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Molecular Mechanism of Angelman Syndrome in Two Large Families Involves an Imprinting Mutation

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Summary

Patients with Angelman syndrome (AS) and Prader-Willi syndrome with mutations in the imprinting process have biparental inheritance but uniparental DNA methylation and gene expression throughout band 15q11-q13. In several of these patients, microdeletions upstream of the *SNRPN* gene have been identified, defining an imprinting center (IC) that has been hypothesized to control the imprint switch process in the female and male germlines. We have now identified two large families (AS-O and AS-F) segregating an AS imprinting mutation, including one family originally described in the first genetic linkage of AS to 15q11-q13. This demonstrates that this original linkage is for the 15q11-q13 IC. Affected patients in the AS families have either a 5.5- or a 15-kb microdeletion, one of which narrowed the shortest region of deletion overlap to 1.15 kb in all eight cases. This small region defines a component of the IC involved in AS (ie., the paternal-to-maternal switch element). The presence of an inherited imprinting mutation in multiple unaffected members of these two families, who are at risk for transmitting the mutation to affected children or children of their daughters, raises important genetic counseling issues.

Introduction

Angelman syndrome (AS) is a neurobehavioral disorder associated with genomic imprinting in chromosome 15q11-q13, characterized by severe mental retardation, lack of speech, ataxic gait, tremulousness, microcephaly, sleep disorder, and inappropriate laughter (Williams et al. 1995). AS occurs in ~1/15,000 births (Clayton-Smith and Pembrey 1992). AS arises from various genetic abnormalities in chromosome 15q11-q13, most commonly a de novo large cytogenetic deletion of maternal origin, paternal uniparental disomy (UPD) in rare patients, a mutation in the imprinting process, or an AS gene mutation (Nicholls et al. 1998). Prader-Willi syndrome (PWS) is also associated with the imprinted 15q11-q13 region but is clinically distinct and involves large deletions of 15q11-q13, UPD, and imprinting mutations (IMs) of parental origin opposite that of the similar genetic abnormalities in AS (Nicholls et al. 1998).

Mutations in the imprinting process were first recognized in AS (Glenn et al. 1993). These patients have neither the typical deletion nor UPD but show biparental inheritance with uniparental DNA methylation (Glenn et al. 1993; Reis et al. 1994; Buiting et al. 1995) and a uniparental gene expression pattern throughout 15q11-q13 (Saitoh et al. 1996). The AS IM patients reported previously include six patients with a microdeletion (Buiting et al. 1995; Saitoh et al. 1996), one patient with a putative point mutation (Dittrich et al. 1996) of a regulatory element termed the "imprinting center" (IC), and a series of patients without any detectable IC mutation (Buiting et al. 1995, 1998; Bürger et al. 1997).

Linkage of the AS gene to 15q was first suggested in one large AS family and two small sibships (Wagstaff et al. 1992, 1993) and by the sharing of maternal haplotypes in affected AS sibs (Clayton-Smith et al. 1992). Subsequent linkage analysis confirmed a chromosome 15q locus for AS (Meijers-Heijboer et al. 1992). The location of the AS gene was refined by use of different-sized deletions and the positions of translocation breakpoints in key patients with AS (Saitoh et al. 1992; Burke et al. 1996) or in patients lacking AS and having a maternal deletion in distal 15q11-q13 (Michaelis et al. 1995). The localization was further supported by the position of genetic crossovers in affected AS sibs (Greger et al. 1994; Nelen et al. 1994). Subsequently, Kishino et

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al. (1997) identified a gene (*UBE3A*) broken by a balanced paracentric inversion in an AS patient and demonstrated mutations of *UBE3A* in additional AS cases, each leading to frameshifts and premature translation termination. That mutations in *UBE3A* could cause AS was confirmed by the finding of de novo mutations in two additional AS patients (Matsuura et al. 1997). Subsequent studies have found maternally inherited or de novo inactivating *UBE3A* gene mutations in a larger series of patients with AS (Malzac et al. 1998). Surprisingly, *UBE3A* mutations were only found in ~30% of AS nondeletion, non-UPD, nonimprinting mutation cases (Malzac et al. 1998), suggesting either that additional mechanisms can silence *UBE3A* or that additional genes may be involved in AS.

We have restudied the large AS family (AS-O) for which linkage to 15q11-q13 was first reported, and we show here that this family has an IM rather than an AS gene mutation. A small microdeletion of only 5.5 kb is identified in the AS-O family in this study. The microdeletions identified previously in AS IM families defined a 2-kb shortest region of overlap (SRO) for the deletions. Abnormalities of this region are sufficient to prevent the paternal-to-maternal imprint switch by the IC in the maternal germline (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996; Nicholls et al. 1998). The microdeletion in the AS-O family narrows the critical region of the AS IC to 1.15 kb, which will help to define the critical genetic function of this region. The segregation of an IM in this large AS family and a larger IC microdeletion in a second large family (AS-F) has important diagnostic and genetic counseling consequences.

Patients and Methods

Clinical Phenotype of AS Patients

Two large families (AS-O and AS-F), each with multiple individuals affected with AS, were ascertained for this study (fig. 1*a* and *b*). Each affected individual has biparental inheritance for microsatellite markers throughout 15q11-q13 (fig. 1). The unique structure of these pedigrees and the small size of genetic aberrations (see the Results section) allows the possibility of useful phenotype-genotype correlations, and thus the clinical features of several affected individuals in each family are given briefly here.

AS-O Family, Case IV-5.—The patient was born at term to a G_1P_0 woman after an uncomplicated pregnancy with no illness or known teratogenic exposures. Growth parameters at birth were normal, with birth weight 3,990 g. The perinatal period was uncomplicated. She was an irritable infant. Global developmental delay was apparent by 6–8 mo of age, and the patient walked alone at 2.5 years. At 15 years of age she is able to feed herself finger foods and to use a fork. She communicates by using 10–20 signs and two single words that are recognizable by her family. She is daytime toilet trained by use of a strict schedule. Her height, weight, and head circumference have tracked along the 75th percentile. Pubertal changes were spontaneous and normal, with onset of menarche at age 14 years. She had strabismus surgery at the age of 3 years but has otherwise been healthy. She has never had a documented seizure; however, an electroencephalogram (EEG) done at the age of 3 years showed a typical pattern seen in children with AS (Boyd et al. 1988), involving high-voltage 3 cycles/s runs. She is very active, easily frustrated, and easily distracted. Problem behaviors include tantrums, pinching, hitting, and pulling hair. She is frequently awake and active during the night. The family set up an alarm system to keep her and her affected sister in the bedroom they share.

The clinical diagnosis of AS was made when she was 9 years old. Family history revealed a normal and an affected maternal half sib (case IV-7), as well as male and female maternal cousins with AS (cases IV-1 and IV-3; fig. 1*a*).

