

ORIGINAL INVESTIGATION

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Relaxation of imprinting in Prader-Willi syndrome

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Abstract We describe two Prader-Willi syndrome (PWS) patients who exhibit maternal uniparental disomy (UPD) of chromosome 15 and unusual patterns of gene expression and DNA replication. Both were diagnosed during infancy as having PWS; however, their growth and development were atypical compared with others with this condition. Weight was below normal in the first patient, and height and development were within normal limits in the second individual. Hyperphagia and polyphagia were not evident in either patient. Genotypes at multiple genomic loci, allele-specific methylation, gene expression, and DNA replication were analyzed at D15S9 [*ZNF127*], D15S63 [*PW71*], *SNRPN*, *PAR5*, *IPW*, and D15S10 in these patients. The maternal imprint (based on the absence of gene expression, synchronous replication, and methylation of both alleles) was retained at *SNRPN* in these patients, as is the case in others with UPD. By contrast, cells from the first individual expressed *PAR5* and *ZNF127*, whereas the second

expressed a single *IPW* allele. Asynchronous DNA replication was observed in both patients at all loci, except *SNRPN*. These findings show that a subset of imprinted genes can be transcribed in some PWS patients with maternal UPD and that asynchronous DNA replication is coordinated with this pattern of gene expression. Relaxed imprinting in these patients is consistent with their milder phenotype.

Introduction

The non-expression or absence of paternally derived genes in 15q11-q13 results in Prader-Willi syndrome (PWS; Nicholls 1993; Cassidy 1995). Several imprinted loci in this genetic interval, including *ZNF127* (Driscoll et al. 1992; R. Nicholls, personal communication), *NECDIN* (McDonald and Wevrick 1997), *SNRPN* (Glenn et al. 1993a; Sutcliffe et al. 1994), *PAR5* (Sutcliffe et al. 1994), *IPW* (Wevrick et al. 1994), and *PARI* (Sutcliffe et al. 1994), are not expressed in these patients. By contrast, some patients with Angelman syndrome (AS) do not express the maternal allele of *UBE3A* in the brain, although this gene is constitutively expressed in other tissues (Rougeulle et al. 1997). The 5' end of *SNRPN* appears to contain an imprinting center (IC) that is involved in imprint resetting in the germline (Sutcliffe et al. 1994; Buiting et al. 1995; Saitoh et al. 1996). Resetting of the imprint produces germline and somatic allele-specific differences in gene expression, genomic methylation, and replication timing that are correlated with the parental origin of the allele, i.e., the epigenotype. PWS patients have a maternal epigenotype resulting from the absence of paternally derived 15q11-q13 alleles (Nicholls et al. 1989; Knoll et al. 1989) or IC mutations that result in paternal chromosomes with a grandmaternal epigenotype (Buiting et al. 1995). For example, in patients with PWS and maternal uniparental disomy (UPD), both alleles at *SNRPN* are methylated (Glenn et al. 1993b), and DNA replication of both chromosomes is synchronous (Knoll et al. 1994; White et al. 1996), whereas differential methylation and asynchronous replication are evident in normal individuals. Although

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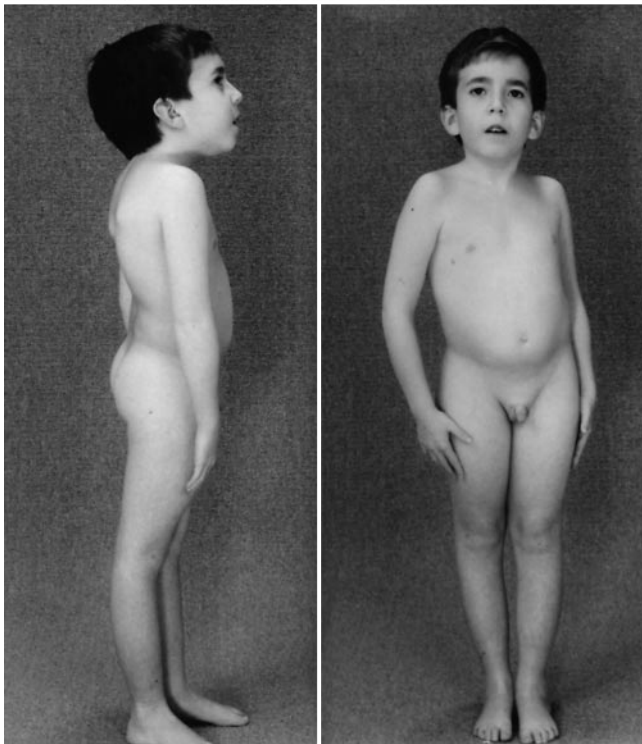
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A



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Fig. 1A, B Clinical presentation. **A** PWS12 at 2 3/4 years and at 8 1/3 years of age. **B** PWS96-019 at 6 years of age

ternal UPD and who were initially diagnosed as having PWS but failed to develop all of the common clinical features. These patients express imprinted genes in 15q11-q13, that are ordinarily transcribed in normal individuals.

