Clinical Spectrum and Molecular Diagnosis of Angelman and Prader-Willi Syndrome Patients With an Imprinting Mutation

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Recent studies have identified a new class of Prader-Willi syndrome (PWS) and Angelman syndrome (AS) patients who have biparental inheritance, but neither the typical deletion nor uniparental disomy (UPD) or translocation. However, these patients have uniparental DNA methylation throughout 15q11-q13, and thus appear to have a mutation in the imprinting process for this region. Here we describe detailed clinical findings of five AS imprinting mutation patients (three families) and two PWS imprinting mutation patients (one new family). All these patients have essentially the classical clinical phenotype for the respective syndrome, except that the incidence of microcephaly is lower in imprinting mutation AS patients than in deletion AS patients. Furthermore, imprinting mutation AS and PWS patients do not typically have hypopigmentation, which is commonly found in patients with the usual large deletion. Molecular diagnosis of these cases is initially achieved by DNA methylation analyses of the DN34/*ZNF127*, PW71 (*D15S63*), and *SNRPN* loci. The latter two probes have clear advantages in the simple molecular diagnostic analysis of PWS and AS patients with an imprinting mutation, as has been found for typical deletion or UPD PWS and AS cases. With the recent finding of inherited microdeletions in PWS and AS imprinting mutation families, our studies define a new class of these two syndromes. The clinical and molecular identification of these PWS and AS patients has important genetic counseling consequences. Am. J. Med. Genet. 68:195–206, 1997.
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INTRODUCTION

Angelman (AS) and Prader-Willi (PWS) syndromes are distinct neurobehavioral disorders, both of which are associated with genomic imprinting in chromosome 15q11-q13 [reviews Nicholls, 1993, 1994]. Genomic imprinting refers to the differential epigenetic modification and subsequent gene expression of a specific set of genes, dependent upon the sex of the parent-of-origin

of the gene. Most PWS patients have either a large paternally derived deletion of 15q11-q13 (70–75% of patients) [Butler et al., 1986; Nicholls et al., 1989a; Robinson et al., 1991; Mascari et al., 1992] or maternal uniparental disomy (UPD) (20–25% of patients) [Nicholls et al., 1989b; Robinson et al., 1991; Mascari et al., 1992]. In contrast, AS patients usually have a large maternally derived deletion of 15q11-13 (65–75% of patients) [Knoll et al., 1989; Magenis et al., 1990; Zackowski et al., 1993; Williams et al., 1990; Saitoh et al., 1994] or, rarely, paternal UPD (2% of patients) [Malcolm et al., 1991; Nicholls et al., 1992; Bottani et al., 1994; Gillessen-Kaesbach et al., 1995b]. Approximately 15–25% of AS patients do not have either a maternal deletion or paternal UPD and occasionally show familial occurrence [Clayton-Smith et al., 1992; Wagstaff et al., 1992; Meijers-Heijboer et al., 1992], and it has been postulated that these patients may have a mutation in the putative AS gene [see Nicholls, 1993, 1994]. The lack of this class in PWS is most consistent with the proposal that at least two genes may be involved in the full expression of the PWS phenotype. Thus, mutation in one of these genes would only result in a specific subset of clinical features and may contribute to the poorly understood group of patients classified clinically as "atypical PWS" or "PWS-like."

Several imprinted genes have been identified in chromosome 15q11-q13: *SNRPN* [Glenn et al., 1993b, Nakao et al., 1994; Reed and Leff, 1994; Sutcliffe et al., 1994; Glenn et al., 1996], *IPW* [Wevrick et al., 1994], and *ZNF127* [Jong et al., 1996], as well as two relatively uncharacterized anonymous transcripts, *PAR-1* and *PAR-5* [Sutcliffe et al., 1994]. Each of these imprinted genes identified within 15q11-q13 is expressed from the paternal chromosome only and is therefore a candidate gene to be involved in the PWS phenotype. The latter finding of multiple imprinted genes within 15q11-q13 is consistent with the hypothesis that PWS results from loss of expression of more than one gene. Two of the imprinted genes, *SNRPN* [Glenn et al., 1993b; Buiting et al., 1995; Sutcliffe et al., 1994; Glenn et al., 1996] and DN34/*ZNF127* [Driscoll et al., 1992; Jong et al., 1996], as well as two other loci within 15q11-q13, PW71 [Dittrich et al., 1992, 1993] and Y48.5 [Buiting et al., 1995], detect parent-of-origin specific DNA methylation (DNA methylation imprints). Moreover, asynchronous DNA replication within 15q11-q13 has also been shown [Kitsberg et al., 1993; Knoll et al, 1994; LaSalle and Lalande, 1995]. The putative single AS gene has not been identified, although it is predicted to be expressed from the maternal chromosome only. Although a recent report described the finding of a small 200 kb deletion near *D15S113* in a single AS patient [Buxton et al., 1994], more recent data suggests that this initial report was incorrect, and that the location is likely to be more proximal [Michaelis et al., 1995; Burke et al., 1996].

Recently, several PWS and AS patients with neither deletion nor UPD were reported to have a DNA methylation abnormality throughout 15q11-q13 [Glenn et al., 1993a; Reis et al., 1994; Nicholls, 1994; Sutcliffe et al., 1994; Buiting et al., 1994, 1995; Saitoh et al., 1996]. These patients appear to have a mutation in the mechanism of genomic imprinting within 15q11-q13. PWS imprinting mutation patients have an exclusively maternal methylation pattern on the paternally derived chromosome, while AS imprinting mutation patients show an exclusively paternal methylation pattern on the maternally derived allele in 15q11-q13. These findings suggest that imprinting mutation patients have a mutation that prevents resetting of imprinting during gametogenesis in a parent and grandparent [Reis et al., 1994; Buiting et al., 1995; Saitoh et al., 1996]. We have very recently discovered microdeletions upstream of the *SNRPN* gene in patients with both PWS and AS imprinting mutations, which indicates that this region (termed the imprinting center, or IC) is essential for the normal resetting of genomic imprinting within 15q11-q13 [Buiting et al., 1995; Saitoh et al., 1996].

Clinical information on two sets of familial PWS have been previously presented [Lubinsky et al., 1997; Örstavick et al., 1992], but it was not known at that time that these families had an imprinting mutation. Reports to date on imprinting mutation patients have not presented clinical information, but simply stated that patients studied for an imprinting mutation "had the typical PWS or AS clinical presentation" [Glenn et al., 1993a; Reis et al., 1994; Buiting et al., 1994, 1995; Sutcliffe et al., 1994; Saitoh et al., 1996]. We present here the detailed clinical features of a series of AS and new PWS imprinting mutation patients, as well as molecular diagnosis by DNA methylation analysis at multiple loci. These findings define a new class of Prader-Willi and Angelman syndrome patients, with important genetic counseling consequences.

CLINICAL REPORTS
PWS-U Family

Clinical findings of three affected sibs in the PWS-U family were reported previously by [Lubinsky et al., 1987] (see Fig. 1a). Briefly, each sib was hypotonic and difficult to feed in infancy, had delayed psychomotor development, developed an insatiable appetite, and at age 21–27 years had a height below the 3rd centile, small hands and feet (below the 3rd centile), uncontrollable behavior, mental retardation, skin picking, and somnolence [Lubinsky et al., 1987]. A fourth sib had the neonatal course of PWS, but died of pneumonia at age 10 months prior to study. High-resolution cytogenetic analysis at the 850 band level showed a normal karyotype [Lubinsky et al., 1987].

