# CYTOGENETIC AND MOLECULAR STUDIES OF CHROMOSOME 15 ABNORMALITIES AND IMPRINTING IN PRADER-WILLI AND ANGELMAN SYNDROMES

by

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## Table Of Contents

**Manuscripts**

**List of Figures**

**List of Tables**

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## ABSTRACT

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are two very distinct genetic syndromes, yet in 60-80% of cases they have a similar cytogenetic deletion of the chromosome 15q11.2-q13. I and others have shown that PWS occurs in cases with a paternally derived deletion and AS occurs in cases with a maternally derived deletion of this region. PWS was first recognized as a distinct disorder in 1956 (Prader et al.) and is characterized by developmental delay, obesity and hyperphagia. AS, first described in 1965 (Angelman) is characterized by more severe mental retardation than seen in PWS and includes features such as a minimal usage of words, ataxic gait and/or tremulous movement of limbs, characteristic frequent laughter and seizures. Both syndromes have an estimated incidence between 1/10,000 and 1/20,000 and occur in all races.

The chromosome 15q11.2-q13 region shows structural variability, for which the clinical consequences are often unknown. This is especially problematic in prenatal diagnosis where there is no clinical phenotype. I have utilized *in situ* hybridization of chromosome 15 PWS/AS region-specific probes and chromosome 15 heteromorphisms, to detect and characterize structural abnormalities as well as predict clinical outcome.

The parent-specific expression of the 15q11.2-13 region defines it as an imprinted domain. As such, there are unique consequences of abnormal segregation. In about 2 percent of PWS and AS non-deletion cases, both chromosomes 15 are of the same parental origin, leading to a phenomenon called uniparental disomy (UPD). In cases of PWS, both homologs are maternal in origin and conversely paternal in origin in cases of AS. Structural rearrangements are known to increase the incidence of nondisjunction. In order to investigate the mechanisms responsible for this phenomenon, I studied two cases of PWS with UPD in which there was a meiosis I nondisjunction error

involving an altered chromosome 15 produced by a translocation event. Results from this study suggested that the chromosome structural rearrangements contributed to disruption of normal segregation of an imprinted region, resulting in UPD and PWS.

The predominant number of AS cases which have no apparent chromosome deletion show biparental inheritance of the chromosome 15 homologs. Approximately 2 percent have an imprinting defect, detectable by an abnormal methylation pattern in the putative "imprinting center" of 15q11.2-q13. In order to establish the gene(s) or defective imprinting mechanisms responsible for AS, a family with two maternal half-siblings, each affected with AS, was studied by cytogenetic and molecular methods. PCR amplification of the D15S113 $(CA)_n$ repeat (commercially obtained) suggested that both patients and their mother carried a deletion of this region of chromosome 15. Further study results indicated that an apparent deletion at the D15S113 locus represented the failure of an allele to amplify.

In summary, in the majority of patients that were studied, the imprinted nature of this region disrupted the normal Mendelian inheritance of the genes located within, leading to parent-specific syndromic expression of PWS and AS.

# INTRODUCTION

Over 15 million people, in America alone, suffer from the consequences of birth defects of varying severity. An estimated 80% of these have a genetic component to their etiology which falls into one of the following categories: single-gene disorders, chromosome disorders and multifactorial disorders (Thompson et al. 1991). Genetic disease due to single gene mutations are individually rare (average: 1 in 10,000 newborns). As a group of disorders, however, the incidence may be as high as 1 in 100. Single gene defects usually follow a simple mode of inheritance with high recurrence risk (Thompson et al. 1991).

Multifactorial diseases are common disorders (approximately 1 in 50) with low recurrence risks. They are obviously familial, but with no clear pattern of inheritance within a single family. They appear to be caused by multiple factors, both genetic and environmental (Thompson et al. 1991).

Chromosome disorders form a major category of genetic disease, resulting from excess or deficiency of the genes contained in whole chromosomes or chromosome segments. They account for a large proportion of all reproductive loss, congenital malformations and mental retardation. Specific chromosome abnormalities are responsible for 60 or more identifiable syndromes, which collectively are more common than all of the Mendelian single gene disorders together (Borgaonkar, 1989). They are present in an estimated 0.7 % of live births (Thompson et al. 1991), in approximately 3% of all pregnancies in woman over 35 years, at 14-18 weeks gestation (Clark et al. 1993) and in 50% of all spontaneous first-trimester abortions (Hassold et al. 1980).

Within this latter group of losses, autosomal trisomies represent the greatest number of chromosome abnormalities (approximately 50 %). Structural rearrangements (deletions, inversions, reciprocal and Robertsonian translocations), however, comprise only about 4% of the abortuses. Depending on the amount of unbalanced chromosomal material, the fetus with a structural chromosome abnormality may be more likely to survive to term, resulting in a live born with clinical consequences. A group of syndromes due to deletion of small chromosome segments resulting in loss of multiple genes at closely linked loci has been described. These "microdeletion" syndromes include Miller-Dieker, Smith-Magenis, Williams, Velo-cardio facial, DiGeorge, Prader-Willi and Angelman.

These latter two syndromes are of particular interest, as they have puzzled and intrigued clinical researchers for over three decades. Not only are these syndromes characterized by unique and unusual phenotypic features, their mode of inheritance deviates from the expected normal Mendelian pattern. The investigations carried out in this thesis attempt to further characterize the genetic mechanisms involved in these syndromes.

## I. PRADER-WILLI SYNDROME (PWS)

### A. Prader-Willi Syndrome Clinical Description

Prader-Willi syndrome (PWS) was first recognized as a distinct disorder in 1956 (Prader et al. 1956). Clinical refinement of the characteristic features has led to a diagnostic standard consisting of major and minor criteria (Holm et al. 1993). The major diagnostic criteria include neonatal and infantile central hypotonia that improves with age,

feeding problems in infancy with failure to thrive, excessive weight gain after 12 months and before 6 years, hypogonadism, global developmental delay with mild to moderate mental retardation, hyperphagia and characteristic facial features of: bitemporal narrowing, almond-shaped eyes, small appearing mouth with thin upper lip, and down turned mouth (See Appendix A for a complete list of the diagnostic criteria, Holm et al. 1993). Utilization of these criteria provide accurate and consistent evaluation of patients. PWS has an estimated incidence of 1 in 10,000 to 1 in 20,000 (Nicholls 1993) and occurs in all races (Butler et al. 1990).

## II. CYTOGENETICS AND PRADER-WILLI SYNDROME

### A. PWS and Chromosome 15 Deletions

The localization of PWS to chromosome 15 did not occur for some time following the delineation of the syndrome. The mapping of this syndrome necessarily awaited the advent of chromosome banding which provided the means to identify chromosomes. Standard G-banding, produced by pretreating chromosomes with trypsin prior to staining with Wright's stain, identified chromosome 15 as an acrocentric consisting of a long arm designated as "q" and a short arm designated as "p", with the two arms separated by a centromere (fig. 1).

The first chromosome analyses of PWS patients were attempted at a time when the state of the art of standard cytogenetic preparations did not routinely produce banded chromosomes long enough to detect a microdeletion. Only when cytogenetic techniques were improved such that a G-band chromosome length of 850 bands per haploid cell was achieved, was the localization of PWS made. At the 850 band length, high resolution chromosome banding (HRCB) provided a banding resolution

which allowed the detection of structural rearrangements and deletions involving as little as a single band or approximately 3-4 Mb of DNA.

The chromosome localization of PWS was made by Ledbetter et al. (1981) who observed that in 8 reported cases of PWS there was an associated chromosome 15 rearrangement, 3 of which were reported to be unbalanced showing loss of the 15 short arm through band q11 of the long arm. In his study of 5 patients, 4 were found by HRCB to have a deletion of 15q11-q13 (Ledbetter et al. 1981). In a subsequent study of 40 PWS patients, 19 had an interstitial deletion of 15q, 1 had an apparently balanced translocation of chromosome 15 to another chromosome 15, and 1 patient had a mosaic karyotype in which 1 cell line was normal and the other had an extra rearranged chromosome 15 ( i.e. extra bisatellited marker) (Ledbetter et al. 1982). From these studies, it was proposed that half of all patients with a clinical diagnosis of PWS had a chromosome abnormality involving chromosome 15q11-q13 detectable by high-resolution chromosome methods.

B. Chromosome 15 Structural Rearrangements in PWS

Chromosome structural rearrangements similar to that which were observed and utilized by Ledbetter et al. (1981) have been subsequently reported in the following studies (fig. 2). In these studies, it was demonstrated that the proximal region of chromosome 15, specifically within the bands q11.2-q13 demonstrated chromosome breakage and rearrangement. With the exception of chromosome 22q11.2 which shows similar chromosome structural instability, the number of different types of chromosomal structural changes that occur within the 15q11.2-q13, region has not been found in any other comparably sized region of a

chromosome (Magenis et al. 1988; Buckton et al. 1985). The mechanism involved in this propensity to break and rearrange is unknown.

PWS and AS cases in the literature have included reports of chromosome 15 structural rearrangements involving *de novo* microdeletions of 15q11.2-q13 (Ledbetter et al. 1981,1982; Magenis et al. 1990; Magenis and Toth-Fejel 1991). One pericentric and two paracentric inversion cases, with at least one breakpoint in the 15q11-q13 region, have been reported (Pembrey et al. 1989; Clayton-Smith et al. 1993). These inversions may be silently passed through a family without phenotypic effect. Alternatively, they may cause clinical abnormalities, such as those observed in PWS or AS, when genetic material is deleted due to misalignment during pairing and unequal crossing over in the inversion of parental chromosomes 15 during meiosis. Translocation to any number of chromosomes including chromosome 1 (R. E. Magenis, personal communication) 3, 5, 7, 8 (3 cases), 9, 10, 12 (2 cases), 14 (2 cases ) 15 (5 cases), 17, 19, and 21. have been reported (Ledbetter et al 1982; Cuoco et al. 1990; Freeman et al. 1993; Smith et al. 1993; Butler 1996; Hawkey and Smithies 1976). These reports include Robertsonian translocations which were fused at the satellite, centromeric or proximal 15q regions with a different acrocentric or another chromosome 15 . In addition, the translocations may be reciprocal or unbalanced and nonreciprocal with the chromosome 15 long arm transferring to another chromosome's terminal end and subsequent loss of the small satellited reciprocal derivative chromosome 15 (Reeve et al. 1993; Jauch et al. 1995). Reeve et al. (1993) reported the unusual finding of the presence of the recipient chromosome's telomere just proximal to the translocation breakpoint.

Breakage and rearrangement of the proximal 15q region may also produce small bisatellited dicentric chromosomes [also referred to as inverted duplications, "inv dup (15)" ] that appear as supernumerary "markers" in a karyotype (Schreck et al. 1977). Marker chromosomes, which are small chromosomes with unknown identity, are estimated to occur in 0.05 % of live births and of these, bisatellited dicentric chromosomes 15 represent approximately 40 % (Buckton et al. 1985). The dicentric chromosomes 15 are morphologically heterogeneous and have been found in patients with variable phenotypes. Large dicentric chromosomes 15 with euchromatin from the most proximal 15q loci, containing two copies of D15Z1 through D15S12 (see fig. 2) have been observed in patients with phenotypes that included mental retardation, developmental delay and seizures. Smaller dicentric chromosomes containing only material from the D15Z1 locus have been associated with a normal phenotype (Cheng et al. 1994).