Subjects and methods

Subjects

PWS12 was the 2850-g (6 lb 5 oz) product of a 41-week gestation to a 26-year-old G1P0 Caucasian female and a 29-year-old Caucasian male (Fig. 1A). Poor fetal movements were noted during pregnancy. Hypotonia, bilateral epicanthus, single transverse palmar crease, small hands and feet with dorsal pedal edema, and a small penis with bilateral cryptorchidism were noted at birth. High-resolution chromosome analysis showed a 46, XY karyotype. At 6 months of age, developmental age was 4 months, head circumference was at the 75th percentile, and length and weight were at the 25th percentile. The clinical diagnosis of PWS was made at 9 months and confirmed 2 years later by demonstrating UPD of chromosome 15 (Mascari et al. 1992; see below). At 2 3/4 years, his length and weight were less than the 5th percentile, and head circumference was at the 8th percentile. Height and weight remained less than two standard deviations below the mean at 5 1/3, 8 1/3 and 12 1/6 years. He had low set, posteriorly rotated ears, epicanthal folds, mild strabismus, mild edema, small hands and feet, and small genitalia (Tanner stage I). His bone age was normal at 8 1/2 years; however, he remained hypotonic and developed thoracic scoliosis (which was exacerbated by treatment with human growth hormone for 6 months). He has not developed the hyperphagia, polyphagia, or obesity typically seen in patients with this condition, and dietary intervention has not been necessary. Cognitive development at age 12 1/6 years was considered normal based on reading skills. He did not meet the minimum diagnostic criteria for PWS (a score of 6 points, with 8 being required; Holm et al. 1993), and the diagnosis was reconsidered. The molecular genetic findings were confirmed and extended by using techniques that were not available in 1990 when UPD was originally identified.

PWS96-019 (Fig. 1B), the second patient, was born after an uncomplicated pregnancy with normal fetal movement. He was hypotonic, required nasogastric feeding, was hypoglycemic, and presented with bilateral clubfeet, dislocated hips, and non-palpable testes. Chromosome analysis showed a 46, XY karyotype. Hypotonia improved

DNA methylation patterns and the level of expression of imprinted genes in 15q11-q13 may vary in degree among different tissues (Mowrey-Rushton et al. 1996a; Glenn et al. 1996), the germline epigenotype within a single individual is consistent.

The way in which the loss of expression of imprinted genes contributes to the PWS phenotype is unknown. Major clinical criteria for PWS during infancy include neonatal hypotonia, poor feeding, and dysmorphic features, including upslanting palpebral fissures, cryptorchidism, and small hands and feet (Holm et al. 1993). In early childhood, reduced head circumference, short stature, severe hyperphagia, and developmental delay are usually noted. This report describes relaxed imprinting in two patients who exhibited ma-

by 2 years of age, and there was a marked weight gain without a change in growth velocity. At 3 1/3 years, down-slanting palpebral fissures, small penis, small hands and feet, and short stature were noted. Hyperphagia and temperament instability were absent, and mental impairment was not detected. PWS was suspected, and the molecular genetic studies were consistent with this diagnosis. Weight was greater than the 95th percentile with a normal caloric intake at 3 1/3 years; however, at 5 1/2 years, his weight and height were at the 50th and 10th percentiles, respectively. PWS96-019 has not exhibited hyperphagia or other behavior patterns often characteristic of PWS.

Clinical specimens

Genomic DNA was analyzed from leukocytes obtained from peripheral blood specimens of PWS12 in 1990 and 1995. A skin biopsy was also obtained from this patient in 1995 and used for cell culture and DNA analysis. A peripheral blood specimen was available for PWS96-019. RNA was analyzed from leukocytes of each patient.

Polymorphic markers

Chromosome 15 inheritance was determined by comparing parental and proband leukocyte and fibroblast genotypes at the following polymorphic genetic loci: D15S541, D15S18, D15S9, D15S11, D15S128, D15S13, *IPW*, D15S97, D15S122, D15S10, *GABRB3*, D15S165, D15S12, D15S24, *ACTC*, D15S118, *FIB*, *CYP19*, D15S103, D15S117, D15S125, D15S108, D15S110, D15S95, D15S111, D15S100, D15S107, D15S87, D15S86, and D15S642. Conditions for amplification or restriction endonuclease digestion of genomic DNA were obtained from the Genome Database (Johns Hopkins University) and from the literature (Mutirangura et al. 1993). Amplified genetic polymorphisms were initially analyzed according to previously established procedures (Weber and May 1989; Woodage et al. 1994). The possibility of additional alleles (>2) because of mosaicism for UPD and biparental disomy (BPD) in PWS12 was assayed by Southern hybridization with probes from D15S18, D15S9, D15S11, D15S13, D15S10, D15S12, D15S24, and D15S86 and by polymerase chain reaction (PCR) amplification at D15S95 in PWS12 and D15S642 in PWS96-019.

Methylation studies

DNA methylation testing is routinely used to confirm the clinical diagnosis of PWS (Gillissen-Kaesbach et al. 1995). Methylation studies were performed with leukocyte DNA by using probes from *SNRPN* (Glenn et al. 1993b) and PW71B (D15S63; Dittrich et al. 1993). Genomic DNA was either digested with the restriction enzymes *XbaI* and *NotI* and hybridized with a DNA probe from the promoter region of *SNRPN* (Glenn et al. 1993b) or with *HpaII* and *HindIII* and hybridized with PW71B (Dittrich et al. 1993).

Gene expression studies

Total RNA was extracted from blood leukocytes with RNAzolB three times to reduce the likelihood of DNA contamination (Cinna/Biotech Laboratories, Tex.). Gene expression was assayed by using 5 µg total RNA isolated from blood leukocytes by oligo dT-primed reverse transcription (RT) with Superscript II (Life Sciences, N.Y.). One tenth of the synthesized cDNA was analyzed by using previously described primers and amplification conditions (Saitoh et al. 1996). Transcription of *SNRPN*, *PAR5*, *IPW*, and *ZNF127* and the constitutively expressed gene *GADPH* (a positive control) was evaluated by RT-PCR of mRNA from patients and in corresponding negative control (PWS deletion and maternal UPD), positive control (AS deletion and paternal UPD), and parental samples. The oligonucleotides used for PCR of the *SNRPN* gene amplified exons 4-10 (Glenn et al. 1996), and those for *ZNF127* amplified the 3' untranslated region of the mRNA

(DD29 and RN153; R. Nicholls, personal communication). Primer sequences and conditions have been described for the amplification of *IPW* (60 C and 60D; Wevrick et al. 1994) and *PAR5* (Sutcliffe et al. 1994). The *HpaI* polymorphism in the *IPW* gene was detected both by restriction digestion and by direct DNA sequencing of the amplified PCR products produced from cDNA and genomic DNA. Restriction digestion products were separated by gel electrophoresis with Nusieve agarose (FMC BioProducts, Rockland, Me.). Primers for amplification of *GADPH* have been described (Sutcliffe et al. 1994).

