

Prader-Willi Syndrome Is Caused by Disruption of the SNRPN Gene

C. D. Kuslich,^{1,*} J. A. Koberi,² G. Mohapatra,³ C. Gregorio-King,^{1,†} and T. A. Donlon^{1,4}

¹Molecular & Cytogenetics Laboratory, Kapiolani Health Research Institute, Honolulu; ²Department of Pediatrics, The Permanente Medical Group, San Jose, CA; ³University of California, San Francisco, San Francisco; and ⁴Department of Genetics and Molecular Biology, University of Hawaii, Manoa

Summary

A Prader-Willi syndrome patient is described who has a *de novo* balanced translocation, (4;15)(q27;q11.2)pat, with breakpoints lying between SNRPN exons 2 and 3. Parental-origin studies indicate that there is no uniparental disomy and no apparent deletion. This patient expresses ZNF127, SNRPN exons 1 and 2, IPW, and D15S227E (PAR1) but does not express either SNRPN exons 3 and 4 or D15S226E (PAR5), as assayed by reverse transcription-PCR, of peripheral blood cells. Methylation studies showed normal biparental patterns of inheritance of loci DN34/ZNF127, D15S63, and SNRPN exon 1. Results for this patient and that reported by Sun et al. support the contention that an intact genomic region and/or transcription of SNRPN exons 2 and 3 play a pivotal role in the manifestations of the major clinical phenotype in Prader-Willi syndrome.

Introduction

Prader-Willi syndrome (PWS) affects 1/10,000–20,000 live births (Holm et al. 1993) and is characterized by neonatal hypotonia and failure to thrive, mental retardation, hypogonadism, short hands and feet, and hyperphagia resulting in obesity (Prader et al. 1956; Holm et al. 1993). Approximately 70% (Ledbetter et al. 1981) of PWS patients have a recognizable deletion (3–4 Mbp) of chromosome 15q11.2, on their paternally-derived chromosome (Butler and Palmer 1983). Most of the remaining cases have maternal disomy (Nicholls et al. 1989), a situation in which the child inherits two chro-

mosomes 15 from the mother but in which there is no contribution from the father. A minority of PWS patients have very small (20–100 kbp) deletions of 15q11.2, in an area proximal to the small nucleoriboprotein N (SNRPN) gene, in a locus that has been proposed as the imprint-control element (ICE) (Sutcliffe et al. 1994; Buiting et al. 1995). This locus plays a major role in erasing the inherited imprint status of genes in 15q11.2 and establishing a new imprint status. PWS patients with such deletions have normal, biparental inheritance of loci within 15q11.2; however, they do not express paternally derived genes. Some of these genes (i.e., ZNF127) can be >1 Mb from the ICE. Several cases of a new rare class of PWS patients have been described that have balanced translocations involving 15q11.2 (Schulze et al. 1996; Sun et al. 1996). We here describe a patient with typical PWS and a balanced translocation (4;15)(q27;q11.2) that breaks between exons 2 and 3 of the SNRPN gene and that will help to define clinical/molecular correlations in this contiguous-gene syndrome.

Subject and Methods

Subject

The patient is a male born to nonconsanguineous parents of African American descent who were 22 years of age. The pregnancy was remarkable because of decreased fetal movement. Apgars were 6/8. The patient has one healthy brother, two healthy half-brothers, and a healthy half-sister, who are all normal. He has a paternal uncle who weighs 300 lbs but who is of normal intelligence. At age 19 mo the patient was diagnosed with IgG-4 deficiency, and therapy with intravenous gamma-globulin was started. He did not respond to beta-human chorionic gonadotropin therapy for undescended testicles. At age 2 years 10 mo the patient was diagnosed with hypertension. At age 3 years 3 mo, the patient was referred for cytogenetic analysis for suspected PWS, because of hypotonicity, small testicles, failure to thrive, and poor sucking reflex during infancy, followed by an eating behavior leading to an increased weight gain at age 2 years and to obesity (>97%-ile for weight, at age 3 years). In addition, the patient was noted to have narrow bifrontal diameter, almond-shaped, up-

Received November 4, 1997; accepted for publication June 30, 1998; electronically published December 14, 1998.