The de novo presence of extra bisatellited chromosome 15 dicentric markers have been found in association with cases of PWS and AS. Three patients, 2 with PWS and 1 with AS (Ledbetter et al. 1982; Robinson et al. 1993b), presented with mosaic karyotypes with 1 cell line containing 46 chromosomes and the other having 47. The impact of the presence of the extra bisatellited chromosome on the PWS and AS phenotype was not fully appreciated when the initial observations of this phenomenon was made. This awaited the elucidation of other genetic mechanisms. Accordingly, this phenomenon will be discussed later in this text.

## III. CHROMOSOME ORIGIN IN PWS

### A. Chromosome Q-band Heteromorphisms and Origin

Q-banding by quinacrine preferentially stains AT rich regions of the chromosome (Casparsson 1970). In addition to providing a useful banding pattern for chromosome identification, this stain highlights the variable blocks of heterochromatin which are referred to as heteromorphisms (Wachtler and Musil 1980; Holmquist et al. 1989). The satellite heteromorphic region is highly variable, stable and inherited in a Mendelian fashion (McKenzie and Lubs 1975). The fluorescence of the heteromorphic regions is scored into 6 categories of size and 7 categories of intensity. Size can range from absent to very large. The intensity scores can range from no fluorescence to brilliant (Olson et al. 1986) (see fig. 1).

In a study of 39 unrelated persons, which were analyzed by means of Q-banding in order to assess the amount of variation and the discriminatory power of Q-band heteromorphisms, the chance of finding two randomly selected persons with an identical set of quinacrine variants was calculated to be 0.0003 (Olson et al. 1986). Twenty different chromosome 15 variants were demonstrated in the group of 39 subjects. It was, thus, shown that the heteromorphic regions of the chromosome 15 short arm are powerful markers for distinguishing between individuals, and that this type of analysis is a highly reliable method in which to follow the parental origin of chromosomes through a pedigree.

B. Parental Origin of Chromosome 15 Abnormalities in PWS

Chromosome 15 Q-band heteromorphisms were used to establish the parental origin of the chromosome deletion responsible for PWS. In 1983 Butler and Palmer examined 11 families in which there was a child with PWS due to a deletion of chromosome 15. They established that in all cases the chromosome 15 donated by the father was identified as the chromosome in which the deletion had occurred. Both sets of parents'

chromosomes were normal and as such the deletion was *de novo* in the child.

## IV. ANGELMAN SYNDROME (AS)

Patient studies by Magenis et al. (1987) indicated that a completely different phenotype was also associated with virtually the same cytogenetic deletion of 15q11.2-q13. This syndrome was recognized as Angelman syndrome.

### A. Angelman Syndrome Clinical Description

Angelman syndrome (AS) was first described in 1965 (Angelman 1965). This syndrome is characterized by more severe mental retardation than seen in PWS, absent or minimal usage of words, ataxia of gait and/or tremulous movement of limbs, a unique behavior of frequent laughter/smiling with a happy demeanor and easily excitable personality, often accompanied by hand flapping movements, microcephaly with a flat occiput, prominent chin, wide spaced teeth, excessive drooling, and seizures. The incidence of AS is unknown, but is thought to occur at a frequency similar to PWS. Williams and colleagues (1995) have produced a consensus diagnostic criteria for AS (see Appendix B for a complete list of diagnostic criteria).

## V. CYTOGENETICS AND ANGELMAN SYNDROME

The sharing of apparently similar chromosome 15 deletions by PWS and AS was initially quite puzzling. The distinctiveness of the two syndromes suggested that the deletions should not be identical. In order to determine if there was a difference in size or whether the deletions overlapped between the two syndromes, Magenis et al. (1990) cytogenetically examined 11 deletion cases of PWS and 10 deletion cases of

AS. Patients were clinically evaluated by clinical geneticists, including R. Ellen Magenis. HRCB studies were performed by technologists in the OHSU clinical cytogenetics laboratory in order to establish the deletion endpoints in each patient.

Chromosomes were also examined by R-banding (Schweizer 1980). R-bands are achieved by staining the chromosomes with the fluorochromes chromomycin and distamycin A, which gives the chromosome a reverse banding pattern to G-banding, and provides an alternate way to view chromosome integrity. The centromeres stain darkly, giving a good demarcation of the adjacent 15q11.2 band (fig. 1). R-banding studies were performed by me except 1 study performed by a graduate student in the research laboratory. The results in this study indicated that in all cases, band 15q11.2 was deleted. In general, the deletion in patients with AS was larger, though variable, and included bands q12 and part of q13. It was postulated from this work that the difference of deletion size might be due to different exchange points in meiosis in males and females or to different mechanisms of breakage in males and females resulting in different breakpoints. Alternatively, it was suggested that the deletion included essentially the same region, with the different syndromic outcomes being due to a differential parent-specific expression of homologous genes in this region. Further studies by Magenis and Toth-Fejel (1991) of 20 PWS and 11 AS patients substantiated that in all 18 PWS and 10 AS patients with a deletion, the deletions were within the bands 15q11.2-q13. In summary, PWS and AS were recognized as distinct genetic syndromes. While the frequency was yet undetermined, an overlapping cytogenetic deletion of 15q11.2-q13 was demonstrable in both syndromes.

## VI. CHROMOSOME ORIGIN IN AS

### A. The Parental Origin of the Chromosome 15 Abnormality in AS

Since it had been shown in PWS that all tested deletion PWS patients had a paternal deletion, it was reasoned that perhaps AS patients would have a maternal deletion. The AS patients reported in Magenis et al. (1990) and Magenis and Toth-Fejel (1991) were studied by Q-banding to determine the parental origin of the deleted chromosomes 15. All, except one origin study, were performed by myself. In all cases the deletion was maternal in origin. At this time, blood samples from 3 of these patients were sent to a molecular laboratory for confirmation of the deletion and maternal origin. Five chromosome 15q11.2-q13-specific DNA segments with known restriction fragment length polymorphisms (RFLPs) were used to examine these three patients, plus 1 more AS patient and 1 PWS patient identified elsewhere (Knoll et al. 1989). This study confirmed a deletion in all cases, but was not able to show distinct nonoverlapping deletions between AS and PWS patients. The origin of the deletion was confirmed as maternal in all cases of AS and paternal in the PWS case. This work established by cytogenetic and molecular techniques that, although PWS and AS share a similar deletion of chromosome 15, the parental origin of the deletion differs.

## VII. MOLECULAR CHARACTERIZATION OF PWS AND AS

### A. The Cloning of Genomic Sequences from 15q11.2-q13

After the cytogenetic deletion of 15q11.2-q13 was established, molecular characterization of the deletion was initiated. The cloning of genomic sequences from the proximal region of chromosome 15 was first achieved by isolating probes from flow sorted libraries of inverted

duplication chromosomes 15 (Donlon et al. 1986). Flow sorting of the inv dup (15), which contain two copies of the proximal 15q genomic sequences, produced libraries enriched for this region, and naturally excluded the cloning of unwanted more distal long arm DNA sequences.

B. Molecular Analysis of PWS and AS and Diagnostic Testing

Deletion analysis, at the molecular level, of both PWS and AS patients was initially achieved by RFLP and quantitative Southern blotting analysis using proximal chromosome 15 specific probes. Work by Donlon et al. (1986) produced four of eight clones that were deleted in 1 of 2 PWS patients tested. Heteroduplex analysis of two of the four clones that localized to band 15q11.2 revealed stem-loop structures in the inserts, indicating the presence of inverted, repeated DNA elements. It was speculated, by this group, that an inverted repeat element might explain many of the deletions and rearrangements involving band 15q11.2. A deletion could result from unequal sister-chromatid exchange or simple looping out of DNA, mediated by direct or inverted repeats. Tantravahi et al. (1989) used genomic DNA probes subcloned from flow-sorted chromosome 15 libraries that mapped to 15q11.2-q12 to test the DNA from 8 patients thought to have PWS. DNA deletion was observed in all 3 patients with cytogenetic deletions of 15q11 as well as a patient with an unbalanced translocation involving chromosomes Y and 15. In three patients with no cytogenetic deletion, no DNA deletions were found. Thus, it was established that these molecular probes could be used in PWS patients to analyze the proximal 15q region. Six of these probes were used to test 4 PWS and 1 PWS- like patients (Nicholls et al. 1989). Results indicated that 2 PWS deletions studied were *de novo* and paternally derived. Two cases involving inv dup (15) showed no deletion, but

indicated maternal origin of the inv dup (15). These studies served to define a molecular critical region for PWS and AS, a region that, when deleted on the paternally derived homolog, resulted in PWS. Deletions in the PWS/AS of the maternal homolog resulted in AS.

While these molecular studies demonstrated the diagnostic application of DNA probes, the order of the DNA segments represented by these probes within the 15q11.2-13 region was yet unknown.

C. The Ordering of 15q11.2-q13 DNA Segments

The ordering of previously mapped DNA fragments utilized yeast artificial chromosome (YAC) clones which allowed larger DNA fragments to be ordered at one time. YACs were isolated from the PWS/AS region using sequence-tagged sites (STSs) (Kuwano et al. 1992) derived from 9 DNA probes (IR39, ML34, IR4-3R, PW71, TD189-1, TD3-21, GABRB3, IR10-1, CMW1) previously mapped to this region (Tantravahi et al 1989). STSs derived from the newly isolated markers LS6-1 and GABRB3 were also used (Kuwano et al. 1992). A total of 51 YAC clones were isolated from two human YAC libraries and ordered into a contig of approximately 1 Mb. STSs derived from the previously mapped YAC, and probes derived from YAC end-clones were used to isolate additional flanking YAC clones and extend the to contig (Mutirangura et al. 1993a). The gene encoding small nuclear ribonucleoprotein-associated peptide N (SNRPN) was localized within this contig between markers PW71 and TDE-21. This work produced an extended YAC contig spanning approximately 3.5 Mb with the following order: cen-IR39 (D15S18)-ML34 (D15S9/ZNF127)-IR4-3R(D15S11)-TD189-1(D15S13)-PW71(D15S63)-SNRPN-TD3-21 (D15S10)-LS6-1(D15S113)-GABRB3-D15S97-GABRA5-IR10-1 (D15S12)-CMW1(D15S24)-tel (see fig 3A).