Allele-specific replication studies

Peripheral blood leukocytes were cultured and fixed by standard cytogenetic methods and prepared for fluorescence in situ hybridization (FISH; White et al. 1996). A fixed cell pellet was available from the initial sample taken from PWS12 (Mascari et al. 1992), and a fresh cell pellet was prepared from PWS96-019. Phage clones 34-10 (D15S9), 3-21-12 (D15S10), and 16β3 (*GABRB3*) were labeled with biotin-11-dUTP or digoxigenin-11-dUTP by nick translation and used for FISH (Knoll et al. 1994). D15S11 and *SNRPN* were digoxigenin-labeled cosmid probes (Oncor, Md.). Hybridization and probe detection were performed on interphase nuclei as previously described (Knoll and Lichter 1994). The proportions of unreplicated (G1 phase), asynchronously replicated, (G1/G2 phase), and completely replicated (G2 phase) homologs were determined at each locus. G1 cells show two single hybridization signals, cells in G1/G2 exhibit one single and one double signal, and those in G2 show two distinct pairs of signals. A minimum of 100 interphase nuclei were scored for each probe by two or more individuals (White et al. 1996). Slides were coded before being analyzed and were randomized with other FISH studies performed in the laboratory so that the identity of neither patient nor probe was evident to the scorers.

Results

Genetic polymorphism studies

Molecular genetic studies of PWS12 at 2 3/4 years of age were originally reported by Mascari et al. (1992) and showed maternal uniparental heterodisomy at D15S86, and heterozygosity consistent with maternal heterodisomy at D15S11 and D15S10. Since the longitudinal development of PWS12 was inconsistent with the initial diagnosis of PWS, additional genetic studies were performed at age 8 years by using DNA isolated from a skin biopsy and another blood sample to examine the possibility that PWS12 was mosaic for both a BPD and a maternal UPD cell line. Maternal UPD was found at D15S11, D15S128, *IPW*, D15S97, *GABRB3*, D15S165, *ACTC*, D15S103, D15S95, D15S100, D15S86, and D15S642 (Table 1). Heterozygosity was also detected at D15S541, D15S122, D15S10, D15S118, *CYP19*, D15S117, D15S125, D15S110, D15S111, D15S107, and D15S87, thus indicating that maternal heterodisomy was present throughout the chromosome. The genotypes derived from the repeat specimens were identical to those obtained in 1990.

Maternal uniparental heterodisomy was identified in PWS96-019 at D15S541, D15S122, *CYP19*, and D15S107. The mother was homozygous, and maternal UPD was found at D15S111 and D15S642 (Table 2). This patient's genotype was also identical to the heterozygous maternal genotype at D15S128, *IPW*, D15S108, D15S110, and D15S100.

Table 1 DNA polymorphism analysis^a: patient PWS12 (*F* fibroblast, *L* leukocyte, *UPD* maternal uniparental disomy, *hUPD* maternal uniparental heterodisomy, *RFLP* restriction fragment length polymorphism, $[CA]_n$ short tandem repeat polymorphism). Paternity was verified with a VNTR probe at the α -globin gene locus on chromosome 16 (Mascari et al. 1992)

Locus	Genotypes			Interpretation
	PWS12	Father	Mother	
15q11				
D15S541(F)	12	11	12	Heterozygous
D15S11: $[CA]_n$ (L, F)	14	23	14	hUPD
D15S11 [RFLP] (L)	12	12	12	Heterozygous
D15S128 (L, F)	12	34	12	hUPD
<i>IPW</i> (L)	11	22	11	UPD
D15S97 (L, F)	34	12	34	hUPD
D15S122 (L, F)	12	23	12	Heterozygous
D15S10 (L)	12	11	12	Heterozygous
<i>GABRB3</i> (L, F)	11	22	11	UPD
D15S165 (L, F)	24	13	24	hUPD
<i>ACTC</i> (L)	12	34	12	hUPD
D15S118 (L, F)	12	11	12	Heterozygous
<i>FIB</i> (L)	11	11	11	Uninformative
<i>CYP19</i> (L, F)	23	13	23	Heterozygous
D15S103 (L)	11	23	11	UPD
D15S117 (L, F)	12	12	12	Heterozygous
D15S125 (L, F)	12	12	12	Heterozygous
D15S110 (L, F)	12	12	12	Heterozygous
D15S95 (L, F)	11	23	11	UPD
D15S111 (L, F)	12	22	12	Heterozygous
D15S100 (L, F)	14	23	14	hUPD
D15S107 (L, F)	12	23	12	Heterozygous
D15S87 (L)	12	12	12	Heterozygous
D15S86 (L)	34	12	34	hUPD
D15S642 (L, F)	24	13	24	hUPD
15qter				

^a Only informative markers are shown

Low-level mosaicism for a BPD cell line was unlikely in these patients, based on the absence of paternally derived chromosome 15 alleles at any polymorphic locus that demonstrated maternal heterodisomy. Furthermore, paternal alleles were not evident either by Southern hybridization at D15S24 (not shown) or D15S86 in PWS12, or by increasing the number of cycles of PCR amplification at D15S95 in PWS12 and D15S642 in PWS96-019. PCR amplification conditions were selected to amplify a rare paternally derived allele, if present (~2% of the predominant alleles; Pangalos et al. 1994). Mosaicism was not observed within and between tissues in PWS12 or within a single tissue in PWS96-019.