Physical examination at age 15 years (fig. 2*b*) revealed height at the 75th–90th percentile, weight at the 90th percentile, and head circumference at the 50th–75th percentile. She had abundant head hair and prominent hair over the temporal aspects of the forehead. She had a flat midface, a wide-appearing mouth, and mild prognathism. There was no scoliosis, heart murmur, or organomegaly. There was bilateral 5th finger clinodactyly. She had normally pigmented skin and hair. Neurologic findings included drooling, increased tone and deep tendon reflexes in all extremities, and jerky movements and hand flapping, both exaggerated with walking. The gait was broad based and unsteady, with arms held in flexion.

AS-O Family, Case IV-7.—The younger maternal half-sister of patient IV-5 (fig. 1*a*) was born at term to a then $G_4P_{2,0,1,2}$ mother after an uncomplicated pregnancy without known teratogenic exposures. Growth parameters were normal at birth, with weight 3,975 g, and there were no problems in the immediate perinatal period. In the patient's first weeks of life, her parents recognized that many of her physical and behavioral characteristics were similar to those of her oldest sister. She was extremely irritable and ate poorly. She had persistence of neonatal reflexes, abnormal tone, and developmental delay, recognized by the age of 4 mo when she had a persistent startle reflex and continued head lag. AS was diagnosed on clinical grounds at age 2 years. Her weight, length, and head circumference have tracked along the 50th percentile. She walked alone at 2.5 years. At 8 years of age (fig. 2*a*) she is able to go up and down steps and to use a fork to feed herself. She babbled at 9–10 mo but never developed speech. She now uses a few signs.

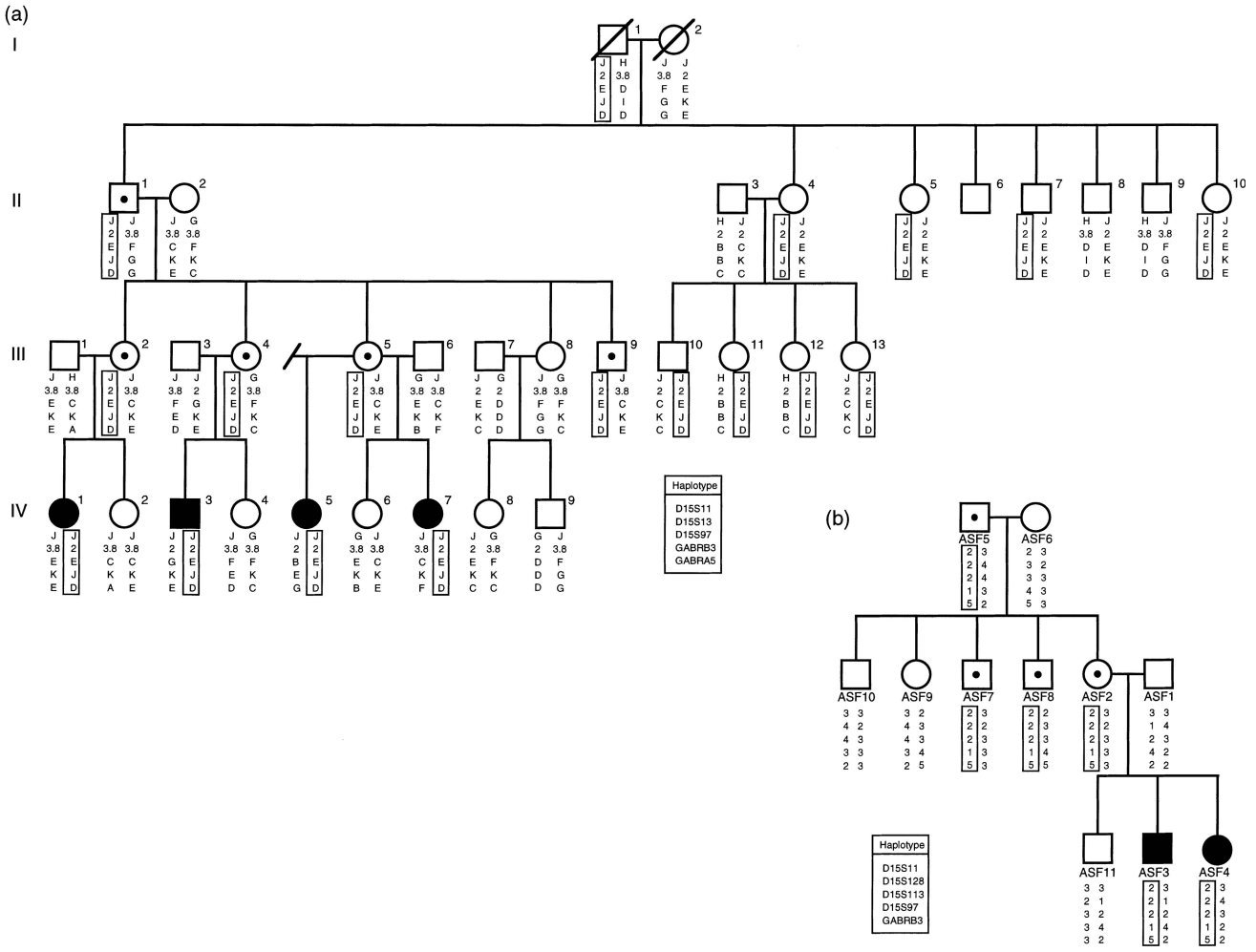

Figure 1 Pedigrees of the two AS families. *a,* AS-O family. *b*, AS-F family. The chromosome 15q11-q13 haplotype data for the AS-O family is from Wagstaff et al. (1993), and the haplotype on which the IC mutation arose in each family is boxed.

Her receptive language is better than her expressive language. The parents note that she is more likely than her affected sister to engage and play with other children and to interact with adults. She uses the toilet on a schedule. Both sisters love to play with toys that make noise. Her personality is generally pleasant, although she has significant behavior problems, with temper tantrums and occasional pinching and pulling hair. Her sleep is abnormal, rarely lasting more than 2 h at a time, and she is awake and playing most nights. She has had no health problems, nor has she had a seizure.

Physical examination revealed height at the 50th–75th percentile, weight at the 75th–90th percentile, and head circumference at the 50th percentile. The head was normally shaped. There was mild midface hypoplasia. The teeth were widely spaced, with a mixture of primary and secondary teeth, and her mandible was prominent. There was no scoliosis, heart murmur, or organomegaly. She had normal pigmentation of the skin and hair. Both hands were small, and she had increased tone in the extremities and brisk deep tendon reflexes throughout. Her gait was broad based, with arms held in flexion and hands flapping.