## VIII. SMALLEST REGION OF DELETION OVERLAP IN BOTH PWS AND AS

Once the YAC clones were ordered and a map of the 15q11.2-q13 region had been produced, the breakpoints of deletions in individual PWS and AS patients could be characterized. By examining the smallest region of deletion overlap among multiple PWS or AS patients with various deletions, a demarcation of the critical regions for each syndrome was produced. The initial boundaries of the PWS critical region was achieved by Robinson et al. (1991) who described a PWS patient deleted for only the loci 189-1 (D15S13) and PW71 (D15S63). Consistent with the data that demarcated the PWS critical region were AS patients with deletions distal to the D15S63 gene. Molecular analysis of a family with three affected AS siblings showed loss of the D15S10 locus (Hamabe et al. 1991) and GABRB3 (Saitoh et al. 1992) in all affected siblings. Wagstaff et al. (1993) reported a family with two siblings affected with AS for which haplotype analysis showed that the patients had inherited different maternal alleles for D15S63 and proximal loci, and the same maternal alleles for GABRB3. These breakpoints which produced a smallest region of deletion overlap designated the AS critical region to be between the D15S63 locus and GABRB3 locus.

At this time, it was recognized that the cytogenetic and molecular deletions of 15q11.2-q13 were demonstrable in approximately 60-80% of both PWS and AS patients (Knoll et al. 1993; Nicholls 1993).

## IX. MOLECULAR CYTOGENETIC ANALYSIS IN PWS AND AS

### A. Molecular Cytogenetics

The cloning of probes for the 15q11.2-q13 region produced the tools that allowed me, as well as all cytogenetics, to view this region at the molecular level. This technique that was used was referred to as "Molecular cytogenetics" and combined karyotype analysis with the technique of *in situ* hybridization of DNA probes. The probes I initially used for in situ hybridization were received from co-investigators (see chapter 1). I then grew, purified and labeled them with tritium prior to hybridization to denatured metaphase chromosomes on microscope slides (isotopic *in situ* hybridization, i.e., isotopic ISH). The slides were then dipped in photographic emulsion, allowed to expose and then developed. A positive signal or localization of a probe was detected by the presence of a silver nitrate grain over a region of a chromosome. Most often, a distribution of grains was seen over the entire cell. Therefore, 100-200 cells were counted and counterstained with R-banding for chromosome identification. The actual site of probe localization was assigned to a region or band having greater than or equal to 25 % of the total grains. This technique worked reasonably well and allowed the detection of the hybridization of small DNA fragments that were under 1000 bp.

Isotopic ISH, for the most part, has been replaced by fluorescent *in situ* hybridization (FISH). In principle, FISH was equivalent to isotopic ISH. FISH, however, employed the usage of probes labeled with biotin or digoxigenin and detected with FITC or rhodamine (Trask and Pinkel 1990). In my studies, chromosomes were counterstained with DAPI or propidium iodide and visualized with a microscope equipped with fluorescence and the appropriate filter set. Background signals, in contrast to isotopic ISH, were virtually nonexistent, and, thus, the localization of a probe could be precisely made.

B. Molecular Cytogenetics of 15q11.2-q13

Commercial (ONCOR) chromosome 15 probes became available that were specific to subregions and were, therefore, quite useful for detecting the presence of a portion of, or the entire, chromosome. These probes were used throughout the investigative work included in this thesis. D15Z is specific for highly repeated centromeric alphoid DNA. D15Z1, a probe which recognizes short DNA repeats related to AATGG in "classical" satellite DNA, is located in pericentromeric heterochromatin, and found in the DA/DAPI positive short arm region of chromosome 15 (fig. 1). The 15q11-q13 specific probes used to detect alterations in this region were: D15S11, SNRPN, D15S10, and GABRB3 (GABRB3).

C. Clinical Cytogenetic Diagnosis Of PWS and AS By HRCB and FISH

By 1992, two commercial FISH probes for the D15S11 and GABRB3 loci in the proximal 15q region were available and being used, albeit in a limited number of laboratories, to substantiate clinical cytogenetic findings. The prevailing smallest region of deletion overlap (deletion SRO) mapping indicated that the D15S11 locus was always lost in deletions that resulted in PWS. Likewise, by deletion SRO mapping, loss of the GABRB3 gene was implicated as a contributing factor responsible for AS. It was apparent, however, that these two loci represented only a fraction of genetic material in the ~ 4 Mb PWS/AS critical 15q11.2-q13 region. Therefore, while loss of either of these two probes could substantiate a deletion of 15q11.2-q13, the use of other 15q11.2-q13 probes which span the PWS/AS critical region was necessary to cover a larger area in order to examine for deletion.

D. Prenatal Diagnosis of PWS and AS by HRCB and FISH

The establishment of the PWS/AS critical region was made by examining 15q11.2-q13 abnormalities in clinically well defined PWS and AS patients. All cytogenetic, molecular and FISH analysis had been made in tissues other than amniotic fluid and in patients for whom there was a distinct phenotype. Therefore, at that time it was unknown whether a prenatal diagnosis of a microdeletion syndrome such as PWS and AS could be established in amniotic fluid. It was not clear if such a small deletion was detectable in a cell type in which the chromosomes are often more compact. In addition, diagnosis was more difficult in an AF sample because at approximately 14 weeks no patient phenotype was available. Therefore, I collected four prenatal cases for which the clinical cytogenetics laboratory had detected an apparent chromosome 15 abnormality and examined them by cytogenetic and molecular cytogenetic methods.

E. Prenatal Diagnosis of Chromosome 15 Abnormalities in the Prader-Willi/Angelman Syndrome Region by Traditional and Molecular Cytogenetics

In order to examine the feasibility of prenatal diagnosis of PWS and AS by cytogenetic and molecular cytogenetic approaches, the following hypothesis was proposed and subsequent study initiated.

1. Thesis Hypothesis 1

Given that the genetic abnormality that causes PWS and AS is a structural deletion of DNA from 15q11.2-q13, which is detectable in chromosomes of blood samples in approximately 60-80 % of patients, this same deletion should be similarly detectable in amniotic fluid cells. The deletion should have consistent breakpoints with those seen in blood samples and be detectable by the same cytogenetic and molecular techniques of high resolution banding, special staining, and fluorescent

and non-isotopic *in situ* hybridization used to examine and assign chromosome origin in blood samples. The results from AF studies should provide consistent and reliable prenatal diagnosis of PWS and AS.

2. The Study

My study included four *de novo* cases of an abnormal-appearing chromosome 15 in amniotic fluid samples referred for advanced maternal age or a history of a previous chromosomally abnormal child. G-, and R-banding, as well as isotopic ISH and FISH studies were performed in each case; Q-banding was performed in 3 of 4 cases. All techniques were performed by me except the G-banding. FISH of commercially obtained probes from D15S11 and GABRB3 showed a deletion in two of four cases with cytogenetic deletions of 15q11.2-q13. In addition, four other DNA sequences specific to 15q11.2-q13 were used in *in situ* hybridization procedures in the remaining two cases. The probes used in isotopic in situ hybridization were from D15S12 and D15S13 and were 0.8kb and 0.9kb in length, respectively. A 6.2kb probe from D15S9, a 37kb probe from D15S13 and a SNRPN genomic clone of approximately 70-80 kb were used for FISH studies. In these 2 cases, one involving a reported cytogenetic deletion of 15q11.2-q11.2 and the other involving an inversion with one breakpoint in 15q11.2 and the other in band q26, all probes hybridized to both homologs. In the inversion case, the probes hybridized to the very distal tip of the chromosome 15 long arm. Q-band variant and R-band analysis, completed in cases 1 and 3 demonstrated paternal origin of the abnormal chromosome. The inversion was *de novo* and maternal in origin. Clinical follow-up was not available on these samples, as in all cases the parents chose to terminate the pregnancies by dilation and evacuation. In order to confirm the results obtained from the amniocytes,

umbilical cord blood was obtained and analyzed in cases 2, 3, and 4. The results obtained in the AF cells were consistant with cord blood results. A skin sample was obtained from case 1, but failed to grow. The results of this work demonstrated that at that time prenatal diagnosis of PWS and AS was still quite difficult. The genes responsible for PWS and AS were unknown. Therefore the approach was to attempt to cover as much material in the PWS/AS region as possible by FISH analysis in order to detect a deletion. While the probes that were used spanned the PWS/AS region they left two gaps of almost 1 Mb each in length (Mutirangura et al. 1993).

The hypothesis tested revealed the following results. The deletions showed loss of the same regions that were consistently lost in clinically diagnosed PWS and AS patients. The deletions found in the AF cells were consistent with the deletions detected in the umbilical cord blood sample from the same patients. Parental origin was established in three of the four examined cases but since clinical follow-up was not possible due to termination of the pregnancy, it cannot be correlated with clinical outcome. These abnormalities detected in AF samples were detectable by the same cytogenetic and molecular techniques of high resolution banding, special staining, and fluorescent and non-isotopic *in situ* hybridization used to examine and assign chromosome origin in blood samples. The results from AF studies did not provide consistent and reliable prenatal diagnosis of PWS and AS. One apparently deleted case was not substantiated by *in situ* hybridization studies. The results obtained in the inversion case, were uninterpretable on a clinical level. No clear prognosis could be made in this case.

The results obtained from this study indicated that accurate prenatal diagnosis required a better understanding of the pericentromeric chromatin structure of chromosome 15. It was known by C-banding, which preferentially stained heterochromatin and by distamycin/DAPI staining that this region could have variable amounts of heterochromatin (see fig. 1). Distal to the centromere is a portion of the p arm that is selectively stained by the combination of distamycin A and DAPI (DA/DAPI) (Schweizer et al. 1978). This is the only acrocentric chromosome that stains positive with DA/DAPI, and thus this chromosome or rearrangements containing this region of chromosome 15 can be identified by staining chromatin with these dyes. Analysis of this region indicates that the predominant sequences stained by DA/DAPI staining technique are tandemly arranged imperfect repeats of the consensus 5'-AATGG-3' (Higgins et al 1985). The relationship of the sequences and the cytochemical staining properties are not known. I and my colleagues acknowledge that an apparent deletion of 15q11.2 might merely reflect a variation in pericentromeric heterochromatin. Chromosome pericentromeric variability was again studied in future work that is a part of this thesis.

Also gleaned from the results of this study was an appreciation of how much more information was needed with regard to the identity of the genes that, when defective, are responsible for PWS and AS. Of equal importance was the necessity to elucidate the mechanisms responsible for the unusual parent-specific inheritance pattern observed in the paternal origin of PWS and the maternal origin of AS, a phenomenon that was termed imprinting (Crouse 1960).

## X. GENOMIC IMPRINTING

The finding that PWS and AS were tied to specific parental inheritance of the proximal 15q region focused attention on the likelihood that this region was differentially imprinted in the paternal and maternal homologs.

### A. Genomic Imprinting Modification

Genomic imprinting was understood as an epigenetic modification of the maternal or paternal genes that were contributed to the zygote which resulted in a differential expression of these parental alleles during development and in the adult (Monk 1988). The modifications were considered epigenetic in nature since they altered phenotypic expression by means other than differences in DNA sequence. Consequently, the genetic information contributed from each parent in an imprinted region was equivalent, but the information itself was not functionally equivalent.