PWS-O Family

Clinical manifestations of the two affected sibs in the PWS-O family were also reported previously [Örstavick et al., 1992] (see Fig. 1a). Briefly, each sib had neonatal hypotonia and feeding problems, delayed psychomotor development, insatiable appetite after 3–4 years of age, temper tantrums, somnolence (in the older child), relative insensitivity to pain, inability to vomit, height below the 25th centile and weight above the 97.5th centile, hypogonadism, mild mental retardation, and facies typical of PWS [Örstavick et al., 1992]. Pigmentation was normal in the PWS-O sibs, as was high-resolution chromosome analysis. A third sib had the prenatal and neonatal course of PWS, but died of respiratory distress
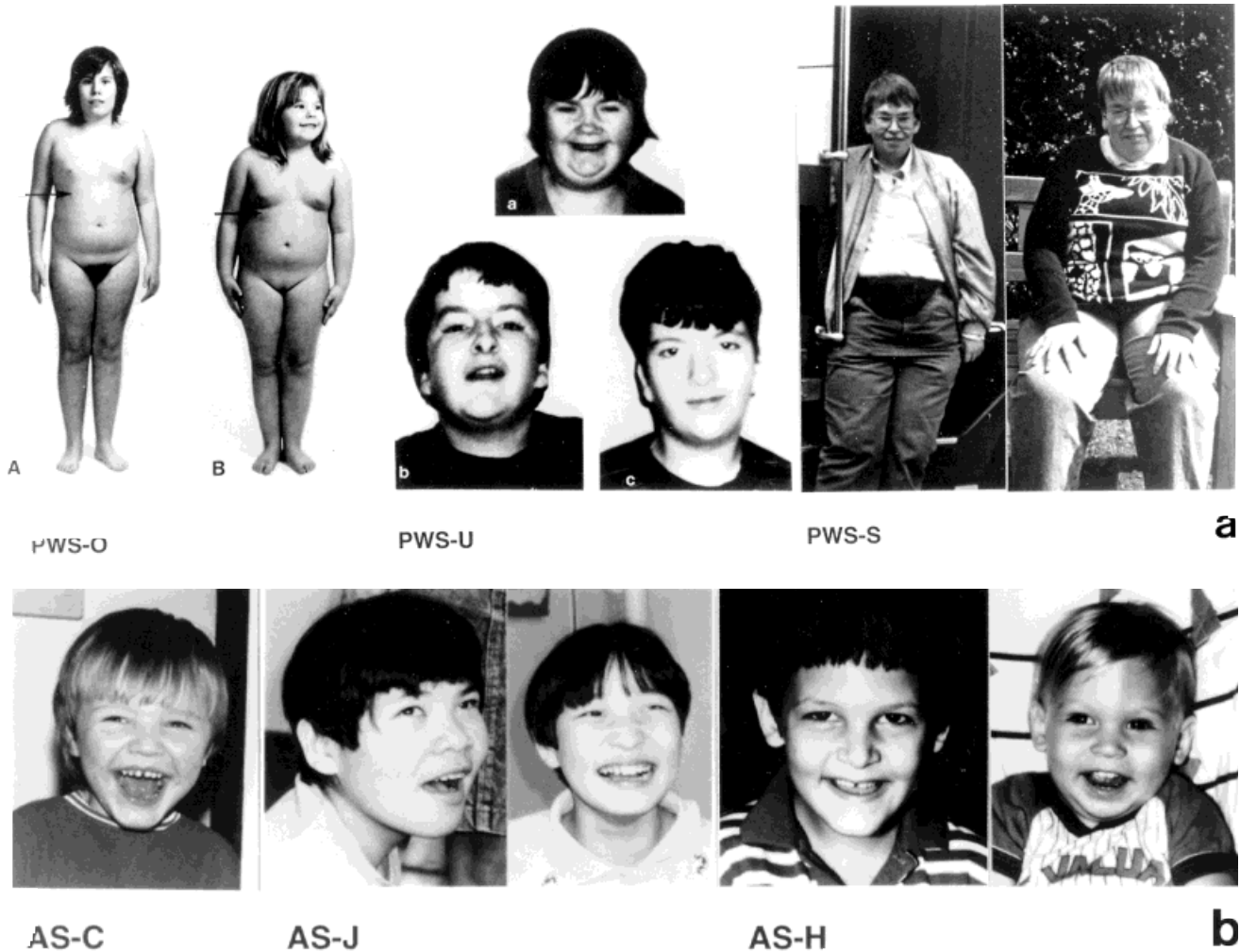
Fig. 1.   Clinical photographs of PWS and AS patients with an imprinting mutation. **a:** The two sibs from the PWS-S family are compared with two previously reported PWS imprinting mutation families [PWS-O, Örstavick et al., 1992; PWS-U, Lubinsky et al., 1987]. **b:** The affected individuals from three AS families (AS-C, AS-J and AS-H) are illustrated. The photographs of PWS-O and PWS-U sibs are reproduced with permission from Örstavick et al., 1992 and Lubinsky et al., 1987, respectively.

at age 7 days [Örstavick et al., 1992]. At least one atypical trait for PWS is the very early menarche in the girl (K. Örstavick, personal communication).

PWS-S Family

The first propositus, a boy, was born after an uneventful pregnancy as the first child of a healthy, non-consanguineous German couple. Fetal movements were normal. Birth weight at term was 3,020 g, length was 50 cm, and OFC (head circumference) was 35 cm, and severe hypotonia, feeding difficulties, cryptorchidism, and scrotal hypoplasia were noted. Obesity developed after his 6th month of life. Development was delayed, with unassisted walking at 2.6 years and active speech starting at 3 years, but severe articulation problems were present. Sagittal synostosis was surgically corrected in the same year. Testosterone injections were given because of hypogonadism, followed by orchidopexy and vasectomy at age 14 years. Now, at the

age of 30 years the patient is mildly retarded (IQ = 65, HAWIK). He has temper tantrums, depression, and anger, especially in situations that overstimulate him. He is working in a sheltered institution and lives with his family. At the age of 3 years the diagnosis of PWS was first considered and was confirmed by DNA methylation testing at age 28 years [Reis et al., 1994]. Physical examination at 29 years (Fig. 1a) documented a height of 165 cm (<3rd centile), weight of 93 kg (>97th centile), and OFC of 59 cm (90–97th centile). Hand length was 16 cm and foot length was 22 cm, both being <3rd centile. He showed obesity, narrow bifrontal diameter, myopia, a high palate, dental caries, downturned corners of the mouth, acromicria, and hypogonadism. He had normally pigmented skin, with multiple scars from skin picking.

A girl with the same syndrome was born to the same couple 3 years later. The diagnosis of PWS was suspected at age 3 months, at the same time the diagnosis

was made in her brother. During pregnancy, reduced fetal movement was noted. Spontaneous birth took place at term, with birth weight of 3,600 g, length of 56 cm, and no recorded OFC. Developmental delay was similar to that of her brother. Active speech started at 3 years and was characterized by articulation problems. She has fewer temper tantrums and behavior problems than her brother. IQ testing was 85 at age 16 (HAWIK). She works in a sheltered institution. Recently, progressive osteoporosis and scoliosis were noted. Molecular testing as above confirmed the diagnosis of PWS at age 26 years [Reis et al., 1994]. Cytogenetic analyses from cultivated peripheral blood lymphocytes showed a normal karyotype at the 650 band level for both sibs. Clinical examination at the age of 26 years (Fig. 1a) confirmed PWS on the basis of obesity, scoliosis, myopia, high palate, narrow bifrontal diameter, acromicria, and hypogonadism. She had primary amenorrhea. Her height was 152 cm (<3rd centile), weight was 83 kg (>97th centile), and OFC was 57 cm (90th–97th centile).