AS-O Family, Case IV-1.—The patient was examined at 19 years of age (fig. 2*c*). Birth weight was 3,890 g. She has no speech and has severe mental retardation, an ataxic unsteady gait, a very happy affect, and inappropriate laughter. She is affectionate and drools frequently. She has a high pain threshold, loves playing with water, and will not wear hats. She has a normal menstrual cycle and has never had seizures. EEG findings have been normal. She sleeps only 5–6 h each night. She can ride a three-wheeled bicycle. She was toilet trained at 8 years. On physical examination, she was nondysmorphic. Head circumference was 53 cm (−1 SD, whereas the father was also −1 SD and the mother was +1 SD). She

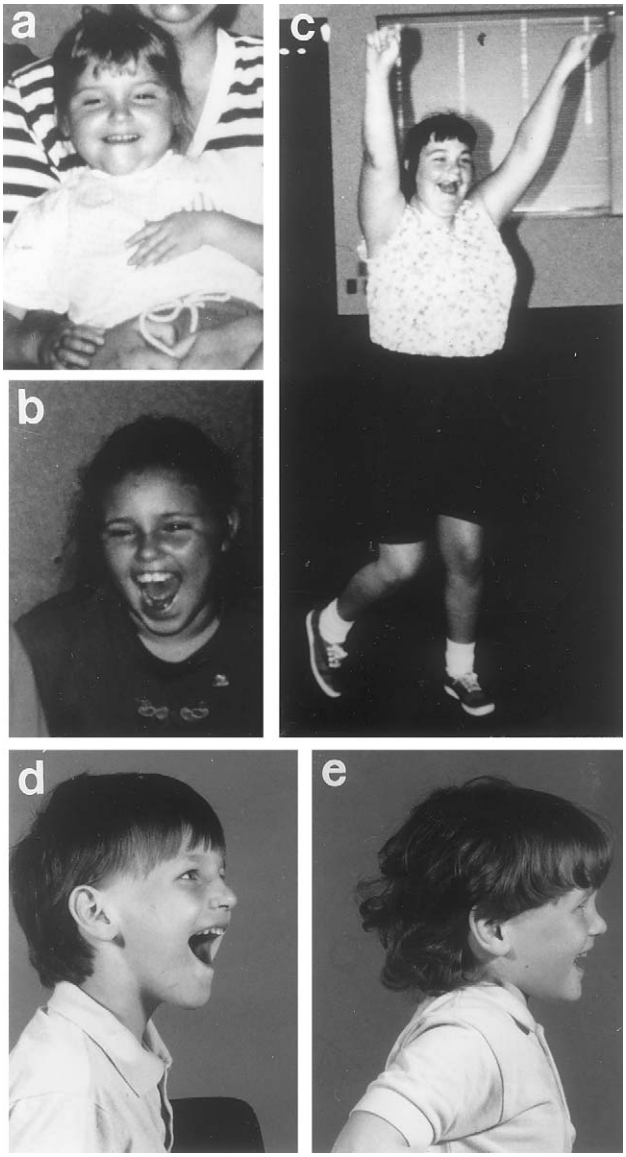

Figure 2 Clinical photographs of AS patients. *a, b,* Maternal half-sisters. *c,* Cousin with AS, in the AS-O family. *d, e,* Affected sibs in the AS-F family at 6 and 4 years of age.

had moderate obesity. There was no heart murmur or organomegaly. Breasts and pubic hair were Tanner stage V. Lordosis was found on spinal examination. She had genu valgum. Skin pigmentation was normal for the family. She had typical AS behavior, with easy excitability, hand flapping, very happy affect, no speech, ataxic gait with uplifted arms, and affectionate behavior with no stranger anxiety. Deep tendon reflexes were normal in the upper extremities and mildly increased in the lower extremities. There was no spasticity.

AS-O Family, Case IV-3.—The patient at the time was aged 14 years and was described by his two aunts (III-5 and III-2) as having severe mental retardation, no speech, a happy affect, inappropriate laughter, easy excitability, and an ataxic gait. In addition, he is a hyperactive child who sleeps very little and receives medication for his hyperactivity and to help him sleep at night. He has a high pain threshold and loves playing with water. He is more aggressive than his cousins (IV-1, IV-5, and IV-7). He was born after a 37-week gestation and weighed 2.7 kg.

AS-F Family, Case ASF3.—The proband, a 16-year-old mentally retarded boy, presented with many features typical of AS (fig. 2*d*, at 6 years of age). He was born after an uneventful pregnancy, with a birth weight of 4,350 g, height of 53 cm, and head circumference of 34 cm. He had sucking difficulties but an otherwise normal neonatal period. His growth has followed the 75th percentile, and his head circumference, the 50th percentile. He walked unsupported at 18 mo. His gait was ataxic. He was very restless, day and night. At the age of 3 mo, the child had staring and "jerking" episodes. He was given antiepileptic medication at the age of 1.5 years because of abnormal EEG findings and progressive convulsions. The epilepsy was classified as Lennox epilepsia and was treated accordingly. Over time, the convulsions have ceased, and he is no longer taking antiepileptic medication.

There are no clear-cut dysmorphic features, but he has slight telecanthus and a wide mouth, with protruding tongue, and some drooling. Pigmentation is normal. He is severely mentally retarded and has very little capacity for abstract thinking. He is hyperactive but is often in a happy mood and, at those times, is not restless. His gait is ataxic but not as severely ataxic as that of his sister. He cannot speak, except for a few words, but has become independent with activities of daily life and has mastered sign language, with 300–350 signs and even some "sentences." His behavior includes nail biting and tearing, and his pain sensitivity is markedly reduced.

AS-F Family, Case ASF4.—The 14-year-old sister (fig. 2*e*, at 4 years of age) of the proband was born after a normal pregnancy, with a birth weight of 3,920 g, length of 51 cm, and head circumference of 35.5 cm. Her neonatal findings were normal, but she was seen at the age of 1 mo for episodes of spasticity. She was also soon noticed to have "easy and loud laughing." A protruding tongue was also noticed, and she was suspected to suffer from a disorder similar to that of her brother. She also developed jerky episodes, did not learn to speak, and showed similar EEG findings. Antiepileptic medication was started at 1 year of age. She presented with restlessness, slow motor development, clumsiness, and an ataxic gait. Her growth has followed the 50th percentile, and her head circumference has been normal. She has a wide nose bridge with slight telecanthus. Pigmentation is normal.

She is somewhat more severely affected than her brother but is independent in her daily activities. She is happy and laughs often. She keeps her tongue out of her mouth and drools. She knows fewer hand signs than does her brother but is somewhat better in "sentences." She can speak a couple of words. She also has reduced sensitivity to pain and a habit of tearing her nails. She is somewhat restless, and she discontinued antiepileptic medication several years ago. Both siblings are fond of water, plastics, and pictures. Both can use a knife and fork for eating. Cytogenetic studies showed normal chromosomes in ASF2, ASF3 and ASF4, whereas FISH with the D15S10 and PML control probes (Oncor) showed no deletion in ASF2, ASF3, or ASF4.