### B. Effect of Genomic Imprinting on Embryonic Development

Nuclear transplantation experiments in mice had shown that both parental genomes were necessary for complete embryogenesis (Barton et al. 1984; McGrath and Solter 1984). In cases where pronuclei were manipulated such that the zygote possessed only maternal or only paternal pronuclei, the developing diploid parthenogenones underwent very limited postimplantation development and never developed to term (McGrath and Solter 1986).

It was proposed that the extensive homozygosity was the cause of parthenogenetic failure (Surani and Barton 1983 ). McGrath and Solter (1986) performed the following experiment to address the nonviability. Pronuclear transfers were made that resulted in the production of embryos containing two female pronclei or two male pronuclei. This resulted in a

zygote with two pronuclei of the same parental origin, but from two different strains of mice. The development of these embryos was compared with the development of control embryos in which the male or the female pronucleus was replaced with a pronucleus from another embryo, but of the same parent of origin. In the control zygotes, the pronuclei represented both parental origins but were of the same mouse strain. Genetic markers were used to verify the genotype of the manipulated embryos. In no case did biparental androgenones (paternal pronuclei only) or the gynogenones (maternal pronuclei only) develop to term. The control nuclear transplant embryos were successful however.

These results indicated that parthegenones did not die because of lethal homozygosity, but rather because a parental genome was missing. Furthermore, the results implied that the maternal and paternal contributions to the embryonic genome were functionally different. The data suggested that the expression of certain genes necessary for development were limited in a parent-specific fashion or that maternal and paternal genomes must interact in a specific way in order for normal development to proceed. It was also realized that reversibility must be an essential feature of all these modifications since the paternal or maternal genome could be the opposite parental genome in the next generation.

C. Imprinting In Hydatidiform Moles And Ovarian Teratomas

An appreciation of the paternal and maternal contribution in eutherian organisms was enhanced by examining the outcome in embryonic development of the human when the chromosome compliment was all of one parental origin. Ovarian teratomas and hydatidiform moles, two grossly abnormal structures are formed when there is absence or abnormal contribution of the maternal or paternal

genomes. A hydatidiform mole may be a true (or complete mole), with no fetus and a disorganized abnormal placenta present, or a partial mole, with remnants of placenta and occasionally a small atrophic fetus. Interestingly, the origins of the two types differ. Partial moles are triploid and thought to have arisen through the fertilization of an egg cell by two sperm (Jacobs et al. 1982). They, therefore, have a maternal and an excess of paternal genomic contribution. With complete moles, the karyotype is 46,XX, with all chromosomes solely paternal in origin (Kajii and Ohama, 1977). It is thought that the mole originates, initially, when a single 23,X sperm fertilizes an ovum that lacks a nucleus, and then endoreduplicates. In contrast, ovarian teratomas originate from germ cells, are solely maternal in origin and are thought to be formed by a failure of polar body formation during meiosis. No extraembryonic tissue is present in the ovarian teratomas. These different parent-of-origin specific abnormalities in embryonic development, indicates that the maternal and paternal genomes are essential. The paternal genome is critical for the normal development of extraembryonic tissue and the maternal genome is critical for embryonic development.

### D. Chromosome Imprinting and Uniparental Disomy in the Mouse

Cattanach and Kirk (1985) demonstrated in the mouse that imprinting occurred, but that not all of the genome showed parental imprinting effects. Parent specific duplication/deficiency of certain chromosomes produced normal offspring, while other apparently imprinted regions produced anomalous phenotypic effects when uniparental in origin. This was shown by taking advantage of strains of mice that carry Robertsonian translocations and exhibited a high level of

nondisjunction. In one such experiment, heterozygotes for the Robertsonian translocation of chromosome 11 and 13, exhibited high levels of nondisjunction for chromosome 11 and 13. When intercrossed, normal disjunction in both parents resulted in the production of chromosomally balanced young. With nondisjunction in one parent, monosomy and trisomy for chromosomes 11 and/or 13 resulted. However, when nondisjunction occurred in both parents and the complementary products united, chromosomally balanced young disomic for chromosome 11 or 13 are produced. If the disomy of chromosome 11 in the offspring was maternal in origin, the young were smaller than their normal sibs. The offspring that had paternal disomy of chromosome 11 were larger than their sibs. Not all chromosomes showed parent-specific origin effects. Mice that had uniparental disomy of chromosomes 1, 3, 4, 5, 9, 13, 14, or 15 were viable and normal. In contrast, uniparental disomy of chromosomes 2, 6, 7 or 8 were found to be lethal to the animals.

In a study of the tabacco mouse it was observed that each of the 7 tabacco mouse Robertsonian chromosomes had been found to lead to nondisjunction when carried heterozygously with the house mouse acrocentrics, but that the frequency of nondisjunction was not the same for each chromosome (Cattanach and Moseley 1973). From the results of the data obtained in this study, it was concluded by these authors that it was the minor chromosomal differences between the Robertsonian chromosomes and their homologous acrocentrics that caused the nondisjunction.

Eichenlaub-Ritter and colleagues (1990) examined the oocytes from mice that were heterozygous for multiple Robertsonian translocations and found that disturbances in chromosome orientation and spindle structure,

rather than a failure in pairing and crossing-over between homologous chromosome arms, was the predominant cause of nondisjunction in those cells.

The nondisjunction of Robertsonian translocations observed in these mice studies was consistent with studies in the human which implicated Robertsonian translocations as contributing factors of nondisjunction (Hamerton et al. 1971; Miller 1981).

## XI. CHROMOSOME 15 IMPRINTING AND UNIPARENTAL DISOMY

The parent of origin specificity of PWS and AS implied, by definition, that the gene or genes involved in the expression of these disorders were imprinted (Hall 1990). At this time the term genomic imprinting was used to refer to the differential expression of genetic material, at either a chromosomal or allelic level. Specific imprinted genes were yet to be identified and the role of uniparental disomy was as yet undefined in the pathogenesis of PWS and AS.

### A. Methods for Detecting Chromosome Origin, Abnormal Segregation and Uniparental Disomy

The parent-specific loss or gain of a chromosome had been traditionally traced by Q-band heteromorphism studies (Magenis and Chamberlin 1981). Q-band heteromorphisms of chromosome 15 had been shown to be inherited in a stable and Mendelian fashion (McKenzie and Lubs 1975; Olson et al. 1986), an effective tool for distinguishing between individuals, and useful in identifying the parental origin of a chromosome 15. Chromosomes 15 of the same parental origin, but with different heteromorphic variants indicated an abnormal segregation of chromosome 15 homologs due to nondisjunction during meiosis I.

Identical heteromorphic markers indicated a nondisjunction error at meiosis II.

The molecular method of determining origin of alleles in previously discussed work was achieved through RFLP analysis. The advent of the polymerase chain reaction (PCR) of microsatellites provided an alternative method to establish the origin of alleles within a family study (Litt et al. 1993). By this method, parent-specific loss of an allele was detected by Southern blotting analysis. In my studies, I used microsatellite data in combination with chromosome origin data and was able to demonstrate whether a pair of chromosomes was from the same parent, as well as whether the pair was the result of a meiosis I or II error. In addition, I was able to detect whether recombination had occurred between homologs from the same parent. A complete lack of recombination indicated a somatic rather than meiotic nondisjunction event.

B. Uniparental Disomy (UPD)

When the techniques described above detected that both homologs or alleles of a given chromosome pair were from the same parent the phenomenon was referred to as "uniparental disomy" (UPD) (Engel 1980). When both homologs were not only from the same parent, but represented the same homolog, the disomy status was referred to as uniparental isodisomy. The disomy status of chromosome homologs representing both of the parental homologs was referred to as uniparental heterodisomy.

C. Trisomy of Chromosome 15, a Possible Prelude to UPD

Chromosome studies of chorionic villi suggested that UPD was the result of an initially trisomic fetus followed by the early loss of one of the three chromosomes 15 (Kalousek et al. 1989). In 2/3 of such instances,

normal biparental inheritance of chromosomes would result. However, in 1/3 of such instances the resulting pair of chromosomes would be uniparental in origin . This hypothesis was supported by several lines of evidence. Trisomy of chromosome 15 was shown to be lethal, with only one reported live born case (Coldwell et al. 1981). UPD was compatible with life. Therefore, if one of the chromosomes 15 was lost from the karyotype early in the zygote, embryonic development could continue. Demonstration that this process could actually occur was achieved by studies of confined placental mosaicism. Confined placental mosaicism in terms of trisomy implies that the abnormal trisomic cell line is confined to the placenta, and that the fetus is disomic. This phenomenon had been demonstrated to occur in about 2 % of chorionic villus samples, and to involve many different chromosomes including chromosome 15 (Kalousek et al. 1991). Eventually, a case of confined placental mosaicism of trisomy 15 that resulted in UPD was reported (Surh et al. 1994), suggesting an initial trisomic 15 cell line with subsequent chromosomal loss.

D. The Detection of Uniparental Disomy of Chromosome 15 in PWS and AS

UPD was initially observed in those PWS and AS patients who did not demonstrate a deletion of chromosome 15. By RFLP analysis in the PWS/AS critical region at the D15S10 and D15S18 loci, it was established that the alleles in the two PWS patients were solely maternal in origin. The alleles were representative of both maternal homologs (Nicholls et al. 1989). Therefore, the uniparental disomy was *heterodisomic* rather than isodisomic. In a study of 30 PWS patients who did not have a cytogenetic deletion, 18 were shown by RFLP analysis at 7 loci within the PWS/AS

critical region to have maternal uniparental disomy. In addition an association of advanced maternal age was observed in this group of 18 patients. Within the total group of 30 patients, 8 had deletions by molecular analysis and 4 patients had normal biparental inheritance for chromosome 15 (Mascari et al. 1992).

Uniparental disomy in AS was first reported in 2 patients with normal cytogenetic findings, but for whom, by RFLP analysis at D15S86 and D15S24, there was loss of the maternal contribution. Alleles at these loci indicated paternal heterodisomy in patient one and paternal isodisomy in patient two for chromosome 15 (Malcolm et al. 1991). With the inclusion of the report of 4 more AS patients with UPD (Robinson et al. 1991), a tabulation of known deletion and nondeletion reported cases of PWS and AS was made.

The conclusion from these studies was that approximately 60-80% of cases of PWS and AS had a cytologic deletion of 15q11.2-q13. In 20-30% of PWS cases, the chromosome 15 pair appeared normal at the cytogenetic level, but in approximately 25% of these, the chromosomes were uniparental in origin (Nicholls 1993). With regard to AS, of the 20-30% of patients with normal appearing chromosomes, only 3-4% had uniparental disomy of chromosome 15. Furthermore, chromosome 15 origin analysis in PWS cases with normal appearing chromosomes had shown that in all cases of UPD, both homologs were maternal in origin. Conversely, in AS cases in which both chromosomes 15 were found to be of the same origin, they were paternal in origin. This phenomenon, in which both homologs of a given chromosome pair were from the same parent, was termed uniparental disomy (UPD) (Engel 1980). Previously described in

the mouse, it was now established as a human phenomenon, and in some cases it was responsible for PWS and AS.