Address for correspondence and reprints: Dr. Timothy A. Donlon, Ohana Genetics, P. O. Box 161052, Honolulu, HI 96816-0923. E-mail: donlon@hawaii.edu

* Present affiliation: School of Public Health and Nutrition, Deakin University, Geelong, Victoria, Australia.

† Present affiliation: Department of Pathology, University of Washington, Seattle.

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Figure 1 Patient at age 3 years 3 mo. Note the midface hypoplasia, almond-shaped eyes, and up-slanted palpebral fissures. The patient's parents also were relatively light in complexion.

slanted palpebral fissures (see fig. 1), narrow arched palate, and undescended testes. At age 11 years the patient had a combined score of 10 points; a minimum of 5 is required for an individual to be considered positive for PWS. The consensus diagnostic criteria (Holm et al. 1993) were evaluated for this patient. The major criteria (1 point each) include hypotonia in the neonatal period, with decreased sucking reflex; failure to thrive during infancy and early childhood; rapid weight gain at age >1 year (in this case, at age 2 years); hypogonadism; developmental delay; hyperphagia (aggressive food-seeking behavior); and deletion or other structural abnormality of 15q11-13. Minor criteria ($\frac{1}{2}$ point each) include decreased in utero activity, behavioral abnormalities (temper tantrums; violent outbursts and obsessive-compulsive, rigid, argumentative, oppositional, and/or stubborn behavior; and/or lying), sleep disturbances/apnea, viscous saliva, articulation difficulty, and skin picking. Supportive (but otherwise not scored) findings include decreased vomiting and normal neuromuscular studies (creatine phosphokinase and aldolase normal). It is of interest that the patient did not have (1) small hands and feet, (2) hypopigmentation, (3) narrow hands with straight ulnar border, (4) or esotropia/myopia. PWS patients of African American ancestry have been reported to have larger hand and foot lengths, compared with Caucasian PWS patients, which is consistent with what was seen in our patient (Cassidy et al. 1996).

Cytogenetic Analysis

A high-resolution, GTW-banded (Seabright 1971) chromosome study was performed on the patient and his parents, as described elsewhere (Yunis and Chandler

1977). Forty cells from the patient and 20 cells from each parent were analyzed in detail.

FISH

YACs were obtained from Craig Chinault and Uta Francke and were grown by standard methods. Correct insert sizes were confirmed by use of contour-clamped homogeneous electric field (CHEF) pulse-field gel electrophoresis (Chu et al. 1986). FISH was performed by standard techniques (Dracopoli et al. 1997), with biotinylated whole-yeast DNA, and visualization was with FITC-streptavidin on a Zeiss Photomicroscope III.

Somatic-Cell Hybrids

A lymphoblastoid cell line from the patient with the translocation (4;15) was established by Epstein-Barr virus (EBV) transformation, was fused with a hypoxanthine phosphoribosyltransferase-deficient Chinese hamster ovary (CHO) cell line (Y21) (a gift kindly provided by Roger Schultz), and was propagated at low density in hypoxanthine-amethopterin-thymidine (HAT) medium for 2 wk, to select for heterokaryons (Dracopoli et al. 1997). Human parental lymphoblastoid cells were removed by shaking the media prior to feeding. Cells were cultured for a minimum of 10 generations and then were dispersed into 80 separate 24-well microtiter plates (i.e., a total of 1,920 cultures), at a dilution that would result in 0.3 cells/well, and were selected for analysis. DNA was isolated and used for PCR analysis using primers for two loci, D15S18 and GABRB3, that are on opposite sides of the PWS chromosomal region. Ten clones from each of two categories were identified; those in the first category, which had segregated der(15) (i.e., the

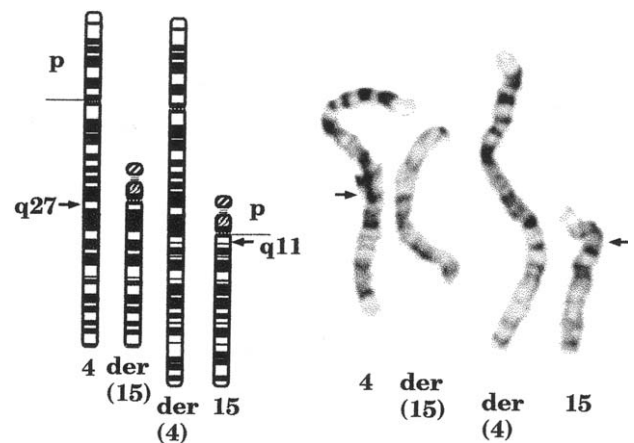


Figure 2 G-banded chromosomes of translocation (4;15)(q27;q11.2). High-resolution chromosome analysis demonstrated an apparently balanced translocation between the long arms of chromosome 4, at band q27, and chromosome 15, at band q11.2 (arrows).