The ASF patients' third cousin, a 4-year-old boy, also has AS, but he has a del(15)(q12)mat. Furthermore, he is related to ASF3 and ASF4 through his father.

DNA Extraction, Southern Hybridization, and Microsatellite Analyses

Blood samples were obtained from AS patients and parents according to protocols approved by the institutional review board at each of the relevant institutions. Many DNA samples from the AS-O family were a kind gift of Drs. J. Wagstaff and M. Lalande. DNA extraction from peripheral blood leukocytes and Southern hybridization with ^{32}P-labeled probes were performed by standard procedures (Buiting et al. 1995; Glenn et al. 1996), as was laser densitometry for dosage determination (Glenn et al. 1993). We used the Y48.5, 1.3XE, L48.33II, and L48.3I probes from the IC phage contig for microdeletion analysis as described elsewhere (Buiting et al. 1995; Saitoh et al. 1996). One new IC probe was developed, kb25XE, a 420-bp *Xba*I–*Eco*RV unique sequence fragment from L48.6I. The L48.3I probe contains repetitive elements and was preassociated with an excess of human placenta DNA and hybridized under competitive hybridization conditions, as described elsewhere (Buiting et al. 1995). Microsatellite analyses (AS-F family) were performed by standard techniques, with markers and PCR conditions from the Genome Database, whereas those for the AS-O family (fig. 1) have been described elsewhere (Wagstaff et al. 1992, 1993).

DNA Methylation Analyses

DNA was digested with *Xba*I/*Not*I, *Bgl*II/*Hpa*II (*SNRPN*) (Glenn et al. 1996), *Hin*dIII/*Hpa*II, or *Cfo*I (PW71B) (Dittrich et al. 1993) and was analyzed by Southern analysis. *Msp*I, an isochizomer of *Hpa*II, was also used for analysis of *SNRPN* in the AS-F family. The *SNRPN* exon 1 (0.6NE, Glenn et al. 1996; or kb17, Bürger et al. 1997) and PW71B probes were labeled and hybridized to each membrane, as described above.

IC Polymorphism and Breakpoint Analyses by PCR

After amplification with primers RN747 (5′-GTGA-AGCGAAGCTCAGACAT-3′) and RN748 (5′-GCTGT-CCTTGGATATATGCC-3′), which are located in the 1.3XE IC subclone, PCR products were digested with *Bst*NI for polymorphism analysis. For PCR, conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (31 cycles). To generate primer sets for breakpoint cloning, we sequenced the microdeletion region (T. Ohta and R. D. Nicholls, unpublished data), including a 2.1-kb *Eco*RI/*Xba*I fragment subcloned from phage subclone L48.6I (Buiting et al. 1995), and the L48.8III clone (Buiting et al. 1995). Primers RN307 (5′-TCAAAGGTG-CACCCTGAGGTCC-3′), within the kb25XE probe, and RN485 (5′-GGTAACTTACATCCTCTGCTG-3′), distal to the AS-SRO, were used for long-range PCR to clone the AS-O breakpoint. Conditions for long-range PCR were 94°C for 30 s, 62°C for 30 s, and 72°C for 3 min (31 cycles). AS-O family DNA and normal control DNA were subsequently amplified with primers RN719 (5′-TCTCCAGTGGAGCTCTCTTC-3′) and RN720 (5′-TGGGTGGCTCACTTTCCTAG-3′), synthesized from the sequences predicted to flank the AS-O breakpoints (T. Ohta and R. D. Nicholls, unpublished data). PCR conditions for the breakpoint PCR were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (31 cycles). Control PCR primers (RN440 and RN441) were from the PWS-SRO (Ohta et al. 1999 [in this issue]).

Results

DNA Methylation in Two Large AS Families

Probe 0.6NE, containing the *SNRPN* exon 1 region, detects allele-specific DNA methylation patterns (Glenn et al. 1996). The maternal, methylated allele is detected as a 4.3-kb band, and the paternal, unmethylated allele is detected as a 0.9-kb band on *Xba*I/*Not*I–digested DNA from normal individuals (fig. 3*a, lane 6*) and the mother of the two AS half-sisters (fig. 3*a, lane 3*). The parental origin of these alleles is defined by analysis of PWS and AS control subjects with a paternally or maternally derived deletion of 15q11-q13, respectively (fig. 3*a, lanes 4 and 5*). The two affected half-sisters in the AS-O family (IV-5 and IV-7, fig. 1*a*) lack the maternal, methylated 4.3-kb band, which is the typical AS pattern (fig. 3*a, lanes 1 and 2*). The intensity of the 0.9-kb band in IV-5 and IV-7 is twice that of this allele in normal individuals or in AS or PWS deletion patients, indicating that two chromosomes 15 are present. The haplotype analyses with five microsatellite markers (D15S11, D15S13, D15S97, GABRB3, and GABRA5) that have been described by Wagstaff et al. (1992, 1993) showed that these patients do not have UPD and have biparental inheritance (fig. 1*a*). Therefore, these two half-sisters

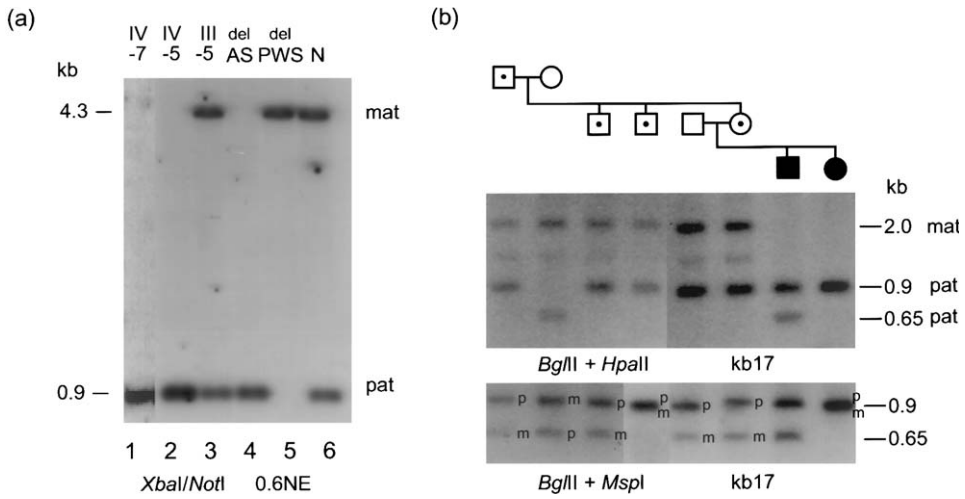

Figure 3 DNA methylation at *SNRPN* identifies an imprinting mutation in each AS family. In *a,* the affected AS-O half-sisters IV-7 (*lane 1*) and IV-5 (*lane 2*) have only the unmethylated band after *Xba*I/*Not*I digestion of DNA, whereas the mother (III-5) has biparental DNA methylation. In *b,* the AS-F family DNA is digested with *Bgl*II/*Hpa*II (*upper panel*) or *Bgl*II/*Msp*I (*lower panel*). *Hpa*II/*Msp*I are isochizomers that detect a polymorphic site in *SNRPN* intron 1 (Saitoh et al. 1997), and comparison of the band patterns allows inference of the parental origin of each band (m or p). See text for further details. mat (m) = maternal; pat (p) = paternal; del = deletion; and N = normal.

have biparental inheritance but a paternal, uniparental methylation pattern, which is diagnostic of an AS IM.