AS-C Family

The propositus was born after a 34 week gestation to a 26-year-old G1 mother and a 28-year-old father. Fetal movements began at the normal time but were not strong. Ultrasound at 11–2 weeks and AFP at 16 weeks were normal. There was premature rupture of membranes and he was delivered vaginally with pitocin induction at 34 weeks. Birth weight was 2,245 g and length was 49.5 cm. Apgar scores were 9 at one and five minutes. He had mild jaundice and was discharged at 3 days of life. He had problems with vomiting and failure to thrive as well as apnea in the first few weeks of life, and was diagnosed at 1 week of age as having reflux, for which he was treated with supportive methods and Reglan without success. At 1 month of age he was admitted for vomiting and was found to have pyloric stenosis, which was surgically corrected. He was noted during surgery to have partial malrotation of the intestine. He also had significant periodic breathing. At 6 months he had bilateral inguinal hernia repairs, and he had exotropia repair at 1½ years. His health has generally been good with the exception of an episode of mononucleosis at 2 years of age, and occasional episodes of reactive airway disease. He has had no seizures. Development was delayed. He walked at 2 years, but gait has remained stiff with some side to side motion.

Because of rapidly enlarging head size, he had two head MRI scans, at 1 year and at 2½ years, and both showed extra axial fluid collections of uncertain etiology and ventricles at the upper limits of normal. He had a barium swallow at 1 year showing poor tongue mobility. He had a chromosome analysis (600 band level) at 2½ years, which was normal. The boy is the only child of his parents. A paternal second cousin died of SIDS. There are no other potential genetic disorders in a four generation family history, and there is no known consanguinity.

At examination at 35 months (Fig. 1b) his height was 94.4 cm (25th–50th centile), weight was 18.2 kg (>95th centile), OFC was 51 cm (50th–75th centile), and blood pressure was 129/77 mm Hg. He was very inquisitive and in constant motion throughout the room. He walked with a broad-base, stiff jerky gait with his arms flexed. He had no words nor true babbling but did make occasional vowel sounds and grunts. He had episodes of unprovoked "hysterical" laughter. Deep tendon reflexes were brisk, tone was diffusely and mildly increased, head was brachycephalic, and the face was very round with a very broad, tall forehead. His eyes were widely spaced (interpupillary distance 6.2 cm, >97th centile). His nose was broad and fleshy and other parts were also somewhat fleshy but structurally normal and not coarse. His mouth was wide and teeth were widely spaced, with the chin somewhat prominent. There was no heart murmur. Limbs appeared normal, with normal hand length, 11.5 cm (50th centile). He has had multiple ophthalmologic evaluations relating to his strabismus, and his retinas were felt to be lightly pigmented but not outside the normal range. Hair coloring is fair, but similar to his mother. He is in an early intervention program and receives OT, PT, and speech therapies. He is hyperactive and sleeps for only a few hours at a time, roaming around the house at night. His mother notes that he loves playing with water, and that he seems to have overly sensitive hearing, but he would not cooperate for formal testing.

AS-J Family

The proposita (now 19 years old, female: Fig. 1b) was born as the first child of a healthy, non-consanguineous Japanese couple at 39 weeks gestation. She had no problems during pregnancy or delivery. Family history was unremarkable. Birth weight was 3,200 g, length was 55 cm, and OFC was 33.5 cm. Developmental delay was noticed by 6 months, and generalized seizures began at 8 months of age. The diagnosis of Angelman syndrome was made at the age of 17 years on the basis of prominent mandible, small head circumference, flat occiput, inappropriate laughter, ataxic gait, and epilepsy. She has never developed meaningful speech. Her IQ was <20 (could not be measured). She was not hypopigmented.

A similarly affected maternal half-sister (now 15 years old, female: Fig. 1b) was born as the third child of her mother. She has a healthy 17-year-old brother from the same father. Her birth weight was 2,470 g, height was 48 cm, and OFC was 30 cm. She had no problems during pregnancy or delivery. Developmental delay was noted at 4 months, and she developed seizures at 5 years. She has not developed any meaningful speech. The diagnosis of AS was made at the age of 13 years. She has a prominent mandible, small head circumference, and ataxic gait. Her IQ was not measured because of poor cooperation, but estimated to be below 20. She does not have hypopigmentation.

AS-H Family

The propositus in this family (now 12 years old, male: Fig. 1b) was born as the first child of non-consanguineous parents. The family history is unremarkable for mental retardation or neurological disorders except his younger brother, who is similarly affected (see below). The parents have also had two spontaneous abortions. The pregnancy and delivery were unremarkable, and birth weight was 3,145 g. At 2 years

of age, developmental delay was noted. A computed tomography (CT) and MRI scan of the head were normal. He began walking age at 2.5 years. At age 9 years, he was referred to the University of Florida Genetics Program where a diagnosis of Angelman syndrome was made. His height was 128 cm (25th centile), weight was 27.5 kg (50th centile), and OFC was 52.0 cm (45th centile). His face was unremarkable except for a tendency for tongue protrusion and somewhat small, wide-spaced mandibular teeth. He had normal pigmentation, and no strabismus nor brachycephaly. His behavior was typical of AS. He was quite curious and active in the room and enjoyed manipulating small objects and turning pages in books. He walked with a slightly ataxic gait with a slight anterior tilt to the pelvis. On occasion, his arms were uplifted but this was not impressive. During excitability, he had excessive facial grimacing suggestive of laughter and he had uplifted waving arms. He developed seizures at age 9 years, and he is treated with anticonvulsive medication. He has five words of speech but cannot speak in sentences. His receptive language is better than his expressive language.

A similarly affected full brother is now 5 years old (Fig. 1b). The pregnancy and delivery were unremarkable. The first several months of life were notable for irritability, spitting up, and regurgitation. Developmental delay and tremulousness were obvious at 6 months. An MRI scan at 18 months of age showed a small area of superficial encephalomalacia in the parasylvian tissue of the right parietal region. This was associated with a tangle of arteries that may be feeding into an A-V malformation. He was first examined by UF Genetics at the age of 19 months, at which time his height was 78.8 cm (5th centile), weight was 10.9 kg (25th centile), and OFC was 46.5 cm (30th centile). He had no craniofacial anomalies, strabismus, or brachycephaly. He did have light colored hair but without impressive skin hypopigmentation. There was generalized hyperreflexia and a movement disorder was evident, consisting of significant tremulousness and truncal ataxia. He has a happy, friendly demeanor, and hand flapping when excited. He began having seizures at 2 years, both generalized seizures and drop attacks, and remains on anticonvulsive medication. At 5 years he is still not walking and he has no speech. He is significantly more severely affected than his brother, with respect to receptive and expressive language, ataxia, tremulousness, and age-of-onset of the seizure disorder.

MATERIALS AND METHODS
DNA Extraction

DNA was extracted either from peripheral blood leukocytes for DNA methylation analysis, or from lymphoblastoid cell lines for other studies, by standard procedures [Nicholls et al., 1989a].