E. UPD Due to Meiotic and Mitotic Nondisjunction Errors

The abnormal segregation that led to UPD was shown to occur at meiosis and mitosis (Robinson et al. 1993a). A study of 27 PWS and 5 AS patients demonstrated by RFLP and microsatellite analysis that UPD presented as heterodisomy, in which the chromosome pair was representative of both of the homologs from a single parent, and was presumably due to a meiosis I nondisjunction error. Alternatively, UPD also presented as isodisomy, in which the chromosome pair represented only a single homolog from a given parent, due to a meiosis II nondisjunction error. Although an isodisomic chromosome pair represented a single parental chromosome, and thus appeared identical, they were not completely so. Due to normal pairing and meiotic recombination, an isodisomic pair had a number of alleles that were heterodisomic. UPD that occurred postzygotically, after meiosis, presented as isodisomy but without any allelic differences. Furthermore, data from this study indicated that 82% of maternal nondisjunction events which led to UPD involved a meiosis I error, whereas most paternal UPD AS cases were meiosis II or mitotic errors. A slight reduction in recombination was associated with maternal nondisjunction. It was unclear from these studies whether the recombination was uniformly reduced throughout the entire chromosome or only for certain intervals.

F. Contributing Genetic Factors to Nondisjunction and UPD

1. Maternal age effect

A maternal age effect with nondisjunction was first documented by Penrose in 1933. Subsequent studies showed that nondisjunction was

primarily attributable to maternal errors at meiosis I and was associated with advanced maternal age (Magenis and Chamberlin 1981; Polani 1981). Nondisjunction of chromosome 15 has been established in cases of UPD of PWS and AS with a suggestion of an increase in maternal age in the PWS population (Mascari et al. 1992).

2. Abnormalities in Chromosome Pairing (Synapsis)

Darlington (1929) suggested that chromosome meiosis I errors might be due to chromosome asynapsis or desynapsis, implying that a relative deficiency of chiasmata would be associated with nondisjunction. Additionally, it was put forth that a reduction in the number and/or alteration in the distribution of chiasmata (Sherman et al. 1994) or the failure of these structures to resolve in a timely fashion might be responsible for nondisjunction (Carpenter 1994). Studying chromosome 15 and UPD, Robinson and colleagues (1993a) noted a slight increase in zero and single recombinations, with meiotic errors occurring primarily in meiosis I in maternal UPD. Trisomy 16 data did not support asynapsis as the causative factor, as recombination was seen in 17 of 22 cases of nondisjunction (Hassold et al. 1991). Studies of trisomy 21 (Antonarakis et al. 1986; Warren et al. 1987; Sherman et al. 1991) and maternal XX nondisjunction (Morton et al. 1990), however, showed an association of nondisjunction and decreased recombination.

3. Structural Rearrangements

Hamerton (1971) reported that 3.5% of his study group of 2594 Down syndrome patients had a Robertsonian translocation involving 21. In addition, it was observed that in normal carriers of Robertsonian translocations involving chromosome 21, where there was no maternal age affect, offspring were at an increased risk of 1 in 10 for having trisomy

21. Normal recurrence risk for live born Down syndrome in the non-advanced maternal age population is approximately 1 in 700. The contributing factors to the nondisjunction in this study are unknown.

4. Extra Bisatellited Dicentric Markers

The *de novo* presence of supernumerary small chromosome 15 dicentric markers was described in cases of PWS and AS (Ledbetter et al. 1982; Robinson et al. 1993). In these cases it was demonstrated that the UPD of the intact chromosomes 15, rather than the presence of the extra dicentric marker, was the cause of PWS or AS. It was submitted that the presence or formation of the extra dicentric marker in a cell line might increase the probability of uniparental disomy. Two theories that were put forth suggested that dicentric markers might represent a by-product of "rescuing" a trisomic fertilization. Alternatively, their presence might cause duplication of a normal homolog in a zygote which had inherited the marker in place of the normal corresponding chromosome and thereby "rescued" an aneuploid cell line.

While multiple contributing factors to nondisjunction and UPD were suggested, just how these factors effected the sequence of events that interfered with proper segregation were unknown. Brown et al. (1994) demonstrated, using a Y chromosome from which chromosome arm segments were separated from the centromere, that α-satellite sequences at the centromere were the most basic material essential for proper chromosome segregation. Since there was a paucity of information regarding the influence of pericentromeric sequences on chromosome segregation and nondisjunction, I chose two PWS patients that were affected due to UPD of chromosome 15 and who had structural

rearrangements of chromosome 15 to study the possible effects of these changes on nondisjunction.

G. The Impact of Imprinting: Prader-Willi Syndrome Resulting From Chromosome Translocation, Recombination and Nondisjunction

In order to investigate the various contributing factors that might be responsible for nondisjunction and result in PWS due to UPD of chromosome 15, the following hypothesis was proposed with the subsequent study initiated.

1. Thesis Hypothesis 2

A substantial number of PWS cases in the literature and in the OHSU cohort (6 %) are due to structural rearrangements of the unstable pericentromeric region of chromosome 15. Nondisjunction, which is associated with chromosome structural rearrangements such as Robertsonian translocations, leads to a unique situation in cases of PWS or AS due to the imprinted nature of this region. If proper meiotic segregation is dependent on timely resolution of chiasmata and/or the proper binding of chromosome-specific spindle fibers to centromeres and their associated kinetochore-related proteins, then rearrangements of pericentromeric or unstable regions of the genome may disrupt normal disjunction and lead to uniparental disomy. Therefore, PWS patients with structural abnormalities of the pericentromeric region and UPD, when studied with FISH, special chromosome staining techniques, and microsatellite amplification, should allow investigation of which chromosome regions are essential for normal segregation as well as indicate any change in recombination frequency from the reported norm.

2. The Study

My study included two cases of PWS demonstrated to have UPD by PCR microsatellite and chromosome 15 heteromorphism analysis, as well as structural abnormalities of chromosome 15. In both cases, HRCB of the long arm was normal and FISH with probes D15S11, SNRPN, D15S10 and GABRB3 indicated no loss of this material. All studies, except the G-banding study of patient 1, were completed by me. I was assisted with a DNA VNTR paternity analysis of patient 1 by the DNA diagnostic laboratory. Chromosome 15 Q-band origin and PCR microsatellite analysis demonstrated that in both patients there had been a meiosis I nondisjunction error involving an altered chromosome 15 produced by both a translocation event between the heteromorphic satellite regions of chromosomes 14 and 15 and recombination. Chromosome 15 heteromorphism analysis showed that each patient had maternal heterodisomy of the chromosome 15 short arm, whereas PCR of microsatellites demonstrated allele-specific maternal isodisomy and heterodisomy of the long arm, indicative of a meiosis I error with recombination.

Patient 1 had a *de novo* translocation involving chromosome 14 and 15 satellite and stalk regions. Special staining using distamycin/DAPI (DA/DAPI) indicated an unusual result. Patient 1 and her mother had chromosome 15-specific material, detected by DA/DAPI staining, translocated on to the chromosome 14 homolog that was involved in the translocation event. In addition, the microsatellite information at loci spanning the chromosome 15 long arm indicated that recombination had occurred.

The structural rearrangement in patient 2 was a Robertsonian translocation of chromosome 14 and 15 which was inherited from his

mother. Chromosome 14 and 15 were fused at the satellite regions of both chromosomes.

In conclusion, nondisjunction was not due to a failure to pair. The number of crossover events was consistent with the number of expected chiasmata along this chromosome (Hultén 1974). In both patients special staining and FISH techniques demonstrated that pericentromeric changes had occurred in both patients. Patient 1, due to a Robertsonian translocation, had chromosome 14 and 15 centromeres placed in close proximity. Patient 2 had structural changes in satellite and stalk regions, as well as chromosome 15-specific pericentromeric material translocated to a chromosome 14 homolog.

These results suggested that it was the pericentromeric structural changes that inhibited proper homolog pairing or metaphase I alignment that ultimately led to nondisjunction at meiosis I. Presumably, there was an early postzygotic loss of the paternal chromosome 15, since no paternal genetic material was detected by chromosome or PCR analysis. In addition, since the frequency of UPD due to structural rearrangements was unknown it was deemed prudent to investigate all cases of structural rearrangements that included an imprinted region in order to detect UPD.

## XII. THE IMPRINTING OF GENES

### A. The Identification of Imprinted Genes

While it was first noted at the chromosome level that a biparental inheritance of chromosomes was essential for normal development, differences in the parent-specific expression of certain genes were soon recognized in both the mouse and humans. The mouse genes included the insulin-like growth factor type 2 gene (Igf2) and receptor gene (Igf2r),

H19, and Snrpn. Igf2r in the mouse was shown to be expressed only from the maternal allele (Barlow et al. 1991). It was demonstrated that Igf2 and H19 were closely linked but are reciprocally expressed, Igf2 showing monoallelic paternal expression and H19 being expressed solely from the maternal allele (Bartolomei et al. 1991; DeChiara et al. 1991). The human homologs of Igf2 and H19, showed a conservation of imprinting. The Snrpn gene was shown to be imprinted in the mouse, as was its human homolog, with both species expressing only from the paternal allele (Cattanach et al. 1992, Leff et al. 1992). The discovery of imprinted genes in mice provided a model system in which to study the mechanisms involved in the imprinting process.

B. Imprinted Genes and Allele-Specific Replication

In addition to showing allele-specific gene expression, imprinted regions were shown to asynchronously replicate the alleles of certain imprinted genes, including IGF2, H19, SNRPN and the non-imprinted gene IGFR2 (Kitsberg et al. 1993). All genes showed early replication of the paternal allele. Allele-specific replication timing was visualized in interphase nuclei with the use of fluorescent *in situ* hybridization (FISH). The presence of a hybridization signal corresponded to an allele on a chromatid from one homolog. The chromosomes were not visible, however, since interphase nuclei were being scored. Synchronous replication appeared as two sets of signal doublets. Asynchronous replication was observed as a single hybridization signal (corresponding to one homolog) and a signal doublet (corresponding to the other chromosome). Additional studies have shown asynchronous paternal early/maternal late replication in the 15q11.2-q13 proximal region (fig. 3B). The D15S63 (PW71), SNRPN, D15S10, D15S113, and GABRB3 loci

replicated the paternal allele first. The maternal allele replicated first at the GABRA5 locus. A mixed pattern asynchrony was observed at the most proximal locus, D15S12 (Knoll et al. 1994).

## XIII. POSSIBLE MECHANISMS OF IMPRINTING

### A. Criteria of Imprinting

Although the mechanisms of genomic imprinting are not completely understood, data obtained in human and mouse studies has indicated that an imprinting mechanism must fulfill the following criteria. First, it must be reversible through generations. Second, it must be involved in the expression or the repression of an imprinted gene. Third, the epigenetic modifications inherited from each parent must be erased and reset during gametogenesis. Finally, the imprint must be faithfully maintained after DNA replication.