In the AS-F family, DNA methylation at *SNRPN* has been analyzed with *Bgl*II/*Hpa*II (fig. 3*b*). In normal individuals, a methylated maternal allele is detected as a 2-kb band, whereas the unmethylated paternal allele is detected as a 0.9- or 0.65-kb band because of a polymorphic *Hpa*II/*Msp*I site (Saitoh et al. 1997). Only the affected ASF3 and ASF4 sibs in the AS-F family show an abnormal methylation pattern characterized by an absence of the methylated band, as is typical for AS (fig. 3*b, upper panel*). On the basis of the *Hpa*II/*Msp*I polymorphism (fig. 3*b, upper and lower panels*), one sib (ASF3) shows heterozygosity for the *SNRPN* exon 1 region, indicating that there is no deletion of this region.

The latter analysis can be taken further to infer parental origin of individual alleles in the AS-F family, by comparison of the band pattern produced by *Msp*I with that produced by *Hpa*II (fig. 3*b*). In normal individuals, the paternal origin is given by the unmethylated *Hpa*II allele. For example, ASF8 is homozygous for the 0.9-kb *Msp*I band, and the 0.9-kb *Msp*I allele inherited from his father (ASF5) is of grandpaternal origin, since it is unmethylated on *Hpa*II analysis in ASF5. Similarly, ASF4 is homozygous for the 0.9-kb *Msp*I band, and the allele inherited from her mother (ASF2) is of grandpaternal origin, since the 0.9-kb *Msp*I allele in ASF2 is unmethylated on *Hpa*II analysis. Furthermore, this methylation analysis shows that the two uncles (ASF7 and ASF8) inherited the same paternal but different maternal chromosomes. These results are consistent with the microsatellite analyses (fig. 1*b*), which also indicate that both affected sibs inherited the same maternal but different paternal chromosomes.

The PW71B probe, which maps ~130 kb upstream of the *SNRPN* gene, also detects abnormal DNA methylation patterns in AS and PWS patients with a deletion, UPD, or IM (Dittrich et al. 1993; Buiting et al. 1995). The two affected sibs in the AS-F family had the typical AS DNA methylation pattern for PW71B, whereas the father and mother had a normal biparental DNA methylation pattern (data not shown).

Identification of a 5.5-kb Microdeletion in the AS-O Family

Some but not all AS patients with an IM have a microdeletion of the IC region ~25–30 kb upstream of *SNRPN* exon 1 (Buiting et al. 1995, 1998; Saitoh et al. 1996; Bürger et al. 1997). A *Bst*NI polymorphic site in the region of the IC that is deleted in AS IM cases (fig. 4*a*) has been identified in the AS-O family and in unrelated individuals (data not shown). This polymorphism was assessed in the two half-sisters (IV-5 and IV-7), mother, and father of IV-7, from the AS-O family. Patient IV-5 has only the lower (digested) allele, and IV-7 has only the upper (undigested) allele, the latter inherited from her father, who is heterozygous (fig. 4*b*). On the basis of the well-established maternal inheritance pattern in AS (Nicholls et al. 1998), the two affected patients must share a common maternal allele, and therefore the results indicate that each patient and the mother

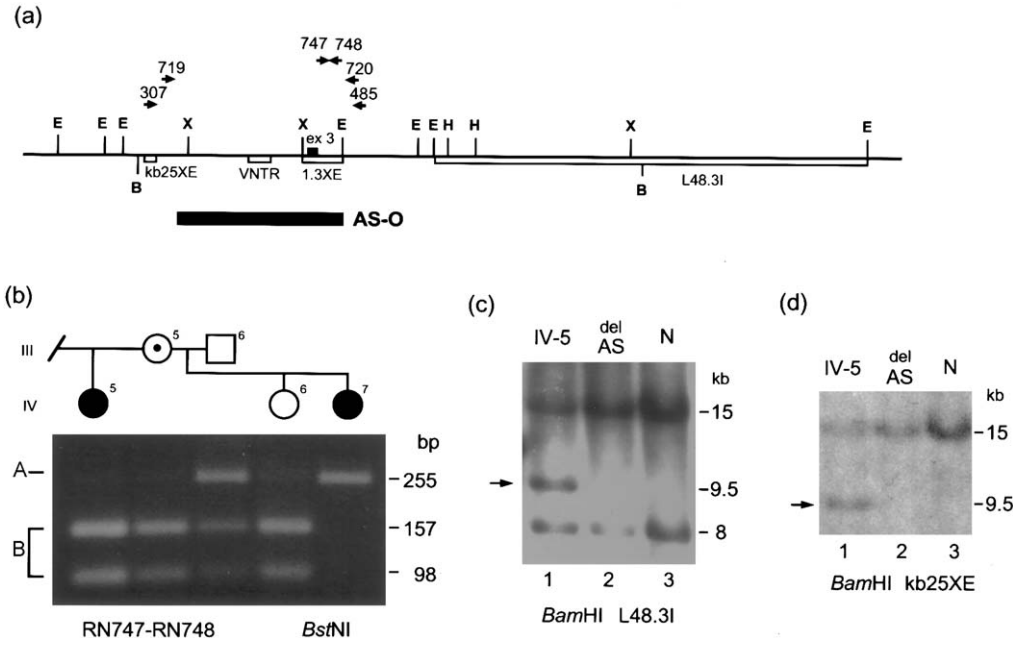

Figure 4 A 5.5-kb microdeletion of the IC in the AS-O family. *a,* Molecular map of the AS IC region and extent of the microdeletion (black bar) in the AS-O family. Open boxes indicate probes and a variable number of tandem repeats (VNTR) sequence (Saitoh et al. 1996); arrows indicate PCR primers; E = *Eco*RI; H = *Hin*dIII; B = *Bam*HI; and X = *Xba*I. *b,* The use of a *Bst*NI restriction fragment length polymorphism (RFLP) in the IC identifies a deletion in IV-7 and IV-5, since these two half-sisters do not share a visible maternal allele. A = undigested allele, and B = *Bst*NI-digested allele. *c,* The L48.3I probe detects the distal breakpoint in *Bam*HI-digested DNA. *d,* The kb25XE probe detects the proximal breakpoint in *Bam*HI-digested DNA.

are most likely hemizygous for the IC region. In other words, this analysis predicts that the maternal allele is deleted, identifying an IC microdeletion in the AS-O family.