Methylation studies

DNA methylation was evaluated in leukocytes of patients and controls at *SNRPN* and PW71B (D15S63). Methylation

Table 2 DNA polymorphism analysis: patient PWS96-019. Paternity was verified at microsatellite DNA polymorphic loci on chromosome 21 (D21S1434, D21S1436, D21S167)

Locus	Leukocyte genotypes			Interpretation
	PWS96-019	Father	Mother	
15q11				
D15S541	12	34	12	hUPD
D15S11: $[CA]_n$	11		11	Uninformative
D15S128	23	13	23	Heterozygous
<i>IPW</i>	12	12	12	Heterozygous
D15S122	24	13	24	hUPD
<i>CYP19</i>	12	33	12	hUPD
D15S108	23	12	23	Heterozygous
D15S110	13	12	13	Heterozygous
D15S111	11	23	11	UPD
D15S100	13	23	13	Heterozygous
D15S107	13	22	13	hUPD
D15S642	11	23	11	UPD
15qter				

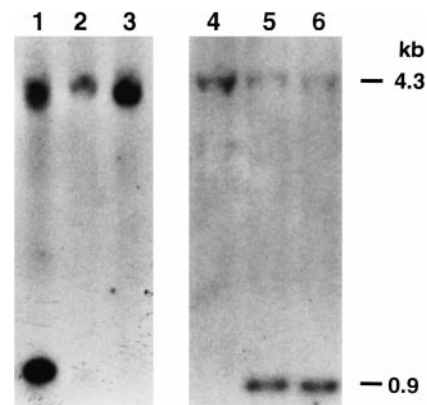


Fig. 2 Methylation analyses. Southern hybridization analysis of patient and control genomic DNA digests hybridized with the *SNRPN* probe. The methylated 4.3-kb *XbaI-NotI* fragment is seen in a deletion-positive PWS control (lane 2), and patients PWS96-019 (lane 3) and PWS12 (lane 4). The normal 4.3-kb and 0.9-kb fragments are evident in the mother of PWS96-019 (lane 1), and the father (lane 5) and mother of PWS12 (lane 6)

profiles for both patients at *SNRPN* (Fig. 2, lanes 3, 4) and PW71B (not shown) were each consistent with a PWS epigenotype (Glenn et al. 1993a; Dittrich et al. 1993). Normal *SNRPN* (Fig. 2, lanes 1, 5, 6) and PW71B (not shown) methylation patterns were seen in parental controls, and the PWS pattern was seen in an individual with a 15q11-q13 paternal deletion (Fig. 2, lane 2).

Gene expression studies

Expression of 15q11-q13 loci - *ZNF127*, *SNRPN*, *PAR5*, and *IPW*, and of the constitutively transcribed gene

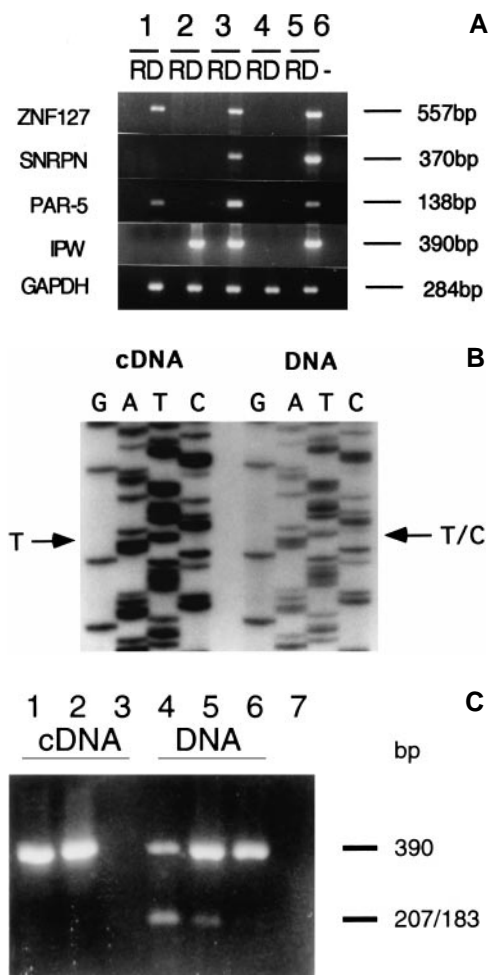


Fig. 3A–C Gene expression studies. **A** RT-PCR amplification of *ZNF127*, *SNRPN*, *PAR-5*, *IPW*, and *GAPDH* loci in PWS12 (lane 1), PWS96-019 (lane 2), a normal individual (lane 3), a PWS patient with a paternal deletion (lane 4), an AS patient with a maternal deletion (lane 5), and a negative control reaction (lane 6). **B** D:PCR amplification of the first-strand cDNA template and the corresponding negative control reaction, R:PCR amplification of RNA without prior cDNA synthesis. **C** Comparison of *IPW* genomic and cDNA sequences in PWS96-019. Arrows: Location of the heterozygous *HphI* (C/T) polymorphism in the genomic sequence and the single T allele in the patient's cDNA sequence. **C** *IPW* genotypes and expression of the *HphI* RFLP. *IPW* cDNAs are restricted with *HphI* in lanes 1–3; lanes 4–6 show the corresponding genomic digests. Results are shown for PWS96-019 (lanes 1, 4), his mother (lanes 2, 5), a PWS patient with a 15q11-q13 deletion (lanes 3, 6), and a negative control reaction (lane 7).