### B. DNA Methylation and Chromatin Structure

DNA methylation and chromatin structure have both been implicated in the imprinting process (Monk et al. 1987; Hendrich and Willard 1995). Methylation of cytosine, which was found exclusively at CpG residues, appeared to play an integral role in gene regulation in the human genome. In vertebrates, a fraction of unmethylated DNA was found clustered throughout the genome (Bird 1986). This DNA was characterized by its cleavage to tiny fragments with the methyl-sensitive restriction enzyme HpaII. The HpaII tiny fragment fraction (HTF islands) differed from bulk DNA by being unmethylated at CpG dinucleotides and contained a relatively high G + C content. HTF islands occured at 5' ends of genes. While HTF islands of active genes were unmethylated, the

majority of CpG sites in the interior of genes in somatic cells were methylated (Driscoll and Migeon 1990).

Mouse studies indicated that adult cell-specific DNA methylation patterns were established in a programmed manner during development (Chaillet et al. 1991). At the blastula stage most DNA was unmethylated. Following implantation of the embryo, a systematic remodeling of DNA methylation occured leaving the CpG islands that are associated with housekeeping genes unmethylated (Kafri et al. 1992). At later stages of embryogenesis the methylation status profile was refined by the demethylation of tissue-specific genes in the cell types in which they were expressed (Neveh-Many et al. 1981).

It was suggested that methylation might inhibit gene expression by affecting the protein-DNA interactions required for transcription. Unmethylated DNA, found at active genes, was sensitive to DNase I digestion, whereas fully methylated DNA was resistant to DNase I (Keshet et al. 1986).

DNA methylation was first suggested to be the epigenetic mechanism for differentially imprinting maternal and paternal genomes by studies on transgenes (Sapienza et al. 1987). Studies of certain transgenes demonstrated that the specific DNA methylation imprint was erased in mouse primordial germ cells and the subsequent methylation imprint that emerged was dictated by whether the germ cell went through spermatogenesis or oogenesis (Chaillet et al. 1991). In the majority of mouse strains studied, the maternally inherited transgene was methylated while the paternally inherited transgene was not. In one study, a direct correlation of paternal inheritance, transgene undermethylation and

tissue specific expression of the transgene was observed for a specific mouse strain (Swain et al. 1987).

C. Imprinting and DNA Methylation in 15q11.2-q13

It was subsequently shown that within the chromosome 15 proximal region, there were parent-specific differences in DNA methylation. Sites of differential methylation were seen at the ZNF127 (Driscoll et al. 1992; Glenn et al. 1993a), D15S63 (Dittrich et al. 1992; Buiting et al. 1994; Lerer et al. 1994; Reis et al. 1994) and SNRPN loci (Glenn et al. 1993b, 1994, 1996; Sutcliffe et al. 1994) (fig. 3A). At these three loci, parent-specific DNA methylation patterns were detected by digesting genomic DNA with isoschizomers, one of which was methylation sensitive, followed by electrophoresis and quantitative Southern blotting analysis. Normal individuals showed two fragments, one from the hypomethylated paternal allele and the other from the methylated maternal allele. At all three loci, DNA methylation patterns of PWS and AS patients was discernible from each other and from normal patients. On quantitative Southern analysis, PWS patients with a deletion, paternal UPD or an imprinting error did not demonstrate the paternal fragment. Similarly, AS patients with a deletion, maternal UPD or an imprinting error did not demonstrate a maternal fragment.

## XIV. CANDIDATE GENES FOR PWS AND AS

### A. The SNRPN Gene as a Candidate Gene for PWS

In 1992 Özçelik et al. reported that the SNRPN gene was deleted in 7 PWS and 2 AS patients studied with cytogenetic deletions in the 15q11.2-q13 region, thus mapping this gene to this region. It had been previously demonstrated that this gene, which was expressed abundantly in brain and

heart, encoded a protein called SmN that was associated with snRNPs (particles essential for pre-mRNA processing) (McAllister et al. 1988; Schmauss and Lerner 1990). Glenn et al. (1993b) demonstrated that in all PWS patients(23 deletions and 5 UPDs) the maternal allele of the SNRPN gene was methylated and that this gene was exclusively expressed from the paternally inherited chromosome. The critical involvement of the SNRPN gene in the pathogenesis of PWS was strengthened by Sutcliff et al. (1994) who reported two PWS siblings with a submicroscopic deletion encompassing only exon α (also referred to as -1 exon) and the promoter region of the SNRPN gene. From the data obtained in these studies, it was postulated that physical or functional loss of the SNRPN gene in PWS patients results in the major features of the syndrome (Reed and Leff 1994) and accordingly the SNRPN gene became a candidate gene for PWS. Futhermore, the mapping of the SNRPN gene and establishment of cases which exclusively involved deletions of this gene in PWS, placed the distal boundary of the PWS critical region proximal to D15S10.

It was originally thought that the SNRPN gene contained 8 exons (Özçelik et al. 1992) but was later shown that there were two exons 5' of the original proposed first exon (Glenn et al. 1996). Hence they were referred to has exon 0 and exon -1. The SmN open reading frame was shown to start at exon -1 and extend through exon 8. The first exon of SNRPN (exon-1) and the transcription start site were embedded within a CpG island. Furthermore, it was demonstrated that this CpG island was extensively methylated on the repressed maternal allele and was unmethylated on the expressed paternal allele showing genomic imprinting in a wide range of fetal and adult somatic cells. Of interest, it is now known that there is also a second 5' open reading frame, in addition

to the SmN open reading frame, that begins in exon 2 and extends through exon 8 (Glenn et al. 1996).

B. AS Candidate Gene(s)

While a candidate gene for PWS had been proposed and a new boundary demarcated the PWS critical region from the AS critical region, no candidate gene for AS had yet been firmly proposed.

C. The Smallest Region of Deletion Overlap in AS

Within the group of AS patients (60-70%) who had an established deletion of 15q11.2-13 (Knoll et al. 1989), molecular refinement of breakpoints redefined the smallest region of deletion overlap. Buiting et al. (1993) indicated that in 60 % of deletion cases, patients had molecular deletions that included all the loci from D15S9 (ZNF127) to D15S12. Five cases of AS demonstrated smaller deletions between D15S10 and D15S113 (Saitoh et al. 1992; Reis et al. 1993) and one case demonstrated a deletion of D15S122 and D15S113 (Greger et al. 1994). Buxton et al. (1994) reported the smallest deletion of 200 kb at the D15S113 locus.

In approximately 25 % of AS patients, however, the genetic defect was yet unknown (Nicholls 1993). Typical of this situation was the reported case of familial AS in which three sisters had given birth to four AS offspring who demonstrated no evidence of deletion or paternal disomy by chromosome and RFLP analysis (Wagstaff et al. 1992). At OHSU, I became aware of two such AS patients that had been clinically evaluated in the genetics clinic. Since these patients were maternal half-siblings, I reasoned that the error that led to the expression of AS in these siblings was familial and that I might be able to detect the abnormality by comparing cytogenetic and molecular 15q11.2-q13 test results of these patients with those of normal family members. In this way, I might be

able to not only establish the defect in this family, but establish the defect that is responsible for AS in general.

D. Cytogenetic and Molecular Findings in a Case of Familial Angelman Syndrome: Reexamination of the D15S113 Locus

In order to investigate the genetic defect responsible for familial AS, a study of two maternal half siblings with AS was initiated, with the proposal of the hypothesis.

1. Thesis Hypothesis 3

A substantial proportion of AS patients do not show a cytologic deletion (approximately 25 %), but may have molecular deletions in areas of the AS critical region that are not fully characterized. A family in which there are two or more affected AS siblings, likely carry a heritable mutation in the AS gene. While this mutation must be maternally inherited in affected individuals, it may be paternally inherited without an abnormal phenotype resulting. A family with two maternal half-siblings, each affected with AS, will be studied for such a mutation. If the two children carry a mutation, their mother may demonstrate a molecular deletion in a blood sample. If she is negative for a mutation she may have gonadal mosaicism for the deletion. The deletion, if found in the mother, will be *de novo* or inherited from her father. Alternatively, this family may have an imprinting defect in the 15q11.2-q13 region, demonstrable by an abnormal methylation pattern at the 5' end of the SNRPN gene. The results from this study will contribute to defining the imprinting mechanism and critical region for AS.

2. The Study

This study included the cytogenetic and molecular examination of two maternal half-siblings affected with AS, and when necessary included

studies of their parents, maternal aunts and maternal grandfather. HRCB was performed by the clinical cytogenetics laboratory. All other studies were performed by me. HRCB and FISH analysis using chromosome 15-specific proximal probes [D15S11, SNRPN, D15S10, GABRB3 (ONCOR)] indicated that the chromosomes were normal. PCR amplification of the D15S113 (CA)n repeat (Research Genetics) suggested that both patients and their mother were deleted at this locus. In order to substantiate this finding, alternative primers for the amplification of the D15S113 (CA)n repeat were obtained. In addition, an (ATTT)n repeat at the D15S113 and a (CTTT)n repeat at the D15S1234 loci were amplified. Biparental amplification of alleles at the D15S113 and D15S1234 loci was achieved by the alternative and additional primers in patients JT and his mother, suggesting that the initial amplification of a single allele in these patients was not a reflection of a deletion, but rather a failure to amplify an allele. It was not clear whether patient CD was hemizygous or homozygous at the D15S113 locus by PCR amplification using alternative primers since the markers were uninformative. She was heterozygous at the D15S1234 locus. The patients did not show abnormal methylation results at the 5' SNRPN locus and thus no abnormality of imprinting was observed. In summary, these two half-siblings affected with AS failed to demonstrate a genetic defect at any of the loci tested. They did not demonstrate a familial imprinting error. By microsatellite analysis these two AS patients were shown to share the same maternal alleles at the D15S11 and possibly the D15S113 loci, but did not have similar maternal alleles D15S1234 locus, indicating that the mutation shared by these siblings must be proximal to the D15S1234 locus. Since this study failed to provide the genetic basis for

AS in this family, it is not possible to infer the mode of AS transmission in this family.

In order to assess the validity of the commercially obtained D15S113 primers, DNA from 50 random samples was amplified (100 alleles). A heterozygosity of .73 was purported by the vendor. In my study, 32 of 50 patients were heterozygous and 17 of 50 were homozygous. One patient's DNA sample failed to amplify even after two attempts. In this study a heterozygosity value of .63 was obtained, indicating no significant difference between the expected and observed values ($p<0.05$).

E. A Putative Imprinting Center (IC) in the 15q11.2-q13 Region

A subset of PWS and AS patients (approximately 2-4%) have apparently normal, biparentally inherited chromosomes, but have abnormal DNA methylation at the D15S9, D15S63 (PW71) and SNRPN loci, presumably caused by an imprinting defect that disrupts the erasure and resetting of parent-specific DNA methylation of alleles (Reis et al. 1994). Further investigation revealed the following results. In 2 PWS patients in which only a maternal methylation pattern was observed at the D15S9 and D15S63, one patient was found to have a deletion of the SNRPN gene. In four cases of AS, two being familial, all were shown to have only a paternal methylation pattern at D15S9 and D15S63 (Reis et al. 1994). In two of these AS families, familial microdeletions between the PW71 and SNRPN loci, representing approximately 50-70 Kb, have been established (Buiting et al. 1995). From the results of these studies, it has been proposed that there is an imprinting center between the PW71 and SNRPN loci that regulates the chromatin structure, DNA methylation and gene expression in *cis* throughout the 15q11.2-q13 region. Mutations of the imprinting center can be transmitted silently through the germline of one

gender, but appear to block the resetting of parent-specific methylation in the germline in the opposite gender. In other words, the methylation pattern does not switch germline patterns. The expression of genes throughout this imprinted region remains defective and results in PWS if passed through the male germline or AS if passed through the female germline.