To confirm and define the extent of the IC microdeletion in the AS-O family, we performed Southern analyses with probes from the IC (fig. 4*a*). To date, all distal breakpoints in AS IM cases cluster in the region detected by the L48.6I to L48.3I probes (Buiting et al. 1995; Saitoh et al. 1996), and therefore these probes were analyzed initially. The L48.3I probe detected bands of 15 and 8 kb on *Bam*HI digestion in the normal control, the AS deletion control, and the IV-5 AS patient, with an additional 9.5-kb band in the AS-O patient IV-5 only (fig. 4*c*). The 15-kb *Bam*HI band showed a 50% reduction in intensity in the AS IM patient IV-5 (fig. 4*c*), but the intensity of the 8-kb band was normal. The L48.3I probe did not detect an extra band or reduced intensity on *Eco*RI digestion, whereas probe 1.3XE showed a 50% reduction in signal on both *Eco*RI and *Bam*HI digestion (data not shown). These results indicate that the IV-5 AS patient has a deletion in the 1.3XE region with a distal breakpoint inside the 15-kb *Bam*HI fragment.

To define the proximal extent of the microdeletion, probe Y48.5, which maps further proximal, was examined but was not deleted in the AS IV-5 patient (data not shown). Probe kb25XE was examined next and is located at the proximal end of the 15-kb *Bam*HI fragment detected by the L48.3I and 1.3XE probes (fig. 4*a*). In *Bam*HI-digested DNA, this probe detected the normal 15-kb band in control DNA and in an AS deletion control, but was of reduced intensity, and also detected the 9.5-kb breakpoint band in the AS-O (IV-5) patient (fig. 4*d*). The kb25XE probe also detects the breakpoint band in *Xba*I digested DNA from the IV-5 AS patient (data not shown). Taken together, these data indicate that the AS-O affected patient IV-5 has a 5.5-kb microdeletion of the IC (fig. 4*a*).

Breakpoint PCR Analysis in the AS-O Family

The breakpoint of the IC microdeletion in the AS-O patient IV-5 was identified by long-range PCR with primers RN307 and RN485 (fig. 4*a*), in which a unique 1,360-bp band was present in the affected patient (IV-5), but not in the controls, and this fragment was cloned and sequenced (T. Ohta and R. D. Nicholls, unpublished data). From this sequence, we developed a proximal primer RN719 and used this with primer RN720 from the distal end of the microdeletion region, to amplify a microdeletion-specific PCR product in the AS-O IV-5

and IV-7 patients, but not in the controls (fig. 5 and data not shown). We then analyzed all relevant members of the large AS-O family (fig. 1*a*) using these breakpoint-specific primers. PCR of II-1, III-2, III-4, III-5, III-9, IV-1, IV-3, IV-5, and IV-7 genomic DNA showed a positive breakpoint-PCR product, whereas PCR of II-2, III-1, III-3, III-6, III-7, III-8, IV-2, IV-4, IV-6, IV-8, and IV-9 individuals showed no amplification (fig. 5, *upper panel*). These results are 100% concordant with haplotype studies in this family reported by Wagstaff et al. (1992, 1993; fig. 1*a*). Control primers from the PWS-SRO part of the IC (fig. 6) showed PCR products from all individuals in the AS-O family (fig. 5, *lower panel*).

Identification of a 19-kb Microdeletion in the AS-F Family

Using probe kb25XE (fig. 4*a*), we detected an abnormal *Eco*RI fragment of 6.2 kb (data not shown). No abnormal *Eco*RI fragment could be identified with several probes that included a PCR probe for the AS-SRO (primers RN285 + IC3), L48.3I, L48.3p1p2 + l48.3P (both subfragments for L48.3I), or L48.33II. For the AS-SRO and L48.3I probes, dosage analyses showed a reduction of intensity to 50%, indicating a deletion (data not shown). In *Kpn*I digested DNA, an abnormal fragment of ~14 kb was detected with kb25XE in both affected sibs, the mother, two uncles, and the grandfather (fig. 7, *upper panel*). By using the 3-kb L48.33II *Eco*RI fragment as a probe, we identified the same *Kpn*I junction fragment (fig. 7, *lower panel*). On the basis of these data, the proximal breakpoint must lie in the 6.8-kb L48.6I *Eco*RI fragment, the distal breakpoint in the L48.33II *Eco*RI fragment, and the size of the deletion is estimated at ~19 kb (fig. 6). The extent of the new microdeletions in the AS-O and AS-F families, and the relationship of these to previously identified cases, are summarized in figure 6.

Discussion

We have identified an inherited IM in each of two large AS families, including one originally reported as the first linkage evidence for an AS gene in 15q11-q13 (Wagstaff et al. 1992, 1993). The finding of an IC mutation in the AS-O family indicates that the initial linkage was for the AS component of the IC to 15q11-q13, rather than to a specific AS gene. IMs were unknown at the time that the initial linkage studies were performed (Wagstaff et al. 1992, 1993), and, therefore, a putative AS gene mutation was the only available hypothesis at that time. Subsequently, an AS gene was identified in 15q11-q13 with the finding of mutations in the *UBE3A* gene in nondeletion, non-UPD, non-IM AS patients (Kishino et al. 1997; Matsuura et al. 1997; Malzac et al. 1998). Initial molecular diagnosis in a clinical laboratory setting for the AS-O family reportedly used the PW71B probe, and we showed recently that DNA methylation with this probe in some AS IM patients indicates the presence of both methylated (maternal) and unmethylated (paternal) bands, albeit the former is reduced in intensity compared to normals, rather than only the unmethylated band typical of AS deletion and UPD patients (Saitoh et al. 1997). As shown here, the *SNRPN* DNA methylation probe clearly identified an imprinting mutation in the AS-O and AS-F families as well as in other IM families (Buiting et al. 1995; Saitoh et al. 1996, 1997). However, we urge caution in the diagnostic interpretation for methylation with any probe if the methylated and unmethylated alleles do not show an equimolar ratio, since such a result may be indicative of an IM, and we recommend further investigation to examine this possibility.