Microsatellite Analysis

Highly polymorphic microsatellite markers within 15q11-q13 were analyzed by PCR using a modification of standard techniques [Weber and May, 1989]. Primer sequences for loci listed in Table I (Operon Technologies Inc., Alameda, CA; Research Genetics, Birmingham,

AL; P.K.R., unpublished data) were obtained from the Genome Database (Johns Hopkins University). For each marker, one of the primers was end-labeled for 1 hour at 37°C in a 10 µl volume consisting of 1.2 µM primer, 25 µCi [γ-^{32}P] ATP at 3,000 Ci/mmol (NEN Research Products, Boston, MA), 50 mmol Tris-HCl, pH 7.5, 10 mmol MgCl$_2$, 5 mmol dithiothrietol, and 5 units T4-polynucleotide kinase (Boehringer Mannheim). PCR amplification was performed with 27–30 cycles of 1 minute at 95°C denaturation, 30–50 second at 56°C annealing (or as appropriate for each primer pair), and 2–10 minutes at 72°C extension in a final volume of 15–25 µl. Each reaction consisted of 30–50 ng genomic DNA, 200 µM each of dATP, dCTP, dGTP, and TTP, 0.4 µM unlabeled primer, 0.06 µM labeled primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, and 0.17–0.32 units *Thermus aquaticus* DNA polymerase. After amplification, the reaction was mixed with an equal volume of formamide loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 5 minutes, and chilled on ice; 3–4 µl of each sample was directly loaded onto 5–8% denaturing polyacrylamide gels (AT Biochem, Malvern, PA), which were processed and autoradiographed according to standard procedures [Sambrook et al., 1989]. Alleles of descending size were consecutively assigned within each family.

DNA Methylation Analysis

Three loci were investigated for DNA methylation. DNA was digested with EcoRI + HpaII (DN34/ *ZNF127*), BglII + HhaI or CfoI (PW71), or XbaI + NotI (*SNRPN* exon -1), run on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized as described previously [Nicholls et al., 1989a]. The probes used were a 1,333 bp subfragment of DN34/*ZNF127* [Driscoll et al., 1992; Jong et al., 1996], PW71B (*D15S63*) [Dittrich et al., 1993], and a 0.6 kb EcoRI-NotI fragment which contains exon -1 of *SNRPN* [Glenn et al., 1996]. DNA methylation using PW71 and *SNRPN* exon -1 was maintained in transformed lymphoblastoid cell lines, but was not maintained at *ZNF127* (data not shown).

RESULTS
Exclusion of a Large Deletion or UPD

Microsatellite analysis demonstrated biparental inheritance in the two affected sibs analyzed in the PWS-U family, at three loci within 15q11-q13 and at two additional loci distal to 15q13 (Table I: *D15S63, D15S122, GABRB3, D15S111, D15S100*). Fluorescent in situ hybridization (FISH) analysis on the AS-C proband using the *D15S10* probe (ONCOR) showed signals on both chromosome 15 homologs (data not shown), indicating no large deletion within 15q11-q13. Microsatellite analysis showed biparental inheritance in the AS-C proband at three loci within 15q11-q13 (Table I: *D15S11, D15S113, GABRB3*). These findings exclude the presence of a large deletion or uniparental disomy (UPD) in the affected individuals of the PWS-U and AS-C families. However, for *D15S128* in the AS-C family, the affected propositus, unaffected mother, and unaffected maternal grandfather (Table I)

TABLE I. Microsatellite Analysis in the PWS-U and AS-C Families[†]

Markers	PWS-U				AS-C				
	P1	P2	F	M	P	F	M	MGF	MGM
D15S18	2/2	2/2	1/2	2/2	ND	ND	ND	ND	ND
D15S11	1/1	1/1	1/1	1/1	3/5*	2/3	4/5	5/5	1/4
D15S63	1/2*	1/2*	1/1	2/2	ND	ND	ND	ND	ND
D15S128	1/1	1/1	1/1	1/1	2/-	1/2	2/-	3/-	2/3
D15S122	2/3*	2/3*	1/2	3/4	ND	ND	ND	ND	ND
D15S10	ND	ND	ND	ND	1/1	1/1	1/1	ND	ND
D15S113	2/2	2/2	1/2	2/2	3/4*	2/4	1/3	3/4	1/5
GABRB3	1/3*	1/3*	1/2	3/4	1/3*	1/4	3/4	2/3	4/4
D15S103	1/2	1/2	1/2	1/1	ND	ND	ND	ND	ND
D15S95	1/1	1/1	1/1	1/2	ND	ND	ND	ND	ND
D15S111	1/2*	1/2*	2/2	1/3	ND	ND	ND	ND	ND
FES	1/2	1/2	1/2	1/2	ND	ND	ND	ND	ND
D15S100	1/2*	1/2*	1/1	2/3	ND	ND	ND	ND	ND

[†] P, patient; F, father; M, mother; MGF, maternal grandfather; MGM, maternal grandmother; *, bi-parental inheritance; -, D15S128 is deleted in the AS-C patient, mother, and maternal grandfather; ND, not determined; markers in bold are within the typical PWS/AS deletion; alleles are denoted 1 to 5 on descending size.

had a deletion of one allele, which is explained by our recent finding of a microdeletion of 60 kb in the AS-C family [Buiting et al., 1995].

Dosage and RFLP data for the PWS-S family [Reis et al., 1994] and the AS-H family [Glenn et al., 1993a], as well as dosage and microsatellite data for the AS-J family [Saitoh et al., 1994], have been reported elsewhere. For each of these three families, these data excluded a typical large deletion or UPD. Since the father's DNA for each of the two affected half-sibs in the AS-J family was not available, paternal UPD has not been formally excluded, although it is a priori extremely unlikely, and a microdeletion has recently been found [Saitoh et al., 1996]. In the PWS-S family, a relatively small deletion which includes SNRPN was the first molecular abnormality found in an imprinting mutation family [Reis et al., 1994]. The centromeric breakpoint is represented by a 9.5 kb BglII junction fragment identified by probe L48.3I [Buiting et al., 1995]. A reduced hybridization signal obtained by a probe for IPW [Wevrick et al., 1994] suggested that the deletion also included this locus. Using a probe for PAR-1 [Sutcliffe et al., 1994], we detected a normal BglII fragment of 6.6 kb and the 9.5 kb deletion junction fragment (data not shown). We estimate the deletion in the PWS-S family to be 200 kb (see Fig. 4 below). These data also indicate that PAR-1 maps distal to IPW (see Fig. 4).