In trying to identify the molecular defect in the AS patients presented above, it was necessary for me to obtain Southern blotting skills in order to perform the molecular assays that would demonstrate parent-specific methylation differences at the 5'SNRPN gene. It was therefore decided by Dr. E. Magenis that I would learn this technique at the University of Florida from Dr. Daniel Driscoll, perfect it so that it could be used at OHSU, and perform this assay on a group of clinically well defined PWS and AS patients. While it had been shown that 5′ SNRPN methylation was abnormal in a small number of patients (6 PWS patients and 2 AS patients) (Sutcliffe et al. 1994), no large studies had been done to test the methylation assay against the cytogenetic results in PWS and AS patients with a clear clinical diagnosis. In addition, it was the decision of the Magenis laboratory at this time to tabulate the number of clinically well diagnosed PWS and AS cases and compare the laboratory results obtained on all of these patients. This data was to be compared with reported data from similar types of studies performed in other laboratories in order to determine if the etiology of genetic abnormalities differed in the PWS and AS population at OHSU when compared with other reported populations. In addition the purpose of this tabulation was to compare the consistency of cytogenetic, FISH and molecular test results within an individual PWS or AS patient.

## XV. TABULATION OF PWS AND AS LABORATORY RESULTS

### 1. Thesis hypothesis 4

Within a selected group of clinically well defined PWS and AS patients, when tested by cytogenetic and molecular methods, the results obtained from these tests will demonstrate similar percentages of patients who are affected with PWS and AS due to, the different etiologies of deletions, UPD and imprinting abnormalities of the 15q11.2-q13 region to those that are reported in the literature. Within the PWS cohort, 60-80% will have a visible cytogenetic deletion of 15q11.2-q13. Of the approximate 30 % of patients with normal appearing chromosomes, approximately 28% will have UPD of chromosome 15 and about 2% will have an imprinting abnormality. Within the AS cohort 60-80% will have a visible cytogenetic deletion of 15q11.2-q13. Of the approximate 30 % of patients with normal appearing chromosomes, less than 5% will have UPD of chromosome 15 and about 2% will have an imprinting abnormality. In addition, PWS and AS patients samples, when tested by cytogenetics, FISH, and origin studies by Q-banding or PCR microsatellite of chromosome 15, should show a consistency of test results. In other words, a PWS patient with a cytogenetic deletion of 15q11.2-q13 should show loss of FISH probes as well as loss of an allele fragment by Southern blotting of the SNRPN gene. Futhermore, the loss of the allele by Southern blotting, Q-banding or PCR analysis should demonstrate a consistant loss of the allele or chromosome 15q11.2-13 region that is paternal in origin. Cases in which the cytogenetics are normal, should demonstrate the presence of all FISH probes. By Southern blotting analysis they should either demonstrate biparental allele fragments or an increased density of a single fragment, suggesting

Origin analysis of these same cases should demonstrate a consistant result with Southern blotting data of biparental inheritance of chromosomes or allele, or alternatively UPD.

2. The Study

This study consisted of a cohort of 51 PWS and 37 AS patients, many of whom who had been followed through the OHSU clinic for some years. These patients were clinically evaluated by Dr. E. Magenis. All patients were examined by HRCB. FISH of proximal chromosome 15 probes were completed on 28 PWS and 25 AS patients. 35 origin studies by Q-banding and PCR amplification of chromosome 15 specific microsatellite were performed on 12 PWS and 24 AS patients. A subset of 8 PWS and 11 AS patients were tested with all techniques. In addition, all techniques but origin were tested in an extra 11 PWS and 5 AS patients. 5' SNRPN methylation assays were performed on 19 PWS and 16 AS patients.

Ninety-three G-band, 17 FISH, and 1 of the special stains studies were completed by the OHSU clinical cytogenetics laboratory. The rest were completed by the Magenis research laboratory technologists (3.5 HRCB, 25 FISH, and 4.5 special stains) with the remaining 7.5 HRCB, 13 FISH and 40.5 special stain studies being completed by me. One HRCB study was completed by Kaiser Permanente cytogenetics laboratory, Portland and two studies were completed by the Idaho State Laboratory, Boise ID. All PCR and Southern blotting studies were completed by me. The number of studies here is higher than the number of studies reported in the tables in chapter 4. This merely reflects that, as HRCB evolved, in some cases were repeated to achieve longer chromosomes for analysis.

A detailed compilation of the results are presented in chapter 4 of this thesis. In summary, however, I was able to demonstrate that with the exception of two false positive results of PWS and AS by HRCB and 1 false negative result for an AS diagnosis, when multiple techniques were tested on a single individual, the test results obtained were consistent with each other. In other words, a cytogenetic deletion was shown to be deleted by FISH, was paternal by origin studies in PWS patients and demonstrated loss of the paternal 5' SNRPN allele. In addition the 5' SNRPN methylation assay detected all 19 PWS cases tested regardless of whether the genetic abnormality was due to a deletion or UPD. All 11 AS patients with cytogenetic deletions and 1 nondeletion case of AS were detected as well. The remaining AS patients tested with normal chromosomes showed a biparental, normal result by Southern blotting analysis. The results of this study were consistant with the results obtained in studies in the literature (see chapter 4). Within the PWS population, 67% had a deletion within 15q11.2-q13, 27% had a normal karyotype and 6% had a structural rearrangement. All PWS deletions were paternal and all UPD were maternal in origin. Within the AS population, 81% had deletion of 15q11.2-q13, and 19% had a normal karyotype. All AS deletions were maternal in origin and the 1 possible case of UPD was paternal in origin. One case was apparently due to an imprinting abnormality. Since I did not have DNA samples from parents of the 1 possible UPD patient, I was not able to determine whether the patient had paternal UPD or an imprinting abnormality.

The results of the test were used to produce a systematic approach to the diagnosis of Prader-Willi and Angelman syndromes. Please see chapter 4 for a complete description of this stratagem.

## XVI. ADDITIONAL IMPRINTED GENES IN 15q11.2-q13

### A. The Imprinted Prader-Willi (IPW) Gene

In recent years it has been shown that there is another gene within the 15q11.2-q13 region that shows parent specific transcription. The human IPW gene (Imprinted Prader-Willi) maps approximately 150 kb distal to the SNRPN gene, and is expressed only from the paternal allele (Wevrick et al. 1994). IPW does not appear to encode a protein and is therefore thought to be analogous to two other imprinted noncoding RNAs, H19 (Brannan et al. 1990) and Xist (Brockdorff et al. 1992). Schulze et al. (1996) have reported a PWS patient with unbalanced *de novo* translocation of chromosomes 9 and 15 with a breakpoint between the SNRPN and IPW gene. This patient shows normal transcription of the SNRPN gene but loss of the IPW transcription, by RT-PCR analysis. These authors conclude that in this PWS patient the SNRPN gene is excluded as the major determinant of his phenotype. For me this patient is difficult to explain and will require further molecular follow-up.

### B. The Expression of Imprinted Genes in Hydatidiform Moles and Ovarian Teratomas

Due to the uniparental chromosome compliment of hydatidiform moles and ovarian teratomas and their presence in early development, the expression of imprinted genes within these tissues was examined in order to substantiate parent-specific expression of these genes. Studies which detected the expression of SNRPN, ZNF127, IGF2 and H19 revealed the following results. SNRPN was expressed in all moles, but surprisingly, this gene was expressed in all ovarian teratomas as well (Glenn et al. 1996). DNA methylation analysis at 5' SNRPN revealed that

all moles and most ovarian teratomas had not maintained a parent-specific imprint. Similarly, functional imprints of the human H19 and IGF2 genes are not maintained in ovarian teratoma and hydatidiform moles (Mutter et al. 1993). In contrast, the paternal DNA methylation imprint was preserved in the ZNF127 gene in hydatidiform moles (Mowery-Rushton et al. 1996).

## XVII. NON-IMPRINTED GENES IN THE 15q11.2-q13 REGION

The SNRPN gene, thus far, is the only imprinted expressed gene in the PWS and AS critical regions. There are, however, three other genes that map to 15q11.2-q13. They are ZNF127, a zinc-finger protein (Glenn et al. 1994), gamma amino butyric acid receptor beta three subunit (GABRB3),gamma amino butyric acid receptor alpha five subunit (GABRA5) (Sinnet et al. 1993; Knoll et al. 1993) and the tyrosinase-positive oculocutaneous albinism (OCA) (Ramsay et al. 1992). The 15q11.2-q13 region in the human which contains the genes, GABRB3, GABRA5, OCA, as well as SNRPN and ZNF127 is syntenic to mouse chromosome 7 and shows conservation of imprinting between species of the SNRPN and ZNF127 genes (Nicholls 1994). The OCA gene in the human is thought to be homologous to the pink-eyed dilute locus, p, in the mouse (Gardner et al. 1992).

Of clinical interest, the hypopigmentation seen in many PWS and AS patients is most likely due to loss of the OCA gene. This gene is located just proximal to the PWS/AS critical regions and is often lost in patients with a observable chromosome deletion (Butler et al. 1989). Correspondingly, patients with a chromosome deletion are often hypopigmented, showing lighter skin than unaffected relatives and

reduced iris and hair pigmentation (Wiesner et al. 1987). PWS patients are able to make melanin pigment but the amount is reduced, demonstrated by the presence of incompletely melanized premelanosomes rather than fully melanized melanosomes in their hair bulb melanocytes (King et al. 1993). This sort of hypopigmentation is not seen in PWS and AS patients with UPD, indicating that OCA gene is not imprinted but is dosage sensitive.

## XVIII. SYNDROMES LOCALIZED TO CHROMOSOME 15

Several genetic disorders , as well as` PWS and AS have been mapped to chromosome 15. They are as follows: Bloom syndrome (Woodage et al. 1994) , Tyrosinemia type I (Phaneuf et al. 1991), Tay-Sachs disease (Takeda et al. 1990), Marfan syndrome (Kainulainen et al. 1990; Magenis et al. 1991), Limb-girdle muscular dystrophy type 2 (Young et al. 1992), Oculocutaneous albinism type II (Ramsay et al. 1992), Angelman syndrome (Magenis et al. 1987) and Prader-Willi syndrome (Ledbetter et al. 1981).