Previously, we detected microdeletions in six AS families with an IM (Buiting et al. 1995; Saitoh et al. 1996). These cases share a 2-kb SRO ~25-30 kb upstream of

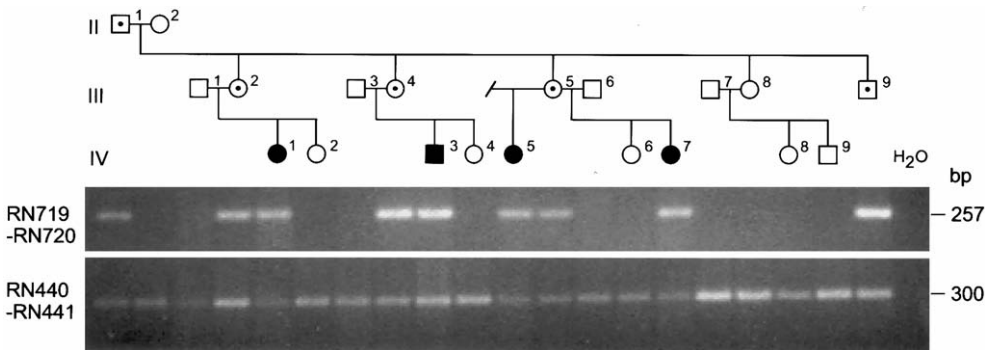

Figure 5 PCR breakpoint analysis of an IC microdeletion in the AS-O family. PCR with primers RN719 and RN720 (*upper panel*) identifies a breakpoint-specific fragment only in II-1, III-2, IV-1, III-4, IV-3, III-5, IV-5, IV-7, and III-9 in the AS-O family. The use of control primers (PWS-SRO) for PCR shows amplification in all DNA samples (*lower panel*).

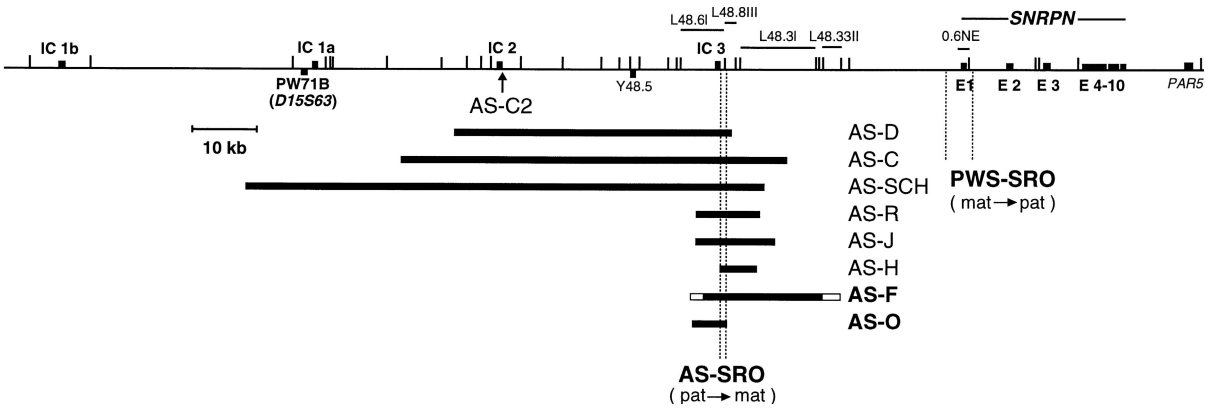

Figure 6 Summary map of AS microdeletions in the IC and minimal definition of the AS-SRO. The extent of deletions in the new AS families AS-O and AS-F are compared to six previously published deletions (Buiting et al. 1995; Saitoh et al. 1996). The vertical arrow identifies a point mutation in the splice donor site of the *IC* transcript exon 2, in family AS-C2 (Dittrich et al. 1996). The location of the PWS-SRO is also shown (Buiting et al. 1995; Ohta et al. 1999 [in this issue]). Microdeletions in AS families affect the paternal-to-maternal (pat→mat) switch in the female germline and microdeletions in PWS families affect the maternal-to-paternal (mat→pat) switch in the male germline. Upper blackened boxes indicate exons, and lower blackened boxes and thin horizontal lines indicate DNA probes.

the *SNRPN* promoter region (Saitoh et al. 1996). In the present study, the distal breakpoint of the 5.5-kb microdeletion in the AS-O family is located 800 bp proximal of the previously identified AS-SRO distal end. Therefore, the new AS-SRO is narrowed to a 1.15-kb region (fig. 6), and deletion of this 1.15-kb region alone may be sufficient to prevent the paternal-to-maternal imprint switch in the female germline (Nicholls et al. 1998). Nevertheless, either identification of such a deletion in an AS patient or development of animal models will be necessary to confirm this suggestion. DNA sequence analysis of the AS-SRO region (T. Ohta, S. Saitoh, K. Buiting, B. Horsthemke, and R. D. Nicholls, unpublished data) demonstrates no obvious features that may suggest an explanation of the molecular basis for the effect of mutations in this region. Previously, we identified a novel alternative 5′ transcript of the *SNRPN* gene (Dittrich et al. 1996), also termed the "*IC* transcript" on the basis of its candidacy for the imprinting control element involved in the paternal-to-maternal imprint switch (Nicholls et al. 1998). Exon 3 of the *IC* transcript is deleted in seven AS IM patients, including the present patients, and a point mutation in the splice donor site of exon 2 was also detected in a single AS IM patient (fig. 6; Dittrich et al. 1996). Nevertheless, the AS-H 6-kb microdeletion and the AS-SRO do not include the *IC* transcript exon 3, suggesting that the AS-SRO encodes another genetic element. This may be a novel exon of the *IC* transcript (C. Färber, B. Dittrich, K. Buiting, and B. Horsthemke, unpublished data), a transcriptional or splice enhancer for the *IC* transcript, or a binding site for a *trans*-acting factor involved in the imprint switch process (reviewed in Nicholls et al. 1998).

Intriguingly, the IC region showing specific mutations in AS IM patients maps ~250–500 kb upstream of the *UBE3A* structural gene (Kishino et al. 1997; Nicholls 1998), which is mutated in a different class of AS patients (Kishino et al. 1997; Matsuura et al. 1997). The IC has been shown to be involved in the parental imprint switch of all imprinted genes over the 2-Mb imprinted domain in 15q11-q13 (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996; Nicholls et al. 1998), including paternal-only and maternal-only expressed genes. Therefore, AS sporadic and familial patients can have specific mutations in two genetic elements located at a significant distance from each other, either a *cis* regulatory sequence that acts in the germline (the IC) or

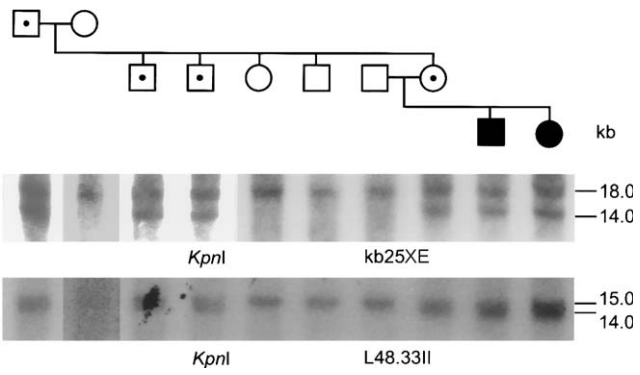

Figure 7 A 19-kb microdeletion of the IC in the AS-F family. The kb25XE probe detects the proximal breakpoint in *Kpn*I-digested DNA (*upper panel*), whereas the L48.33II probe detects the distal breakpoint in *Kpn*I-digested DNA (*lower panel*).

in a structural gene (*UBE3A*) important for proper brain development and function.