GAPDH- was analyzed by RT-PCR of leukocytes from PWS12 and PWS96-019. The imprinted loci are not expressed from the maternally derived chromosome 15 and are thus not transcribed in PWS patients (Fig. 3A, lane 4, and results not shown; Saitoh et al. 1996; Ohta et al., in press) but are expressed in cells of normal (Fig. 3A, lane 3) and AS individuals (Fig. 3A, lane 5). *SNRPN* and *IPW* are not transcribed in cells from PWS12 as expected, but *ZNF127* and *PAR5* are unexpectedly expressed (Fig. 3A, lane 1). Since the genetic polymorphism studies indicated that PWS12 is not mosaic for a cell line of biparental origin,

expression of *ZNF127* and *PAR5* is consistent with relaxation of the genomic imprint at these loci. This finding prompted us to analyze the expression of imprinted loci in other individuals referred for chromosome 15 UPD testing. This screen identified patient PWS96-019, who expressed *IPW*, but not *SNRPN*, *PAR5*, or *ZNF127* (Fig. 3A, lane 2). Both PWS96-019 and his mother are heterozygous for a polymorphism in *IPW* (Table 2; Fig. 3B, T/C; Fig. 3C, lanes 4, 5; Wevrick et al. 1994), but analysis of the patient's *IPW* cDNA revealed that only one of these alleles is transcribed (Fig. 3B, T; Fig. 3C, lane 1).

Allele-specific replication studies

Results of allele-specific DNA replication studies for PWS12 and PWS96-019 are presented in Table 3. Patients with UPD for chromosome 15 generally show synchronous replication, since both chromosomes are inherited from the same parent (White et al. 1996). Synchronous replication (9%–13% of cells at the G1/G2 phase of the cell cycle) was observed at *SNRPN* in both patients, a finding that is consistent with their methylation and expression profiles at this locus. In contrast, asynchronous replication was observed in both patients at D15S9 (21%–22% of cells), D15S11 (30%), D15S10 (24%–26%), and *GABRB3* (27%). The level of asynchronous replication at these imprinted loci was comparable to that seen in control individuals with biparental inheritance. D15S9 and *ZNF127* are colocalized and proximal to *SNRPN*. Moreover, D15S11 is proximal to *SNRPN*, whereas D15S10, *IPW*, and *PAR5* are tightly linked and distal to *SNRPN* (Robinson et al. 1997). Thus, the asynchronously replicating domains adjacent to *SNRPN* contain imprinted loci that are expressed in these patients.

Discussion

We report two patients who exhibit atypical PWS and maternal UPD and who express chromosome 15 alleles that are ordinarily silent in typical PWS patients. Their molecular genetic and cytogenetic findings are consistent with relaxed imprinting at *ZNF127*, *PAR5*, and *IPW*. *PAR5* and *ZNF127* are expressed in PWS12 and *IPW* in PWS96-019. Relaxation of imprinting refers to the failure either to reset germline imprinted epigenotypes during gametogenesis or to maintain each of the parental imprints in somatic tissues (Brown et al. 1996; Morrison et al. 1996; Taniguchi et al. 1995). A milder PWS phenotype may result from the expression of maternal genes as a consequence of relaxed imprinting. Expression of specific imprinted loci may be associated with appetite regulation and growth in these patients. Both individuals lack the hyperphagia typical of PWS and have normal or decreased body weight. Analysis of expression in other tissues (which was not possible in these patients) would be necessary to corroborate the relationship between these clinical findings and relaxed imprinting. However, if relaxation of imprinting occurs postzygotically,

Table 3 Replication timing. Replication analyses at the loci given below have been previously reported in control individuals and patients with UPD (Knoll et al. 1994; White et al. 1996). In control individuals, 25%–40% of cells show asynchronous replication (G1/G2) in this imprinted domain, whereas 3%–11% of cells in patients with UPD show this level of asynchrony. Hybridizations were scored independently by two or three individuals (A–C)

Locus (probe) Subject	Scorer (no. scorings)	Mean percent of cells at cell cycle stage (range)		
		G1	G1/G2	G2
D15S9 (34–10) PWS96–019	A, C (3)	67 (65–70)	21 (21–22)	11 (8–13)
D15S11 (D15S11) PWS96–019	A, C (2)	56 (53–59)	30 (30)	14 (11–17)
<i>SNRPN</i> (<i>SNRPN</i>) PWS12	A, B, C (4)	87 (86–87)	9 (9–10)	4 (4)
PWS96–019	A, C (2)	76 (75–78)	11 (10–13)	12 (12)
D15S10 (3–21–12) PWS12	A, B, C (3)	68 (66–69)	24 (22–26)	8 (8–9)
PWS96–019	A, C (2)	61 (60–62)	26 (25–28)	13 (12–13)
<i>GABRB3</i> (16β3) PWS12	A, C (2)	64 (62–67)	27 (27)	9 (6–11)
PWS96–019	A, C (2)	68 (67–69)	27 (27)	6 (5–6)

it is possible that some tissues would not express these imprinted genes.