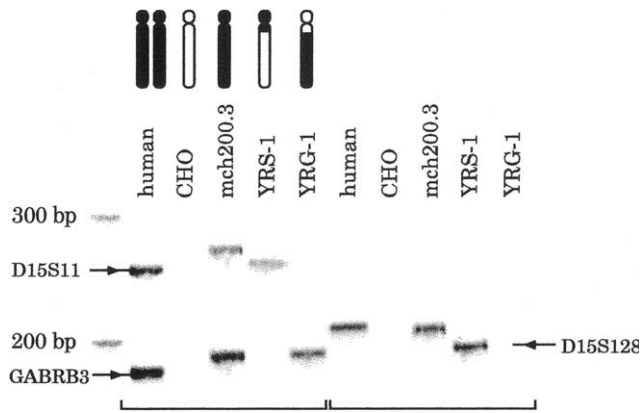


Figure 3 Map of translocation breakpoint. Mapping panels were constructed for both PCR and Southern blotting, to refine the breakpoint in the proband. These panels consisted of an unaffected human control, a CHO control, mch200.3, YRS-1 a somatic cell hybrid containing der(15) (15pter→q11.2) but not the normal 15, and YRG-1 a somatic cell hybrid containing der(4) (15q11.2→qter) but not the normal 15. The locus D15S11 (~260 bp) was amplified in the human, mch200.3, and YRS-1 samples but not in the CHO or YRG-1 samples, indicating that this locus is proximal to the breakpoint. The same was observed for locus D15S128 (~200 bp). Conversely, locus GABRB3 (~180 bp) was amplified in the human, mch200.3, and YRG-1 samples but not in the YRS-1 sample, indicating that it is distal to the breakpoint. These loci are polymorphic and therefore have different-sized products.

derivative chromosome 15) from both the normal chromosome 15 and der(4) and were positive only for D15S18, were designated “YRS,” and those in the second category, which segregated der(4) from both the normal 15 and der(15) and were positive only for GABRB3, were designated “YRG.” A mouse-human hybrid cell line (mch200.3) (a generous gift from Eric Stanbridge), containing chromosome 15 as its only human contribution, also was used, for the construction of the mapping panel.

Southern Blotting

Plasmid probes D15S18 (pIR39), ZNF127 (pTD34), D15S11 (pIR4-3), D15S13 (pTD189-1), D15S10 (pTD3-21), and P (pIR10-1) were from Donlon et al. (1986). The probe for SNRPN exons 4–10 (previously referred to as exons “2”–“8”) was from Stuart Leff (Özçelik et al. 1992). The probe for D15S63 (pW71B) was from Bernhard Horsthemke (Dittrich et al. 1993). The probe for D15S24 (pCMW-1) was from David Ledbetter (Rich et al. 1988).

Methylation Studies

Methylation studies were performed on loci DN34/ZNF127 (*EcoRI/HpaII* digested), SNRPN exon 1 (previously “–1”) (*NotI/XbaI* digested), and D15S63

(*HindIII/HpaII* digested), as described elsewhere (Dittrich et al. 1993; Glenn et al. 1993a, 1996).