DNA Methylation Studies

DN34/ZNF127 DNA methylation. For the DN34 probe [Driscoll et al., 1992], which detects DNA methylation at the ZNF127 gene [Jong et al., 1996], normal individuals have four bands detected by an EcoRI/HpaII double digest: a completely methylated 5.2 kb band, partially methylated bands of 4.3 and 4.0 kb, and an unmethylated band of 3.5 kb (Fig. 2a, lanes 1, 5) which are also detected in the parents of each family analyzed in this study (lanes 8, 12) [also see Glenn et al., 1993a]. PWS patients with a typical large deletion or UPD show a reproducible reduction in intensity of the 3.5 kb band (lane 2, Fig. 2a), whereas AS patients with a

typical large deletion (lane 3, Fig. 2a) or UPD show a significant reduction in the 4.3 and 4.0 kb bands [Driscoll et al., 1992]. The affected propositus in the AS-C family (lane 4), the affected sibs in the AS-J family (lanes 6, 7) and the affected sibs in the AS-H family [Glenn et al., 1993a] all show the typical reduction in intensity of the 4.3 and 4.0 kb bands (Fig. 2a). In the PWS-U family, one sib showed a typical PWS pattern with reduction of the 3.5 kb band (lane 10), whereas the affected brother (lane 9) and the father (lane 11) showed an abnormal partially unmethylated pattern which was also not typical of either syndrome (Fig. 2a). DNA methylation using the DN34 probe has not been studied in the PWS-S family, although DNA methylation was normal when analyzed with the p34 (D15S9) probe [Reis et al., 1994], which detects sites within 2–10 kb from those detected by the DN34 probe [Driscoll et al., 1992].

PW71 (D15S63) DNA methylation. The PW71B probe detects DNA methylation at the D15S63 locus: normal individuals have two bands of 8.0 and 6.6 kb when DNA is digested with BglII and HhaI, as do the parents in each family studied (Fig. 2b, lanes 3, 5, 6, 8, 9, and 12). The lower and upper bands are of paternal and maternal origin [Buiting et al., 1994], respectively, as demonstrated by typical AS (lane 1) and PWS (lane 2) deletion patients. PWS patients with an imprinting mutation have only the maternal band, as shown by affected sibs in the PWS-U family (lane 4, Fig. 2b and data not shown) and the PWS-S sibs [Reis et al., 1994]. The probands in the AS-C (lane 7) and AS-J (lanes 10, 11, Fig. 2b) families have a significant reduction in intensity of the 8.0 kb maternal band, which is consistent with the typical AS pattern. The affected sibs in the AS-H family also had the typical AS pattern at this locus [Glenn et al., 1993a].

SNRPN exon −1 DNA methylation. A paternally derived unmethylated band of 0.9 kb and a maternally derived methylated band of 4.2 kb are detected for XbaI/NotI digestion by the SNRPN exon −1 probe [Glenn et al., 1996], as shown by normals (lane 3, Fig.
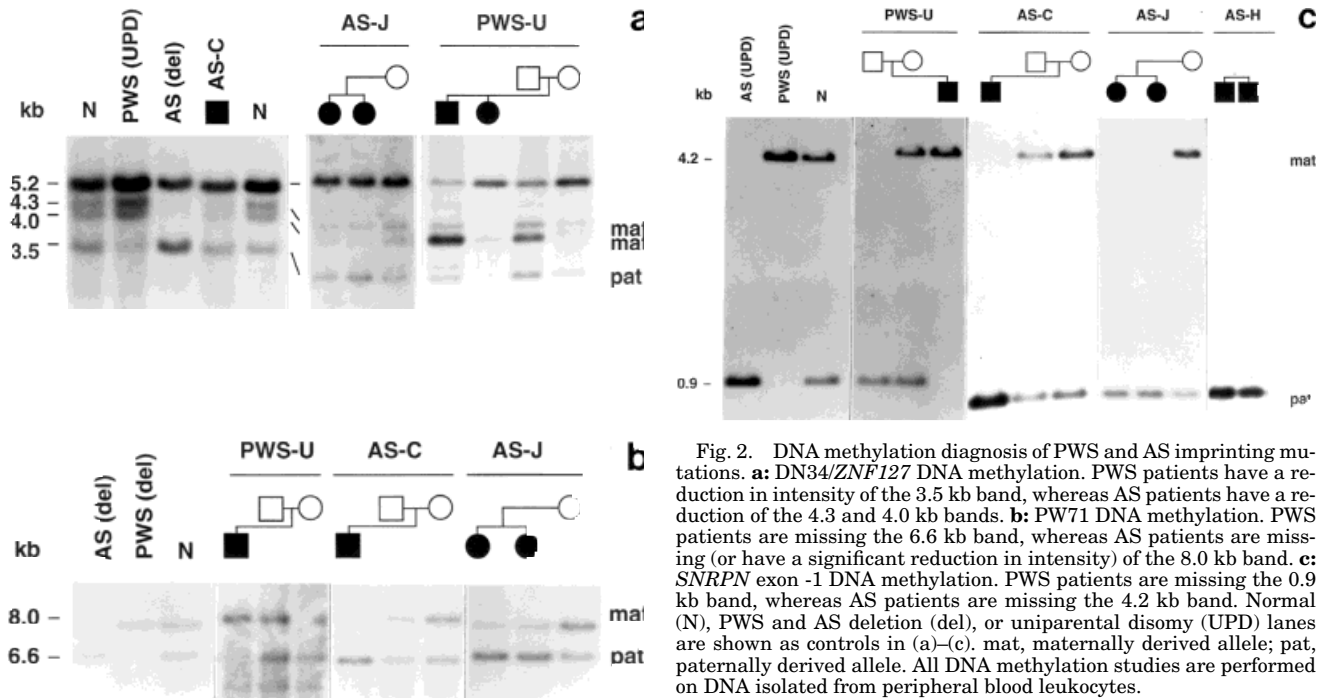
Fig. 2.   DNA methylation diagnosis of PWS and AS imprinting mutations. **a:** DN34/*ZNF127* DNA methylation. PWS patients have a reduction in intensity of the 3.5 kb band, whereas AS patients have a reduction of the 4.3 and 4.0 kb bands. **b:** PW71 DNA methylation. PWS patients are missing the 6.6 kb band, whereas AS patients are missing (or have a significant reduction in intensity) of the 8.0 kb band. **c:** *SNRPN* exon -1 DNA methylation. PWS patients are missing the 0.9 kb band, whereas AS patients are missing the 4.2 kb band. Normal (N), PWS and AS deletion (del), or uniparental disomy (UPD) lanes are shown as controls in (a)–(c). mat, maternally derived allele; pat, paternally derived allele. All DNA methylation studies are performed on DNA isolated from peripheral blood leukocytes.

2c) and AS (lane 1) or PWS (lane 2) patients with UPD. The affected propositus in the three AS imprinting mutation families, AS-C, AS-J, and AS-H, all show the typical AS methylation pattern (lanes 7, 10, 11, 13, and 14; Fig. 2c), whereas the parents have the normal pattern (lanes 8, 9, and 12). The PWS-S and PWS-U families have been previously studied, and in both families, the *SNRPN* exon -1 probe is deleted from the paternal allele [Buiting et al., 1995]: the PWS-U results are reproduced here for comparison to the AS imprinting mutations and to results with other methylation-sensitive probes. It should be noted that due to the deletion of this locus, the parental origin of the deletion can be determined (see lanes 4, 6, Fig. 2 c). The presence of the maternal band only in the PWS-U proband demonstrates a paternal deletion, and in the father, the presence of the paternal band only indicates that he has a maternally derived deletion at this locus [Buiting et al., 1995].

A HpaII/MspI RFLP in the SNRPN gene. During the course of our investigation of *SNRPN* exon -1 methylation, we found a novel MspI/HpaII restriction fragment length polymorphism (RFLP) (Fig. 3a,b,c). Analysis of 31 independent chromosomes demonstrated an allele frequency of 0.39 for the 1.4 kb allele and of 0.61 for the 0.6 kb allele. Since the polymorphic site is subject to parent-of-origin specific DNA methylation, HpaII can only be used to determine the paternal allele (Fig. 3b), but MspI can be used to determine both parental alleles as shown in a two generation family (Fig. 3c). The use of this novel RFLP may aid identification of microdeletions close to the *SNRPN* gene in PWS imprinting mutation families [Buiting et al., 1995], as well as identification of the methylation status

of a particular parentally inherited chromosome associated with either polymorphic allele.