The AS gene, *UBE3A*, encodes an E3 ubiquitin protein ligase likely to be involved in protein degradation pathways (Huibregtse et al. 1993; Scheffner et al. 1995; Hochstrasser 1996). Recently, evidence has been reported for tissue-specific imprinting of *UBE3A,* with maternal-only expression in human brain (Rougeulle et al. 1997; Vu and Hoffman 1997). Expression of the mouse *Ube3a* gene is also maternal-only in cerebellar Purkinje cells, hippocampal neurons, and mitral cells of the olfactory bulb (Albrecht et al. 1997). Similarly, evidence for maternal-only *Ube3a* expression in mouse cerebellum and hippocampus has been obtained by analysis of an inherited deletion of this gene (K. C. Goss, J. Schryver, M. Dhar, R. D. Nicholls, E. M. Rinchik, and D. K. Johnson, unpublished data) or a gene knockout (Jiang et al. 1998). Although the parental-specific expression of the *UBE3A* gene in brain might be controlled by a differentially regulated promoter (Kishino et al. 1997) or enhancer (Tilghman et al. 1998; see Schumacher et al. 1998 for evidence against this proposal) or by differential splicing (Yamamoto et al. 1997), evidence suggests that the most likely explanation is inhibition of paternal allele expression by antisense gene regulation (Nicholls et al. 1998; Rougeulle et al. 1998). The AS-SRO in the 1.15-kb IC region may therefore be proposed to act through setting the gamete-specific epigenetic mark at the oppositely imprinted antisense gene rather than directly at *UBE3A* itself. Similarly, the IC controls the gametic methylation mark of the paternally expressed *SNRPN* (Glenn et al. 1996) and *ZNF127* (Jong et al., in press) genes.

Interestingly, in the AS-F family, a paternal cousin also has AS as a consequence of a maternal 15q11-q13 deletion. A similar finding of maternal 15q11-q13 deletions in affected AS cousins, related through the fathers, was recently made in two pedigrees (Connerton-Moyer et al. 1997). These findings are consistent with an AS frequency of ~1/15,000 births and likely occur by chance (Connerton-Moyer et al. 1997).

All four affected AS individuals in the AS-O family and the two AS sibs in the AS-F family meet the clinical criteria for AS (Williams et al. 1995), with severe mental retardation, severe speech impairment, ataxic gait, and typical AS behavior, including an inappropriately happy affect, sleep disturbance, and easy excitability, with hand flapping. However, several features distinguish these individuals from the classic AS deletion patients, including the lack of seizures in several individuals and milder seizures in the others, absence of microcephaly, larger birth size and more body and muscle mass, better fine and gross motor control, and normal pigmentation. These differences may arise because genes subject to haploinsufficiency may contribute to the AS phenotype in deletion patients (Minassian et al. 1998; Nicholls 1998), or IM patients (and UPD) may be milder as a consequence of the *UBE3A* gene showing incomplete imprinting in brain (Albrecht et al. 1997; Rougeulle et al. 1997; Vu and Hoffman 1997), and thus they would have a double dose of paternal allelic expression of this gene. Analyses in these large families may also allow appreciation for the intrafamilial variability in the clinical phenotype produced by the same mutation in different individuals, particularly in the neurologic and behavioral aspects. In the AS-O and AS-F IM families, the affected siblings do not differ significantly in the severity of their AS features, apart from some differences in aggressiveness, ataxia, and hyperactivity. In contrast, we previously described one AS IM family (AS-H) in which the two siblings' conditions differed significantly in severity (Saitoh et al. 1997). The clinical differences in these cases may be caused by genetic background effects or by stochastic differences in imprinted or developmental gene expression.

Our molecular results allow explanation of the inheritance patterns in these large AS families. In the AS-O family, the grandfather (II-1) has the same microdeletion as the patients, in his leukocyte DNA. His sisters (II-4, II-5, and II-10) and brother (II-7) also inherited the same great-grandpaternal haplotype (fig. 1*a*; Wagstaff et al. 1993), but no microdeletion was detected in these individuals in our microdeletion-specific PCR assay (data not shown; results of breakpoint-specific PCR were also negative in brothers II-8 and II-9). These results therefore directly confirm the previous prediction (Wagstaff et al. 1993) that the mutation must have arisen as a de novo event on the J2EJD haplotype in the germline of I-1 (fig. 1*a*). This mutational event must have occurred after setting of the paternal imprint, and thus a paternal epigenotype was fixed on the allele (Nicholls et al. 1998). Individuals III-2, III-4, III-5, and III-9 inherited the mutated allele from their father (II-1), but their phenotype is normal because of inheritance of a biparental epigenotype. In the female germline of III-2, III-4, and III-5, the paternal epigenotype cannot be reset into a maternal imprint, because of the IC mutation. Therefore, the patients have biparental inheritance but a uniparental paternal imprinted epigenotype.

The presence of an inherited IM in multiple unaffected members of the AS-O and AS-F families, who are at risk of transmitting the mutation to their children or to the children of their daughters, raises important genetic counseling issues. Individual III-9, in the AS-O family, and individuals ASF7 and ASF8, in the AS-F family, are male carriers of the IC mutation. In our IM inheritance model (Nicholls et al. 1998), silent transmission occurs whenever offspring are of the same sex as the transmitting ancestor in whom the epigenotype was first fixed, because of inheritance of biparental imprints in such

individuals. In AS families, paternal transmission occurs silently, as explained above. Thus, the children of these male carriers in the AS-O and AS-F families will have a normal phenotype, but they have a 50% risk of being a carrier of the IC mutation. Male offspring who are carriers of the mutation similarly have a 50% chance of transmitting the mutation silently, but female offspring who are carriers have a 50% risk of having children who inherit the mutation and hence develop the AS phenotype (because of maternal inheritance of a paternal epigenotype).

In such IM families, all individuals at risk for carrier status should be offered molecular testing and genetic counseling. Prenatal diagnosis is possible and genetic counseling is recommended for those individuals who are at risk for having a child with AS. Such families present a real challenge to the genetic counselor, since carrier males are not at risk for having a child with AS, but their daughters have a 50% risk of inheriting the mutation and their grandchildren have a 25% risk of having AS. Similar considerations also theoretically occur for PWS IM families, although the parental origins are opposite those described here for AS. We recommend that such individuals and families obtain genetic counseling, which would include explanation of the patterns of inheritance and discussion of (1) the possibility of "at risk" molecular genetic testing specific to each family, (2) the availability of services of a molecular laboratory familiar with IC analysis (because this is a difficult region to analyze), and (3) the importance of careful consideration of possible genetic options, including appropriate reproductive choices.

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Electronic-Database Information

URL for data in this article is as follows:

Genome Database, http://www.gdb.org (for markers and PCR conditions used for microsatellite analyses)

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