Although different imprinted loci are expressed in each patient, both exhibit asynchronous DNA replication at the same loci. In contrast, other patients with maternal UPD demonstrate synchronous replication (Kitsberg et al. 1993; Knoll et al. 1994; White et al. 1996). Interestingly, *SNRPN*, which is not expressed in either of these patients, demonstrates synchronous replication. The asynchronously replicating loci reside in adjacent regions containing imprinted loci that are expressed in our patients. Expression and replication remain coupled at *IPW* in PWS12 and at *ZNF127* and *PAR5* in PWS96–019, despite the maternal origin of these alleles. Changes in chromatin structure can potentiate gene activation at other loci in the genome, regardless of parental origin (Tuan et al. 1992; Yoo et al. 1996), and these changes can be related to replication timing. Gene expression has been correlated with the timing of DNA replication for genes with both constitutive and tissue-specific expression profiles (Goldman et al. 1984; Hatton et al. 1988; Gunaratne et al. 1995; Yeshaya et al. 1998).

Monoallelic expression of *IPW* in PWS96–019 is consistent with the possibility that this locus is transcriptionally regulated. The expression of a single allele could conceivably be optimal for normal development, since abnormal phenotypes result from the failure to express imprinted genes in PWS and AS (Sutcliffe et al. 1994; Saitoh et al. 1997) and from extra gene copies in individuals with duplications of chromosome 15q11–q13 (Leana-Cox et al. 1994; Cheng et al. 1994; Cook et al. 1997; Repetto et al. 1998).

Inheritance of the *IPW* allele that is expressed in PWS96–019 may provide a clue to the developmental and replication timing of relaxed imprinting. Expression of a single allele from a maternal chromosome pair can result from a grandparental allele having either germline failure to complete imprint switching (Buiting et al. 1998) or somatic relaxation of the imprint. Failure to reset a grandpaternal imprint during oogenesis transmits a paternal epigenotype

and produces normal imprinting. Once the switch has occurred, partially relaxed imprinting in the germline could result from failure to effect imprinting at loci adjacent to the IC. If the expressed allele were grandmaternally derived, relaxation of imprinting should have occurred in somatic tissues and result in failure to maintain the silence of this allele.

In theory, somatic mosaicism for UPD and BPD could result in the expression of imprinted genes. Mosaicism in these cases is unlikely for several reasons. They include: (1) paternally derived chromosome 15 alleles were not detected; (2) only a subset of imprinted loci were expressed, instead of all loci, which would be expected if the paternal chromosome were present; (3) synchronous DNA replication was found only at *SNRPN*; (4) the phenotype of mosaic trisomy 15 was not apparent (Milunsky et al. 1996; Buhler et al. 1996); and (5) UPD and BPD cell lines present in a single individual would have required independent postzygotic errors (Cassidy et al. 1992). Expression of imprinted genes is more likely to be the result of the relaxation of maternal alleles in the 15q11–q13 interval.

The PWS phenotype appears to be determined both by the loss of IC function and by the expression of individual imprinted genes in this region. Expression of the IC (within the 5' untranslated region of *SNRPN*) is required for normal development and imprinting of other 15q11–q13 loci, but the *SNRPN* coding region itself appears to be dispensable (Yang et al. 1998). However, some clinical features of PWS are present in patients with an intact IC and translocation breakpoints within the PWS critical region (Schulze et al. 1996; Conroy et al. 1997) or smaller than typical deletions (Mowrey-Rushton et al. 1996b). Schulze et al. (1996) have reported a non-hyperphagic patient with a balanced (9;15) translocation breakpoint distal to *SNRPN* and who exhibits normal *SNRPN* expression and methylation but who does not express either *IPW* or *PAR-1*. Another atypical patient with a balanced paternal (2;15) translocation breakpoint between *IPW* and *SNRPN* expresses *SNRPN* and *PAR-5* but not *IPW* (Conroy et al. 1997). Individuals with deletions

distal to *SNRPN* (PW4 and PW10 in Mowrey-Rushton et al. 1996b) are also atypical. In these individuals and the present UPD patients, imprint switching appears to have occurred correctly at *SNRPN* but either does not occur or is not maintained at adjacent loci. Patients who lack some findings of PWS and express imprinted genes may be useful in defining chromosomal intervals associated with specific clinical features.