PCR

PCR on somatic-cell hybrid DNA was performed for loci D15S18, D15S13, D15S10, D15S113, D15S12, D15S97, and GABRB3, as originally described by Kuwano et al. (1992) and Mutirangura et al. (1993). SNRPN exons have been renamed to reflect the discovery of two new exons, 1 and 2 (previously exons “–1” or “α” and “0” or “β,” respectively). SNRPN exon 1 was amplified by means of the following set of primers: SNRPN-1F (5'-GTG GAG CGG CCG CC-3') and SNRPN-1R (5'-CTT GCC CGC TCC ATC G-3'), which produced a 70-bp product. Exon 2 was amplified by means of primers SNRPN0SHF (5'-GCA GTC TAC CAA ACA AAT GCC) and SNRPN0SHB (5'-CCC AAC ACA GTC TGT ACT CAC-3'), which produced a 154-bp product. Exon 3 was amplified by means of primers SNRPN1AF (5'-GTT CTC AGC AGC AGC AAG-3') and SNRPN1AR (5'-CAG ATT CCT CGC TAC TC-3'), which produced a 112-bp product. Primers for exons 1 and 3 are within the coding segment, whereas the primers for exon 2 are present in flanking intronic DNA. Primers for D15S225E, D15S226E (PAR5), D15S227E (PAR1), D15S228E (PAR4), and D15S229E (PAR7) are from Sutcliffe et al. (1994). IPW is from Wevrick et al. (1994). Primers for loci 71B11L, A156E1R, 30A12L, B58C7R, 132D4R, B230E3L, B230E3R, 189 1-CA, and 307ARL were generous gifts from David Ledbetter and Susan Christian. The remaining primer sequences were obtained from the Genome Database. All primers were synthesized by Operon Technologies. PCR conditions for the SNRPN primers sets were 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 10 s, and a final elongation at 72°C for 7 min.

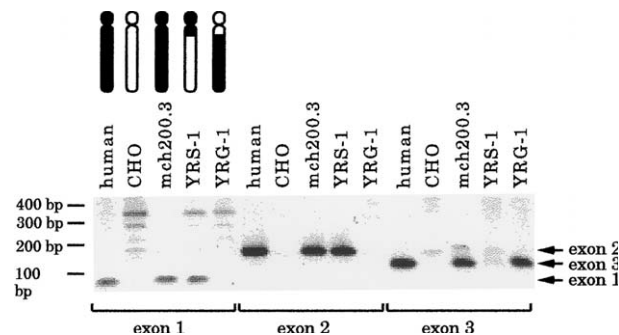


Figure 4 Map of SNRPN relative to translocation breakpoint. SNRPN exons 1–3 were amplified in each of the DNA samples, which showed that exons 1 and 2 are proximal to the breakpoint, since they were present in YRS-1 but not in YRG-1, whereas exon 3 was distal to the breakpoint, since it was present in YRG-1 but not in YRS-1.