DISCUSSION
Clinical Spectrum of Imprinting Mutation Patients

Since the first molecular description of two sibs with a new molecular class of Angelman syndrome [Glenn et al., 1993a], termed imprinting mutations, several additional AS and PWS imprinting mutation patients have been found [Reis et al., 1994; Buiting et al., 1994; Buiting et al., 1995; Saitoh et al., 1996]. However, there have been no previous clinical reports of AS patients with an imprinting mutation, and only two previous reports of PWS families [Lubinsky et al., 1987; Örstavick et al., 1992] later shown to have an imprinting mutation. Here, we have described the clinical details of five patients from three AS imprinting mutation families, and two patients from a new PWS imprinting mutation family. One clue to the identification of such families is the recurrence of the syndrome in sibs (discussed further below). Since the first child born to parents may have an imprinting mutation, it would be of immense value if PWS and AS patients with an imprinting mutation could be recognized clinically as being distinct from the typical PWS and AS patients with a deletion or UPD. Furthermore, this is essential to understanding the clinical phenotype-genotype correlations in each molecular class, particularly for the eventual goal of developing therapeutic intervention for the various clinical symptoms of each disorder.

The five affected individuals from the AS-C (one patient), AS-J (two), and AS-H (two) families all fulfill the diagnostic criteria for classical AS (Table II) pro-
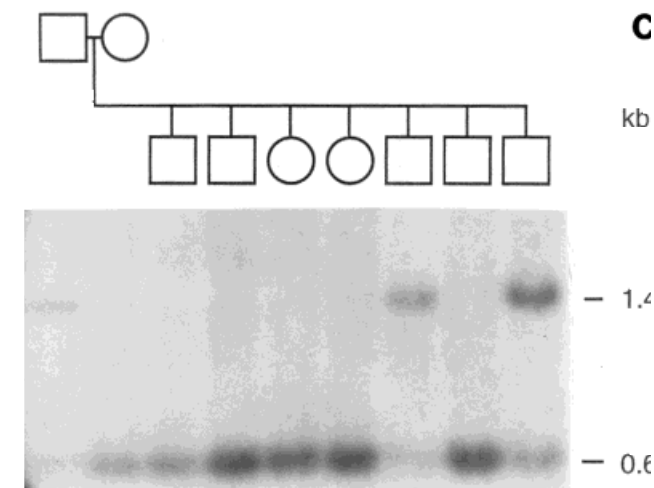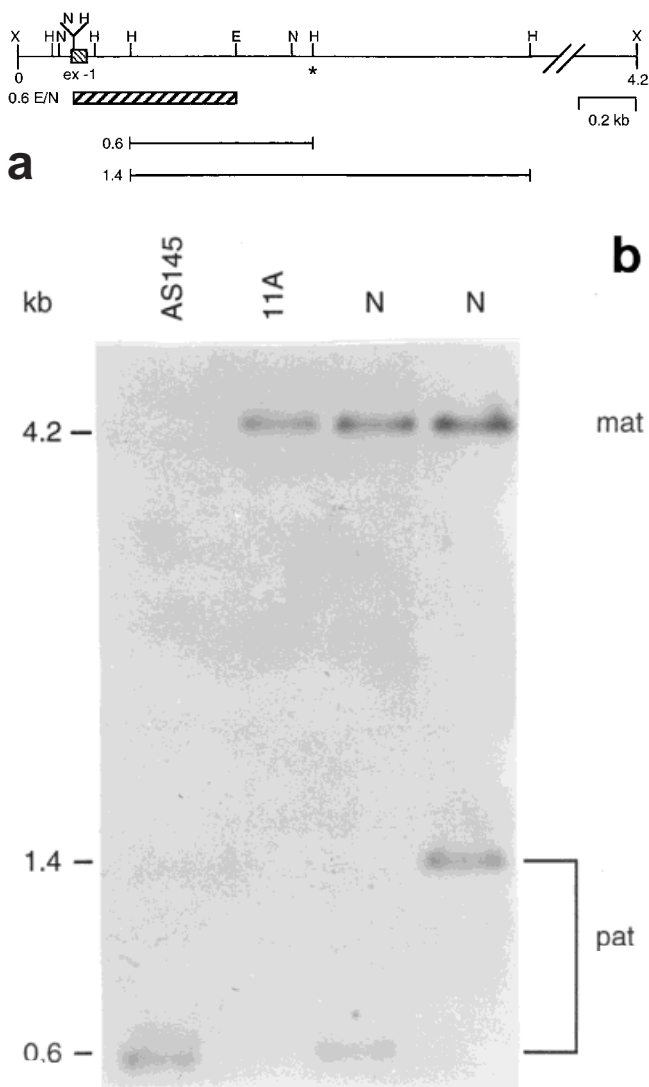
Fig. 3.   Identification of a HpaII/MspI restriction fragment length polymorphism (RFLP) in the *SNRPN* gene. **a:** Restriction enzyme map of the 5′ end of the *SNRPN* gene illustrating the HpaII/MspI RFLP. Also shown are exon -1 and the 0.6 kb DNA probe (hatched boxes); polymorphic fragments (horizontal lines); the polymorphic HpaII (H) site (asterisk); E, EcoRI; N, NotI; X, XbaI. **b:** HpaII RFLP. Genomic DNA was digested with XbaI and HpaII and hybridized with the *SNRPN* exon -1 probe. The HpaII RFLP is only seen on the unmethylated paternally derived allele, as seen in the AS patient, AS145 (lane 1). 11A (lane 2) is a PWS deletion control, and N represents normal controls. **c:** Mendelian inheritance in a two generation pedigree. Genomic DNA was digested with MspI and hybridized with the *SNRPN* exon -1 probe. The father is heterozygous and the mother is homozygous for the lower allele. HpaII is methyl-sensitive, whereas the isochizomer MspI is not.

posed by [Williams et al., 1995], as each patient has all the four clinical signs present in 100% of AS cases (developmental retardation, lack of speech, movement/balance disorder, and behavior usually involving frequent laughter). Each affected individual (Table II) also has many of the frequent (>80% of patients) or associated (20–80% of patients) AS features [Williams et al., 1995]. However, the patient in the AS-C family has an above average head circumference (Table II) and has not yet developed seizures at 3 years, but he does have many other associated AS characteristics, such as wide spaced teeth, prognathia, and strabismus. Similarly, the first sib in the AS-H family also lacks microcephaly and had delayed onset of seizures (9 years of age). His younger brother is more severely affected and has all the typical characteristics, although he has more tremulousness than most AS patients. He developed seizures at 2 years. He also lacks microcephaly, although the two patients from the AS-J family do have microcephaly. Indeed, microcephaly is not universal in AS, and seizure onset

varies. As a group, the five AS imprinting mutation patients described here have the typical neurobehavioral pattern and cannot easily be distinguished from patients in other AS classes, although it must be noted that only 2/5 of these AS imprinting mutation patients have microcephaly and none has hypopigmentation (Table II).