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References

- Brown KW, Villar AJ, Bickmore W, Clayton-Smith J, Catchpoole D, Maher ER, Reik W (1996) Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic IGF2 expression through an H19-independent pathway. *Hum Mol Genet* 5:2027-2032
- Buhler EM, Georg Bienz, Straumann E, Bosch N (1996) Delineation of a clinical syndrome caused by mosaic trisomy 15. *Am J Med Genet* 62:109-112
- Buiting K, Saitoh S, Gross S, Dittrich B, Schwartz S, Nicholls RD, Horsthemke B (1995) Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. *Nat Genet* 9:395-400
- Buiting K, Dittrich B, Gross S, Lich C, Farber C, Buchholz T, Smith E, Reis A, Burger J, Nothen MM, Barth-Witte U, Janssen B, Abeliovich D, Lerer I, Ouweland AM van den, Halley DJ, Schrandt-Stumpel C, Smeets H, Meinecke P, Malcolm S, Gardner A, Lalonde M, Nicholls RD, Friend K, Horsthemke B, et al (1998) Sporadic imprinting defects in Prader-Willi syndrome and Angelman syndrome: implications for imprint-switch models, genetic counseling, and prenatal diagnosis. *Am J Hum Genet* 63:170-180
- Cassidy SB (1995) Uniparental disomy and genomic imprinting as causes of human genetic disease. *Environ Mol Mutagen* 26:13-20
- Cassidy SB, Lai LW, Erickson RP, Magnuson L, Thomas E, Gendron R, Herrmann J (1992) Trisomy 15 with loss of the paternal 15 as a cause of Prader-Willi syndrome due to maternal disomy. *Am J Hum Genet* 51: 701-708
- Cheng SD, Spinner NB, Zackai EH, Knoll JH (1994) Cytogenetic and molecular characterization of inverted duplicated chromosomes 15 from 11 patients. *Am J Hum Genet* 55:753-759
- Conroy JM, Grebe TA, Becker LA, Tsuchiya K, Nicholls RD, Buiting K, Horsthemke B, Cassidy SB, Schwartz S (1997) Balanced translocation 46,XY,t(2;15)(q37.2;q11.2) associated with atypical Prader-Willi syndrome. *Am J Hum Genet* 61:388-394
- Cook EH, Lindreg V, Leventhal BL, Courchesne R, Lincoln A, Shulman C, Lord C, Courchesne E (1997) Autism or atypical autism in maternally but not paternally derived proximal 15q duplication. *Am J Hum Genet* 60:928-934
- Dittrich B, Buiting K, Gross S, Horsthemke B (1993) Characterization of a methylation imprint in the Prader-Willi syndrome chromosome region. *Hum Mol Genet* 2:1995-1999
- Driscoll DJ, Waters MF, Williams CA, Zori RT, Glenn CC, Avidano KM, Nicholls RD (1992) A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader-Willi syndromes. *Genomics* 13:917-924
- Gillessen-Kaesbach G, Gross S, Kaya-Westerloh S, Passarge E, Horsthemke B (1995) DNA methylation based testing of 450 patients suspected of having Prader-Willi syndrome. *J Med Genet* 32:88-92
- Glenn CC, Porter KA, Jong MT, Nicholls RD, Driscoll DJ (1993a) Functional imprinting and epigenetic modification of the human *SNRPN* gene. *Hum Mol Genet* 2:2001-2005
- Glenn CC, Nicholls RD, Robinson WP, Saitoh S, Niikawa N, Schinzel A, Horsthemke B, Driscoll DJ (1993b) Modification of 15q11-q13 DNA methylation imprints in unique Angelman and Prader-Willi patients. *Hum Mol Genet* 2:1377-1382
- Glenn CC, Saitoh S, Jong MT, Filbrandt MM, Surti U, Driscoll DJ, Nicholls RD (1996) Gene structure, DNA methylation, and imprinted expression of the human *SNRPN* gene. *Am J Hum Genet* 58:335-346
- Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A (1984) Replication timing of genes and middle repetitive sequences. *Science* 224:686-692
- Gunaratne PH, Nakao M, Ledbetter DH, Sutcliffe JS, Chinault AC (1995) Tissue-specific and allele-specific replication timing control in the imprinted human Prader-Willi syndrome region. *Genes Dev* 9:808-820
- Hatton KS, Dhar V, Brown EH, Iqbal MA, Stuart S, Didamo VT, Schildkraut CL (1988) Replication program of active and inactive multigene families in mammalian cells. *Mol Cell Biol* 8:2149-2158
- Holm VA, Cassidy SB, Butler MG, Hanchett JM, Greenswag LR, Whitman BY, Greenberg F (1993) Prader-Willi syndrome: consensus diagnostic criteria. *Pediatrics* 91:398-402
- Kitsberg D, Selig S, Brandeis M, Simon I, Keshet I, Driscoll DJ, Nicholls RD, Cedar H (1993) Allele-specific replication timing of imprinted gene regions. *Nature* 364:459-463
- Knoll JHM, Lichter P (1994) In: Dracopoli NC, Haines JL, Korf BR, Moir DT, Morton CC, Seidman CE, Seidman JG (eds) Current protocols in human genetics, vol 1. Green-Wiley, New York, unit 4.3
- Knoll JHM, Nicholls RD, Magenis RE, Graham JM Jr, Lalonde M, Latt S (1989) Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion. *Am J Med Genet* 32:285-290
- Knoll JH, Cheng SD, Lalonde M (1994) Allele specificity of DNA replication timing in the Angelman/Prader-Willi syndrome imprinted chromosomal region. *Nat Genet* 6:41-46
- Leana-Cox J, Jenkins L, Palmer CG, Plattner R, Sheppard L, Flejter WL, Zackowski J, Tsien F, Schwartz S (1994) Molecular cytogenetic analysis of inv dup(15) chromosomes, using probes specific for the Prader-Willi/AS region: clinical implications. *Am J Hum Genet* 54:748-756
- McDonald HR, Wevrick R (1997) The *Necdin* gene is deleted in Prader-Willi Syndrome and is imprinted in human and mouse. *Hum Mol Genet* 6:1873-1878
- Mascari MJ, Gottlieb W, Rogan PK, Butler MG, Waller DA, Armour JA, Jeffreys AJ, Nicholls RD (1992) The frequency of uniparental disomy in Prader-Willi syndrome. Implications for molecular diagnosis. *N Engl J Med* 326:1599-1607
- Milunsky JM, Wyandt HE, Huang XL, Kang XZ, Elias ER, Milunsky A (1996) Trisomy 15 mosaicism and uniparental disomy (UPD) in a liveborn infant. *Am J Med Genet* 61:269-273
- Morrison IM, Becroft DM, Taniguchi T, Woods CG, Reeve DG (1996) Somatic overgrowth associated with overexpression of insulin-like growth factor II. *Nat Med* 2:311-316
- Mowery-Rushton PA, Driscoll DJ, Nicholls RD, Locker J, Surti U (1996a) DNA methylation patterns in human tissues of uniparental origin using a zinc-finger gene (*ZNF127*) from the Angelman/Prader-Willi region. *Am J Med Genet* 61:140-146
- Mowery-Rushton PA, Hanchett JM, Zipf WB, Rogan PK, Surti U (1996b) Identification of mosaicism in Prader-Willi syndrome using fluorescent in situ hybridization. *Am J Med Genet* 66:403-412
- Mutirangura A, Greenberg F, Butler MG, Malcolm S, Nicholls RD, Chakravarti A, Ledbetter DH (1993) Multiplex PCR of three dinucleotide repeats in the Prader-Willi/Angelman critical region (15q11-q13): molecular diagnosis and mechanism of uniparental disomy. *Hum Mol Genet* 2:143-151
- Nicholls RD (1993) Genomic imprinting and uniparental disomy in Angelman and Prader-Willi syndromes: a review. *Am J Med Genet* 46:16-25