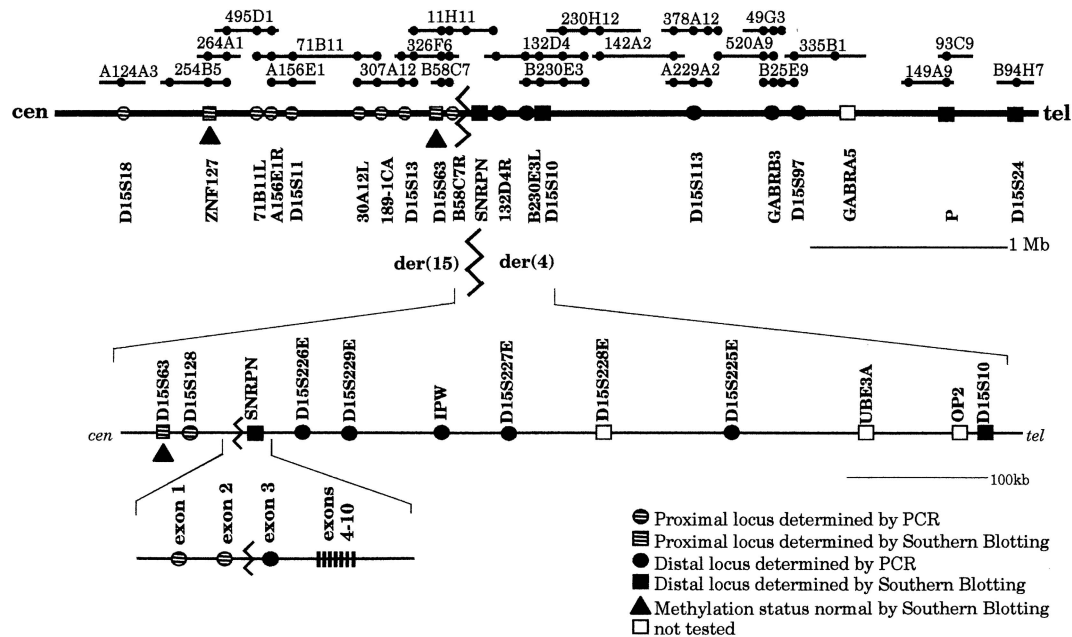


Figure 5 Localization of translocation breakpoint. The locations of 28 loci relative to the translocation (4;15)(q27;q11.2) breakpoint are shown. The breakpoint was localized to the region between SNRPN exons 2 and 3. The horizontal bars represent YACs and their respective anchor points (i.e., sequence-tagged sites). (Redrawn from Mutirangura et al. 1993)

Gene expression was assayed by reverse transcription-PCR (RT-PCR), as described elsewhere (Özçelik et al. 1992; Glenn et al. 1993b; Sutcliffe et al. 1994; Wevrick et al. 1994). SNRPN exon 2 was assayed by means of intraexonic primers SNRPN0AF (5'-AGA ACA GCA CGT ACC AGA GG-3') and SNRPN0AR (5'-TCT TGG TTG CTC AGT GAG GC-3'), which produced a 68-bp product. The transcript including SNRPN exons 1 and 2 was assayed by means of primers SNRPN-1F (5'-CTG ACG CAT CTG TCT GAG GA-3') and SNRPN0R (5'-TTC GTC TCA GGT GTA AGC GA-3'), which produced a 72-bp product. The transcript including SNRPN exons 2 and 3 was assayed by means of primers SNRPN0F (5'-GGA AGT CCA AGT CAA ACG C-3') and SNRPN1R (5'-CCT CGC TAC TCC AAT ATG GC-3'), which produced a 175-bp product. RT-PCR was performed on ZNF127, SNRPN (exon 1), SNRPN (exon 2), SNRPN (exon 3), SNRPN (exons 1 and 2), SNRPN (exons 2 and 3), SNRPN (exons 4 and 5), D15S226E (PAR5), IPW, and D15S227E (PAR1). All RNA samples were treated with 200 U RNase-free DNase I (Sigma) for 1 h at 37°C, followed by a heating to 68°C for 10 min, prior to first-strand cDNA synthesis. The integrity of RNA and the absence of genomic DNA were assayed by means of primers for β -actin (CloneTech) and for SNRPN exons 4 and 5, both of which are interexonic primer sets. cDNA was generated by incubation of 1 μ g RNA in a volume of 20 μ l containing 1.5 mM MgCl₂, 1.0 mM of each dNTP, 2.5 μ M reverse primer, and 25

U Moloney murine leukemia virus (New England Biolabs), at 42°C for 1 h. This cDNA was then amplified by 35 cycles of PCR, by addition of the cDNA to a volume of 100 μ l, which included 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μ M forward primer, and 1.25 U *Taq* polymerase (Perkin Elmer Cetus), with denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and elongation at 72°C for 30 s, in a volume of 100 μ l.

Results

High-resolution chromosome analysis demonstrated an apparently balanced translocation between the long arms of chromosomes 4 and 15, with breakpoints tentatively localized to 15q11.2 (fig. 2). Parental chromosomes were normal, indicating that the child's translocation was de novo (not shown). FISH was performed

Table 1

Parental Origin of Translocation

SAMPLE	ALLELE(S) AT LOCUS ^a				
	D15S11	D15S128	D15S113	GABRB3	D15S98
Father	<u>B</u> /C	A/ <u>C</u>	A/ <u>B</u>	A/ <u>B</u>	B/C
Mother	A/ <u>D</u>	A/ <u>B</u>	A	<u>C</u> / <u>D</u>	A/C
Patient	<u>B</u> / <u>D</u>	A/ <u>C</u>	A/ <u>B</u>	A/ <u>C</u>	A/C
YRS-1	<u>B</u>	<u>C</u>
YRG-1	<u>B</u>	<u>A</u>	C

^a Paternal alleles are underlined.