The two sibs from the new PWS family, PWS-S, fulfill the criteria for classical PWS (Table III), as with the previously described affected individuals in the PWS-U [Lubinsky et al., 1987] and PWS-O [Örstavick et al., 1992] families. All affected individuals in each of these families score 9–10.5 points on the PWS consensus diagnostic criteria scale, where a score of 8 is required for diagnosis [Holm et al., 1993]. None of the affected patients with PWS or AS in these families has hypopigmentation (Tables II, III). However, hypopigmentation in these disorders appears to be associated with deletion [Butler et al., 1986] of the non-imprinted *P* gene [Lee et al., 1994; Spritz et al., submitted], and thus imprinting mutation patients would be expected

TABLE II. Summary of Manifestations in AS Imprinting Mutation Patients, in Addition
to Severe Developmental Delay/Mental Retardation, Ataxic Gait and/or Tremulousness,
Abnormal EEG, Episodes of Unprovoked Laughter, Hyperactivity, and Sleep Disturbances

Patient (age in years)	Presence of clinical characteristic[a]				
	Microcephaly[b]	Brachycephaly	Hypo-pigmentation[b]	Speech[c]	Seizures[d]
AS-C (3)	−	+	−	0	−
AS-J P1 (19)	+	+	−	0	8 mo
AS-J P2 (15)	+	ND	−	0	5 yr
AS-H P1 (12)	−	−	−	5	9 yr
AS-H P2 (5)	−	−	−	0	2 yr

[a] +, Clinical feature is present in patient; −, clinical feature is not present in the patient at the age shown; ND, not determined.
[b] Clinical feature showing a significant difference from AS patients with a typical large deletion.
[c] Number of words of speech is shown.
[d] Age of onset of seizures, where mo, months; yr, years.

to have similar pigmentation to unaffected family members.

In conclusion, clinical analysis of seven PWS patients in three families [PWS-S, this report; PWS-U, Lubinsky et al., 1987; PWS-O, Örstavick et al., 1992], and five AS patients in three families (AS-C, AS-H, and AS-J: this report) suggests that the clinical presentation in PWS and AS patients with an imprinting mutation is not easily distinguishable from the clinical phenotype of the deletion and UPD classes of PWS and AS patients, respectively. The exception is the lack of microcephaly in some AS imprinting mutation patients.

Several authors [Driscoll et al., 1993; Bottani et al., 1994; Gillessen-Kaesbach et al., 1995b; Williams et al., in preparation] have recently described more mildly affected patients in AS cases with paternal UPD, including the lack of typical facial changes, late onset and milder seizures, as well as milder or absent ataxia and motor dysfunction. However, the spectrum of phenotype of patients with UPD is not well delineated. The AS cases with an imprinting mutation would be predicted to be equivalent to the UPD cases (see below), but based on the five patients described here, the phenotypic spectrum is not significantly different from that seen in patients with a deletion.

DNA Methylation Analysis in Imprinting Mutation Patients

All imprinting mutation patients show DNA methylation abnormalities at multiple loci within 15q11-q13, including the PW71 (*D15S63*), *SNRPN*, and DN34/*ZNF127* loci. Nevertheless, the DNA methylation pattern is uniparental and typical for the syndrome: i.e., maternal in PWS and paternal in AS. As a consequence, abnormal DNA methylation is at present the diagnostic tool of choice for the imprinting mutation class of PWS and AS patients, when combined with normal results for chromosome 15q11-q13 deletion (using FISH or molecular studies) or UPD analysis (e.g. using microsatellite studies). This is emphasized in a recent report of a joint American Society of Human Genetics/American College of Medical Genetics statement [Cassidy et al., 1996].

Although the PWS-S sibs have been described as having normal DNA methylation at p34 [Reis et al.,

1994], p34 measures methyl-sensitive sites in the region 2–10 kb upstream of the *ZNF127* gene, whereas the DN34 probe that is commonly used measures sites within the *ZNF127* promoter and gene body [Driscoll et al., 1992; Mowery-Rushton et al., 1996; Jong et al., 1996], and thus DN34 is a more reliable indicator than p34. Furthermore, it is likely that peripheral blood leukocytes are not the relevant tissue for testing the *ZNF127* gene, since at least 50% of cells show complete DNA methylation, rather than the 100% differential methylation imprinting of parental alleles that is seen in brain and germ cells [Jong et al., 1996]. Of course, the latter tissues cannot be tested for diagnostic purposes. In our studies, analysis of DNA methylation at *ZNF127* has been difficult (e.g., one PWS-U sib: Fig. 2a), although it was clear for the PWS-O sibs [Reis et al., 1994] and AS cases [this report; Glenn et al., 1993a]. Analysis of DNA methylation at PW71 was reliable, although several AS patients (e.g., AS-J sibs and the AS-C proband, Fig. 2b) show a partially methylated band. In comparison, DNA methylation analysis using the *SNRPN* exon -1 probe always demonstrated only one band, either unmethylated or methylated for AS or PWS, respectively. We conclude that PW71 and *SNRPN* exon -1 probes detect imprinting mutations. In addition, PW71 has been reliably used on over 167 PWS patients [Gillessen-Kaesbach et al., 1995a] and the *SNRPN* exon -1 probe in over 190 PWS and AS patients [Glenn et al., 1996; Conroy et al., unpublished data]. Therefore, we recommend the use of both PW71 and/or *SNRPN* exon -1 probes for accurate molecular diagnosis in PWS and AS.

Microdeletions Upstream of the *SNRPN* Gene

It was initially found that the *SNRPN* gene was completely [Reis et al., 1994; PWS-S] or partially [Sutcliffe et al., 1994; PWS-O] deleted in two PWS imprinting mutation families, but the extent of these deletions was not determined. Subsequently, we [Buiting et al., 1995] identified microdeletions located upstream of *SNRPN* in the PWS-S, PWS-U, PWS-O, and AS-C families (and an additional family, AS-D). The extent of the microdeletions in these five PWS and AS imprinting mu-

TABLE III. Summary of Clinical Findings in PWS Imprinting Mutation Patients,
in Addition to Decreased Fetal Movement and Hypogonadism

Patient (age in years)	Presence of clinical characteristic[a]								
	Neonatal hypotonia	Feeding problems	Obesity	Typical facies[b]	IQ[c]	Behavior	Short stature	Small hands	Hypopigmentation[d]
PWS-S P1 (29)	+	+	+	+	65	+	+	+	−
PWS-S P2 (26)	+	+	+	+	85	−	+	−	−
PWS-O P1 (12)[e]	+	+	+	+	Mild	+	−	−	−
PWS-O P2 (7)[e]	+	+	+	+	Mild	+	+	+	−
PWS-U P1 (27)[f]	+	+	+	+	54	+	+	+	−
PWS-U P2 (26)[f]	+	+	+	+	50	+	+	+	−
PWS-U P3 (21)[f]	+	+	+	+	57	+	+	+	−

[a] +, Clinical feature is present in patient; −, clinical feature is not present in the patient at the age shown; ND, not determined.
[b] Typical facies including narrow bifrontal diameter, almond-shaped eyes, small appearing mouth with thin upper lip, down-turned corners of the mouth.
[c] IQ for PWS-S family determined using the HAWIK scale, for PWS-U using the WAIS scale. Mild, mild mental retardation, IQ not available.
[d] Clinical feature showing a significant difference from PWS patients with a typical large deletion.
[e] Örstavick et al., 1992.
[f] Lubinsky et al., 1987.

