Therefore, the *IC* transcript would not be required for paternal imprinting in spermatogenesis, and *IC* mutations would have no effect upon paternal transmission, as was observed in families with an AS IM.

The above model assumes a role for a silencing mechanism in the imprint-switch process. By use of *Drosophila* as a model system in vivo, fragments from the 5′ regulatory region of *SNRPN* have been demonstrated to function as a silencer in transgenic flies and have been interpreted to indicate that the imprinting mechanism may involve evolutionarily conserved components (Lyko et al. 1998). Whereas the observed silencing in somatic cells of transgenic flies was bidirectional and occurred over distances of only several kilobases, it was parent-of-origin independent (Lyko et al. 1998). Furthermore, *SNRPN* expression is predominantly in neurons (Schmauss et al. 1992; Grimaldi et al. 1993; Glenn et al. 1996), and it is well known that many neuronal-specific genes have silencer elements within the gene promoter that silence gene expression in nonneural tissues (for a review, see Schoenherr and Anderson 1995). Therefore, it is quite likely that the *SNRPN* gene has promoter silencer elements that extinguish high levels of expression in nonneural tissues, and such a silencer element that is active in somatic cells could provide an alternative explanation for the *Drosophila* results obtained by Lyko et al. (1998).

An enhancer-competition model was first described for the β-globin locus control region (LCR), in which differential transcription of the five β-like globin genes during erythroid development is controlled by promoter competition of each of these genes, for interaction with the LCR enhancer components (Choi and Engel 1988; Crossley and Orkin 1993; Dillon et al. 1997). An enhancer-competition model may also explain the differential imprinting of the maternally expressed *H19* and paternally expressed *Igf2* genes in somatic cells (Bartolomei et al. 1993; Leighton et al. 1995). A similar enhancer-competition model, involving a shared neuronal-specific enhancer, was recently proposed as a basis for IMs in PWS and AS (Tilghman et al. 1998). Nevertheless, this hypothesis is unlikely, since (1) the PWS and AS genes are unlikely to be expressed in the same cell type, as required by this hypothesis; (2) the AS SRO is not a maternal epigenetic mark (Schumacher et al. 1998); and (3) the imprint switch occurs in germ cells, not postfertilization (Dittrich et al. 1996; Saitoh et al. 1996; Nicholls et al. 1998; this study). Antisense gene regulation is an alternative form of transcription competition that could play a role in imprint switching, since it is used in transcription control by several imprinted genes (Wutz et al. 1997; Rougeulle et al. 1998; Jong et al., in press).

In an alternative model, during spermatogenesis the binding of a TF(s) to one or more of the conserved binding sites on the methylated, maternally derived *SNRPN* allele may allow direct activation of transcription of the *SNRPN* gene, which represents the paternal imprint. TFs that can bind unmethylated and methylated DNA are known—for example, Sp1 (Harrington et al. 1988)—although none of the sites on the *SNPRN* promoter appear to represent Sp1-recognition motifs. Alternatively, the TF may serve to prevent silencing, by displacement of the silencing proteins. In either model, concomitant changes in DNA methylation must occur in the *SNRPN* gene, to establish the gametic methylation pattern (paternal loss of methylation at the 5′ end and methylation at the 3′ end of the gene) (Glenn et al. 1996; Shemer et al. 1997), by an unknown mechanism. These events are then followed by bidirectional spreading of the IC signal to each imprinted gene in 15q11-q13.

We currently are using oligonucleotides containing

each of the phylogenetically conserved motifs in the *SNRPN* promoter to identify TFs, from various cell types, that may bind to these sites. Identification of patients with a PWS IM who have smaller deletions or targeted mutagenesis in mice would also further define which of these putative TF binding sites are involved in the maternal-to-paternal imprint switch during spermatogenesis. This IC-switch element is mutated in patients with PWS, but it has no role during oogenesis, since, in families with PWS, mutations of this element transmit silently through female ancestors (Buiting et al. 1995; this study). Silent maternal transmission of *SNRPN* mutations is also consistent with the transcription inactivity of this gene from the maternal chromosome. In addition to a *cis* role for the *SNRPN* promoter during spermatogenesis, it is possible that the *SNRPN* transcription unit may play a role in the clinical features of PWS, since AS IM microdeletions have not spanned closer than 20 kb from the 5′ end of *SNRPN* (Buiting et al. 1995; Ohta et al. 1999). These AS mutations transmit silently through male ancestors, but, if a deletion extended into promoter or enhancer elements of *SNRPN,* such individuals may show PWS features, including hypogonadism and sterility.

In conclusion, our studies of PWS and AS IMs have identified a novel mechanism of human disease, in which the mutation blocks the switch of the grandparental imprint in the parental germ line and which is the consequence of inheritance of an incorrect parental imprint that leads to the clinical manifestations in offspring. Nevertheless, these PWS and AS IMs identify only the first step of imprint switching in the germ line. To fully understand the molecular basis of imprint switching in the germ line, it will be necessary to identify mutations, in patients or mouse models, that block successive stages of imprint switching at the IC, bidirectional spreading of the IC signal, recognition by each imprinted "target" gene, and switching of the gametic methylation patterns by each target gene. A detailed understanding of the molecular basis and consequences of mutations in the imprinting process, in PWS and AS, may also lead to insight into similar IMs or "loss of imprinting" in Beckwith-Wiedemann syndrome (Reik and Maher 1997) and in many childhood and adult cancers (Feinberg et al. 1994).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/Web/Genbank/ (for *SNRPN* [U41384], *GAPDH* [J04038], and *FBN* [L13923])
Genome Database, http://gdbwww.gdb.org/ (for the primers used for the polymorphic marker studies)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PWS [MIM 176270] and AS [MIM 105830])

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