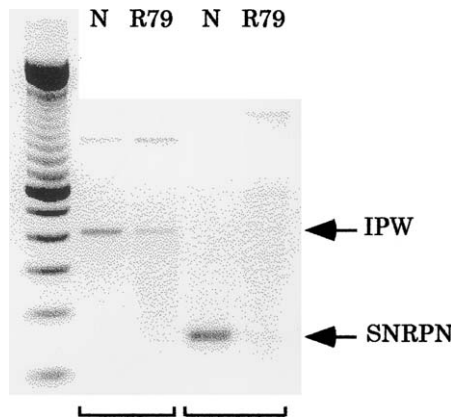


Figure 6 RT-PCR of genes IPW and SNRPN. The patient expresses the IPW gene but not the SNRPN gene. From left to right, the lanes show a 100-bp size ladder (leftmost, unlabeled lane) and the IPW RT-PCR product (~410 bp) from a normal control (first lane N), results for patient PWS (lane R79), SNRPN exons 4 and 5 RT-PCR product (~160 bp) from a normal control (second lane N), and results for the patient in the present study.

with YAC DNA as probe and demonstrated that the breakpoint lies between D15S18 and D15S10 (data not shown).

Somatic-cell hybrids were constructed by use of the patient's chromosomes, to separate the normal chromosome 15, der(4), and der(15), thereby facilitating the construction of mapping panels to refine further the breakpoint in the patient's chromosome 15. Figure 3 shows an example of a mapping panel indicating that loci D15S11 and D15S128 are proximal (i.e., present in hybrid YRS-1 but not in YRG-1) whereas GABRB3 is distal. Southern blot analysis of six loci and PCR amplification of 22 loci revealed that the breakpoint lies between D15S128 and SNRPN exons 4–10. PCR primers were constructed for SNRPN exons 1 (previously exon “–1” or “ α ”), exon 2 (previously exon “0” or “ β ”), and exon 3 (previously exon “1”) and show that the breakpoint lies between exons 2 and 3 (fig. 4). The mapping results for all 28 loci are shown in figure 5.

The parental origin of this translocation was determined by comparison of five polymorphic loci in the parents versus those in the somatic-cell hybrids (table 1). Informative loci include D15S11, D15S128, D15S113, and GABRB3 and demonstrate a paternal origin of the translocation chromosome; the paternal allele present in the hybrid is shown underlined in table 1. Methylation studies were conducted to ascertain the imprint status of loci ZNF127, SNRPN, and PW71B (Dittrich et al. 1993; Glenn et al. 1993a, 1996). All three loci showed normal methylation patterns, indicating normal biparental inheritance. (i.e., methylated on the

maternal chromosome 15 but unmethylated on the paternal 15; data not shown).

RT-PCR was conducted to assay the expression of genes in the PWS chromosomal region—that is, ZNF127, SNRPN exons 1–5, D15S226E (PAR5), IPW, and D15S227E (PAR1). Figure 6 shows that SNRPN exons 4 and 5 were not expressed, whereas IPW was. In addition, this patient did express ZNF127, SNRPN exon 1, SNRPN exon 2, SNRPN exons 1 and 2, and D15S227E (PAR1) but did not express SNRPN exon 3, SNRPN exons 2 and 3, and D15S226E (PAR5), as shown in table 2.

Discussion

The ICE (Sutcliffe et al. 1994; Buiting et al. 1995) contains at least five transcription units that are part of the SNRPN gene and that are alternately spliced during fetal development (Dittrich et al. 1996). These upstream transcripts appear to control the paternal→maternal imprint switch and are defective in Angelman syndrome patients with imprinting mutations (i.e., two paternally imprinted chromosomes), whereas deletions of SNRPN exon 1 are involved in the maternal→paternal imprint switch and are defective in PWS patients with imprinting mutations (i.e., two maternally imprinted chromosomes). The paternal pattern of imprinting appears to be the default status for the genes that have been characterized thus far (Dittrich et al. 1996). The breakpoint in our patient was found to lie between SNRPN exons 2 and 3 and therefore is believed to be outside the ICE. We have shown both normal expression of three of five genes examined and normal methylation at three of three loci. Although we know that there are both methylated and unmethylated versions of each gene, we assume that the unmethylated (paternal) alleles are on the derivative, translocation chromosomes, since we have determined it to be paternal in origin. It is not possible to determine