- Nicholls RD, Knoll JHM, Butler MG, Karam S, Lalande M (1989) Genetic imprinting suggested by maternal heterodisomy in non-deletion Prader-Willi syndrome. *Nature* 342: 281–285
- Ohta T, Gray TA, Rogan PK, Buiting K, Gabriel JM, Saitoh S, Muralidhar B, Bilienska B, Krajewska-Walasek M, Driscoll DJ, Horsthemke B, Butler MG, Nicholls RD (1998) Imprinting mutation mechanisms in Prader-Willi syndrome represent a new paradigm for genetic disease. *Am J Hum Genet* (in press)
- Pangalos C, Avramopoulos D, Blouin JL, Raoul O, deBlois MC, Prieur M, Schinzel AA, Gike M, Abazis D, Antonarakis SE (1994) Understanding the mechanism(s) of mosaic trisomy 21 by using DNA polymorphism analysis. *Am J Hum Genet* 54:473–481
- Repetto GR, White LM, Bader PJ, Johnson D, Knoll JHM (1998) Interstitial duplication of chromosome 15q11q13: clinical and molecular characterization. *Am J Med Genet* 79:82–89
- Robinson WP, Horsthemke B, Leonard S, Malcolm S, Morton C, Nicholls RD, Ritchie RJ, Rogan P, Schultz R, Schwartz S, Sharp J, Trent R, Wevrick R, Williamson M, Knoll JHM (1997) Report of the Third International Workshop on Human Chromosome 15 Mapping 1996. *Cytogenet Cell Genet* 76:1–13
- Rougeulle C, Glatt H, Lalande M (1997) The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain. *Nat Genet* 17:14–15
- Saitoh S, Buiting K, Rogan PK, Buxton JL, Driscoll DJ, Arnemann J, Konig R, Malcolm S, Horsthemke B, Nicholls RD (1996) Minimal definition of the imprinting center and fixation of chromosome 15q11-q13 epigenotype by imprinting mutations. *Proc Natl Acad Sci USA* 93:7811–7815
- Saitoh S, Buiting K, Cassidy SB, Conroy JM, Driscoll DJ, Gabriel JM, Gillissen-Kaesbach G, Glenn CC, Greenswag LR, Horsthemke B, Kondo I, Kuwajima K, Niikawa N, Rogan PK, Schwartz S, Seip J, Williams CA, Wizenitzer M, Nicholls RD (1997) Clinical spectrum and molecular diagnosis of Angelman and Prader-Willi Syndrome patients with an imprinting mutation. *Am J Med Genet* 68:195–206
- Schulze A, Hansen C, Skakkebaek NE, Brondum-Nielsen K, Ledbetter DH, Tommerup N (1996) Exclusion of SNRPN as a major determinant of Prader-Willi syndrome by a translocation breakpoint. *Nat Genet* 12:452–454
- Sutcliffe JS, Nakao M, Christian S, Orstavik KH, Tommerup N, Ledbetter DH, Beaudet AL (1994) Deletions of a differentially methylated CpG island at the *SNRPN* gene define a putative imprinting control region. *Nat Genet* 8:52–58
- Taniguchi T, Sullivan MJ, Ogawa O, Reeve AE (1995) Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms tumor. *Proc Natl Acad Sci USA* 92:2159–2163
- Tuan D, Kong S, Hu K (1992) Transcription of the hypersensitive site HS2 enhancer in erythroid cells. *Proc Natl Acad Sci USA* 89:11219–11223
- Weber J, May P (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44:388–396
- Wevrick R, Kerns JA, Francke U (1994) Identification of a novel paternally expressed gene in the Prader-Willi syndrome region. *Hum Mol Genet* 3:1877–1882
- White LM, Rogan PK, Nicholls RD, Wu BL, Korf B, Knoll JHM (1996) Allele-specific replication of 15q11-q13 loci: a diagnostic test for detection of uniparental disomy. *Am J Hum Genet* 59:423–430
- Woodage T, Prasad M, Dixon JW, Selby RE, Romain DR, Columbano-Green LM, Graham D, Rogan PK, Seip JR, Smith A, Trent RJ (1994) Bloom syndrome and maternal uniparental disomy for chromosome 15. *Am J Hum Genet* 55:74–80
- Yang T, Adamson TE, Resnick JL, Leff S, Wevrick R, Francke U, Jenkins NA, Copeland NG, Brannan CI (1998) A mouse model for Prader-Willi syndrome imprinting-centre mutations *Nat Genet* 19:25–31
- Yeshaya J, Shalgi R, Shohat M, Avivi L (1998) Replication timing of the various FMR1 alleles detected by FISH: inferences regarding their transcriptional status. *Hum Genet* 102:6–14
- Yoo J, Herman LE, Li C, Krantz SB, Tuan D (1996) Dynamic changes in the locus control region of erythroid progenitor cells demonstrated by polymerase chain reaction. *Blood* 87:2558–2567