tation families are shown in Figure 4: our new data presented here determine the distal boundary of the PWS-S family deletion, as well as allow mapping of the *D15S128* marker within the region affected by microdeletions. The latter finding may aid detection of a microdeletion in informative AS imprinting mutation families. These microdeletions affect the function of an element that we term the imprinting center (IC) which is involved in resetting imprinting within 15q11-q13, and thereby causes the disease phenotypes [see Buiting et al., 1995; Saitoh et al., 1996]. The same deletions have been found in the phenotypically normal father and paternal grandmother in PWS imprinting mutation families, and in the phenotypically normal mother and maternal grandfather in AS imprinting mutation families [Buiting et al., 1995; Sutcliffe et al., 1994; Saitoh et al., 1996]. Therefore, the microdeletions do not directly cause disease phenotypes but appear to fix an "epigenotype" [Buiting et al., 1995; Saitoh et al., 1996] and prevent resetting of the imprint during gametogenesis. The disease phenotype only arises when the deleted chromosome passes through gametogenesis (i.e., resetting of the imprint) of the opposite sex from that in which the deletion occurred. Recently, we have shown that small microdeletions of the IC region are also found in the AS-J and AS-H families [Saitoh et al., 1996].

Significance of Imprinting Mutations for Genetic Counseling

Large deletion and UPD cases of AS and PWS have a recurrence risk less than 1%, although advanced maternal age in maternal UPD does contribute a theoretical increased risk [Robinson et al., 1991; Mascari et al., 1992]. However, PWS or AS cases due to imprinting mutations do have a significant recurrence risk for not only the relevant parent but also certain other close relatives, and thus it is important to determine the molecular defect in a new patient. To date, most AS and PWS patients with an imprinting mutation contain a familial microdeletion in the im-

printing center, although there are several families without a detectable microdeletion [Buiting et al., 1995; Driscoll et al., unpublished data]. Thus, the carrier parent (mother in AS, father in PWS) has a 50% recurrence risk for subsequent pregnancies. Sibs of the same sex as the carrier parent have the same risk if identified to also be silent carriers of the deletion, or an a priori risk as high as 25% if untested. Theoretically, sibs of the other sex than the carrier parent (who are shown to be silent carriers) would not be at known risk of having an affected child, but their children would be at risk to be silent carriers, and thus PWS or AS would be significant risk factors for future generations. However it should be noted that we have not to date observed any families with more than one carrier individual. In the case of a new mutation in the germline or in early embryogenesis [Saitoh et al., 1996], the risk to the individual or future generations depends on the sex of the patient and the grandparental origin of the chromosome on which the mutation arose. Since microdeletions can now be identified in many of these families [Buiting et al., 1995; Saitoh et al., 1996], genetic counseling concerning these risks, and the offer of DNA testing (in a research laboratory performing these analyses) of at-risk relatives and pregnancies, should be made available to affected families.
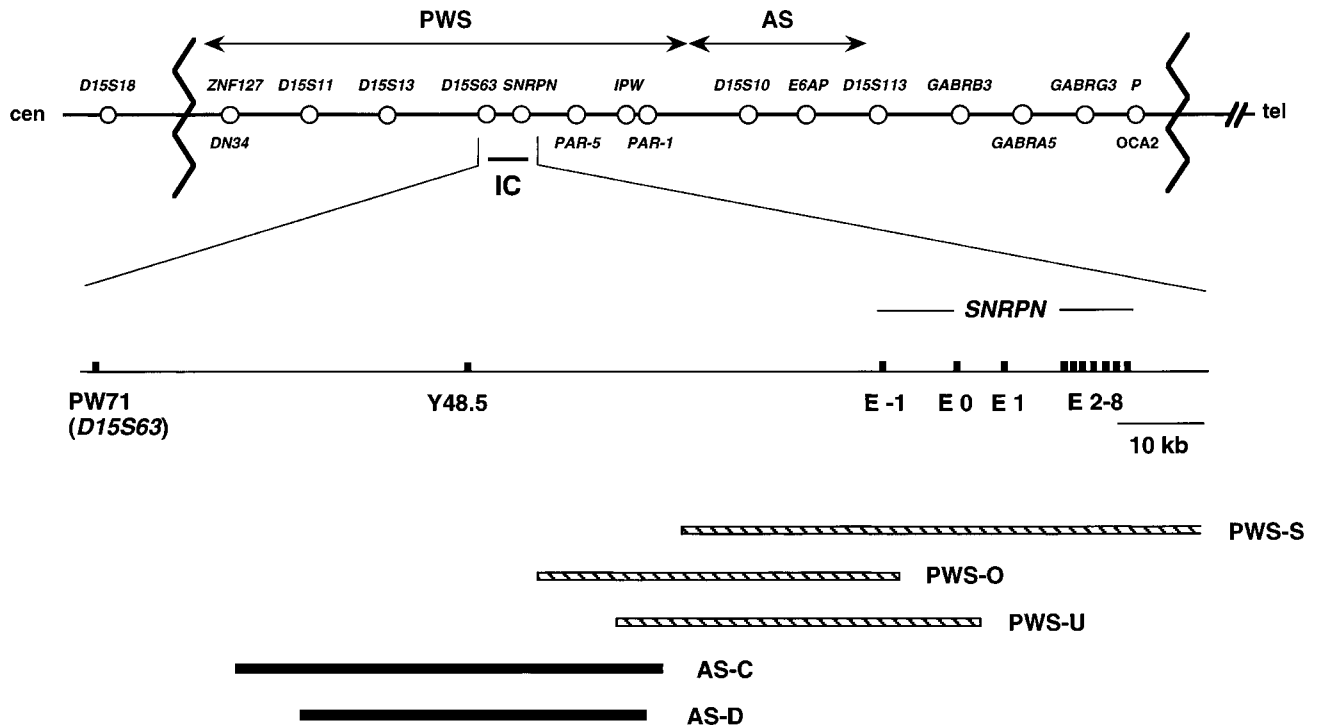
Fig. 4.   Summary of microdeletions in chromosome 15q11-q13 in PWS and AS imprinting mutation families. The location of inherited microdeletions within 15q11-q13 in the PWS-U, PWS-S, PWS-O, AS-C, and AS-D families are shown [adapted from Buiting et al., 1995]. The location of DNA methylation imprints detected by PW71, Y48.5 and *SNRPN* exon -1 probes are shown, along with the position of various genes and DNA markers within 15q11-q13. PWS and AS critical regions are also indicated (arrows). The *ZNF127*, *SNRPN* and *IPW* genes, and *PAR-1* (location from data in this report) and *PAR-5* transcripts, are all expressed from the paternal chromosome only. No candidate AS gene expressed from the maternal chromosome only has yet been identified. The zigzag lines indicate the position of common deletion breakpoints in the majority of PWS and AS patients with a 15q11-q13 deletion; OCA2 represents oculocutaneous albinism type 2 which is due to mutations of the P gene [Lee et al., 1994]. See text for further details.

NOTE ADDED IN PROOF

We have recently described 6-80 kb microdeletions in an additional 4 AS families [Saitoh et al., 1996]. Paternal gene expression (imprinted genes) is silenced in PWS imprinting mutation parents, but is biparental in AS imprinting mutation parents, providing additional support for the hypothesis that the IC functions in germ line resetting of imprinting throughout 2 Mb of 15q11-q13 [Saitoh et al., 1996].

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