Table 2

RT-PCR of 11 Transcripts from PWS Chromosomal Region

Locus	Expressed?
ZNF127	Yes
SNRPN:	
Exon 1	Yes
Exons 1 and 2	Yes
Exon 2	Yes
Exons 2 and 3	No
Exon 3	No
Exons 1–3	No
Exons 4 and 5	No
D15S226E (PAR5)	No
IPW	Yes
D15S227E (PAR1)	Yes

whether this translocation disrupts the imprint status of these three loci, because we do not have the opportunity to study further generations of this *de novo* rearrangement. Likewise, we will not be able to address issues of imprint initiation, spreading, or maintenance in our patient. The translocation breakpoint is presumably far enough from the ICE that it should have little or no impact on the functions of the ICE. There is only one gene, UBE3A, distal to this patient's breakpoint, that is known to be maternally imprinted; however, UBE3A is imprinted only in brain and during a short time during embryogenesis, thus precluding analysis in our patient.

Since we have shown both normal methylation patterns of three loci on this patient's chromosomes 15 and normal expression of three of five loci, we surmise that the full PWS phenotype can be manifested by the lack of SNRPN (exons 3–10) and/or D15S226E (PAR5) gene expression. The fact that our patient did not express D15S226E and SNRPN exons 4–10 suggest that D15S226E is under the same regulatory control of SNRPN and possibly is another 3' exon. A similar PWS patient has been described, by Sun et al. (1996), as having a *de novo* balanced translocation breakpoint between SNRPN exons 2 and 3; however, Sun et al.'s patient expressed not only SNRPN exons 1 and 2 but also SNRPN exons 3–10 from cultured fibroblast cells. Sun et al. proposed that this expression of exons 3–10 was the result of a fusion with an unidentified promoter on chromosome 19. Similarities between that patient and our patient include decreased fetal movement, hypotonia, poor sucking reflex, narrow bifrontal diameter, almond-shaped eyes, down-turned mouth, failure to thrive in infancy and early childhood, rapid weight gain at age >2 years, undescended testes, and lack of hypopigmentation. One difference was that our patient had normal length hands and feet, but this presumably is due to his racial background (Cassidy et al. 1996).

Another report (Schulze et al. 1996) has described an atypical PWS patient with a *de novo* balanced translocation breakpoint between D15S226E and IPW. This patient expressed SNRPN and D15S226E but neither IPW nor D15S227E (PAR1). It should be noted, however, that EBV-transformed cells, which, with respect to methylation and gene expression, are notorious for differing from *in vivo* cell types, were used for the analysis of this patient. Another report (Conroy et al. 1997) has described an "atypical" PWS patient with a balanced *de novo* translocation breakpoint between SNRPN and IPW, with expression, from EBV-transformed lymphoblasts, of SNRPN (distal exons) and D15S226E but with no expression of IPW and D15S227E. Collectively, these cases indicate that both the expression of SNRPN exons 1–3 and colinearity with distal loci are necessary to prevent the PWS core phenotypic features. It appears that expression of both SNRPN/D15S226E and IPW/

D15S227E are necessary for prevention of PWS. The four mutations described thus far, which include that in our patient, have occurred on the father's chromosome 15, but it is not known whether they occurred on the father's maternal or paternal copy. Loci that are distal to such a translocation would be expected to retain the grandparental imprint pattern. Although our patient expressed IPW and D15S227E, our analyses were performed on RNA extracted from peripheral blood and may not reflect the levels found in brain during fetal development. Similarly, other reports of PWS patients with balanced translocations have dealt with expression from either cultured fibroblasts or peripheral blood and may not reflect the levels found in brain tissues during early development.

Acknowledgments

We thank Mr. Dana Bangs for providing the original expert cytogenetic analysis, and Nicki Chun, M.S., Susan Schelley, M.Ph., and Susan Donlon, M.S. for follow-up and genetic counseling on this patient. This research was supported in part by funding from the National Institutes of Health (HD28015).

Electronic-Database Information

URLs for data in this article are as follows:

Genome Database, <http://gdbwww.gdb.org>

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