While these genes have not been shown to be imprinted, these syndromes may be expressed in the offspring with chromosome 15 uniparental isodisomy when a parent is a carrier of a mutation in one of the genes responsible for these syndromes. Accordingly, PWS and AS patients are at a higher risk for having these syndromes. Woodage et al. (1994) have reported a case of Bloom syndrome in a PWS patient. Previous studies have mapped Bloom syndrome to distal 15q (McDaniel and Schultz 1992). Molecular analysis of chromosome 15 in this patient demonstrated maternal isodisomy at 15q25, and it is the homozygosity of

this region that is proposed as responsible for the expression of Bloom syndrome in this patient.

## XIX. SUMMARY

In summary, the chromosome 15q11.2-q13 region is commonly involved in chromosome structural rearrangements including deletions and duplications, translocations, inversions, and extra bisatellited chromosomes. These structural rearrangements not only lead to clinical malformations due to duplication and deficiency of gene products, but may also disturb meiotic and mitotic segregation. Malsegregation, such as a nondisjunction event, may result in UPD, and due to the imprinted nature of this region, abnormalities of epigenetic gene regulation occur and result in the mental retardation and clinical malformations included in Prader-Willi and Angelman syndromes. The 15q11.2-q13 region has not been completely sequenced nor have the mechanisms responsible for the regulation of imprinted and non-imprinted genes within been fully characterized. While a candidate gene for PWS has been postulated, the gene(s) responsible for AS remains illusive. Molecular analysis in the clinically well defined, non-deletion AS patients is necessary to determine if they are affected with AS due to an imprinting defect in the putative imprinting region, or a mutation located elsewhere in the 15q11.2-q13 region.

The studies included within this thesis have sought to address particular questions:

1. What is the feasibility of prenatal diagnosis of PWS and AS?

2. Are particular alterations in chromosome structure or changes in recombination frequency responsible for abnormal meiotic segregation and UPD in PWS?
3. What is the molecular defect in AS patients with normal appearing chromosomes 15?

The studies pertaining to these questions and the results obtained are presented in the following chapters.

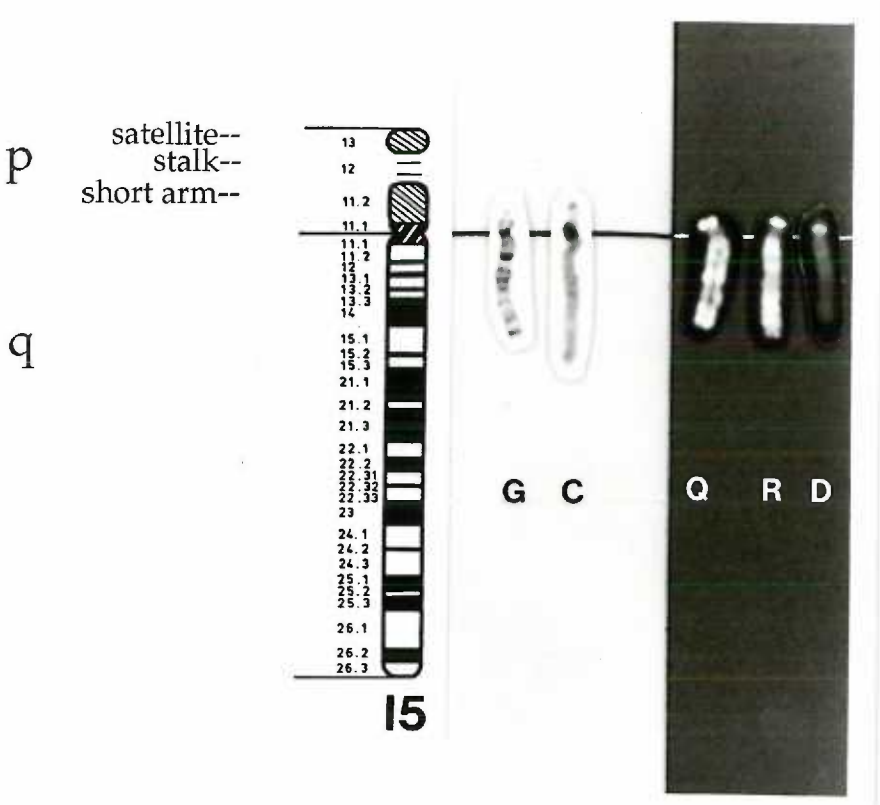


Fig. 1 Ideogram of chromosome 15 at 850 band stage. Various techniques are illustrated. G = G-banding at 800 band stage, C = C-banding, R = R-banding, Q = Q-banding, D = Distamycin/DAPI staining.

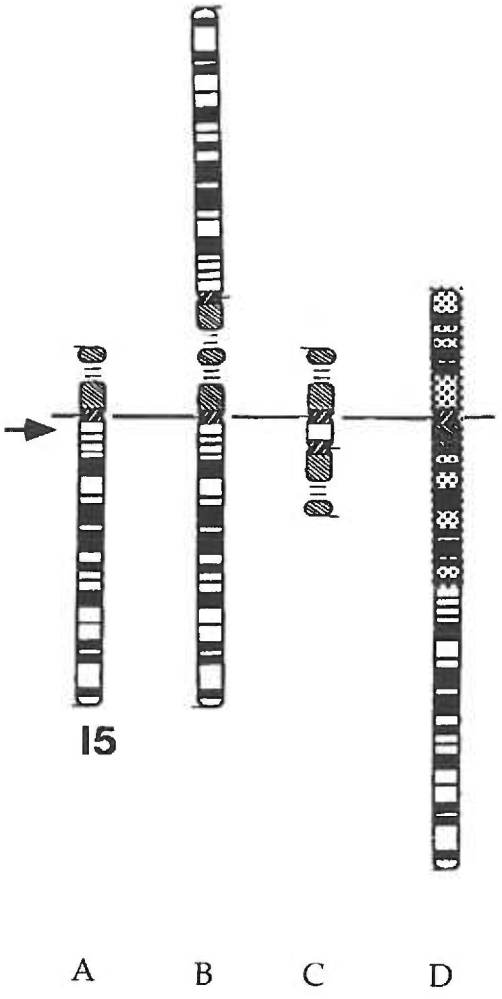


Fig. 2 Illustration of various structural rearrangements of chromosome 15. A. Normal chromosome 15 at the 850 band stage. Common breakpoint in 15q11.2-q13 is denoted by an arrow. B. Robertsonian translocation. C. Extra bisatellited chromosome 15. D. Translocation of chromosome 15 to distal long arm of chromosome 12. Chromosome 12 is indicated by stippling.

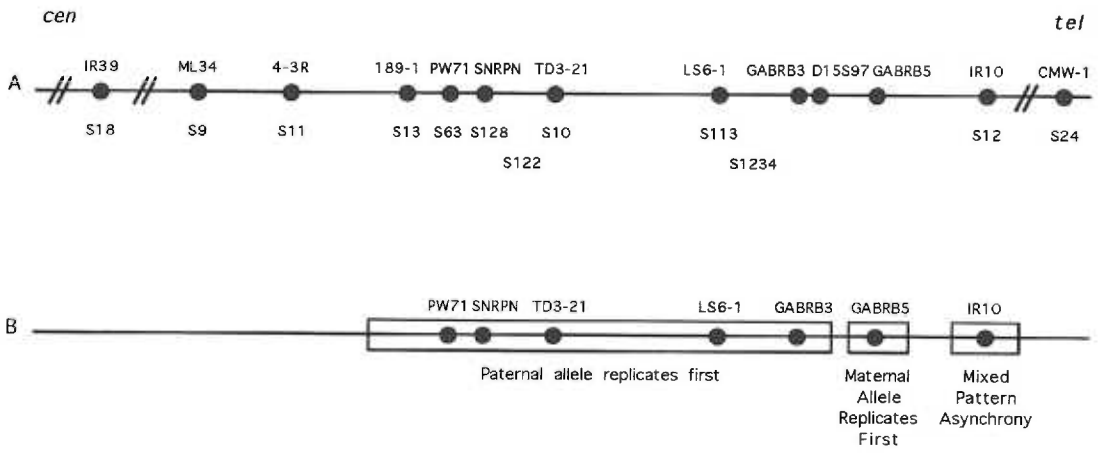


Fig. 3 A. Physical map of 15q11-q13. Loci are indicated by dots with the gene name labeled above the line and the designation number given below the line. B. Replication map.

Prenatal Diagnosis of Chromosome 15 Abnormalities in the Prader-Willi / Angelman Syndrome Region by Traditional and Molecular Cytogenetics

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## ABSTRACT

With improvements in culturing and banding techniques, amniotic fluid studies now achieve a level of resolution at which the Prader-Willi syndrome (PWS) and Angelman syndrome (AS) region may be questioned. Chromosome 15 heteromorphisms, detected with Q- and R-banding and used in conjunction with PWS/AS region-specific probes, can confirm a chromosome deletion and establish origin to predict the clinical outcome.

We report four *de novo* cases of an abnormal appearing chromosome 15 in amniotic fluid samples referred for advanced maternal age or a history of previous chromosomally abnormal child. The chromosomes were characterized using G-, Q- and R-banding as well as isotopic and fluorescent *in situ* hybridization of DNA probes specific for the proximal chromosome 15 long arm.

In two cases, one chromosome 15 homolog showed a consistent deletion of the ONCOR PWS/AS region A and B. In the other two cases, one of which involved an inversion with one breakpoint in the PWS/AS region, all of the proximal chromosome 15 long arm DNA probes used in the *in situ* hybridization were present on both homologs. Clinical follow-up was not available on these samples, as in all cases the parents chose to terminate the pregnancies.

These cases demonstrate the ability to diagnose prenatally chromosome 15 abnormalities associated with PWS/AS. In addition, they highlight the need for a better understanding of this region for accurate prenatal diagnosis.

Key Words: Prader-Willi Syndrome, Angelman Syndrome, Chromosome 15, FISH, Prenatal diagnosis, Imprinting.

## INTRODUCTION

Prader-Willi syndrome (PWS) is characterized by infantile hypotonia, obesity, short stature, small hands and feet, hypogonadism, a characteristic face and mental retardation [Prader, et al., 1956; Butler, 1990]. Angelman syndrome (AS) patients have more severe mental retardation, absent speech, inappropriate laughter, microcephaly, a large mandible and open mouth with a protruding tongue, seizures and ataxic gait [Angelman, 1965; Magenis et al., 1987]. These syndromes are associated with a microdeletion of the proximal chromosome 15 long arm. [Ledbetter et al., 1981; Magenis et al., 1987; Knoll et al., 1989]. Figure 1 illustrates the region of chromosome 15 in which most deletions occur in both PWS and AS. Consistently achieving the very high resolution chromosomes necessary for identifying this microdeletion in peripheral blood samples has been difficult. This has been even more so the case when attempting to analyze amniotic fluid (AF) chromosomes.

However, with the appropriate manipulations of improved culturing and banding techniques, AF studies now can achieve a clarity and band length enabling evaluation of the PWS/AS region. Use of DNA probes specific to the PWS/AS region aid in interpretation of the cytogenetic findings. In addition, R- and Q-banded chromosome 15 heteromorphisms, which are stably inherited in a Mendelian fashion and variable enough in the population to distinguish homologs [Olson et al., 1986], can be used to successfully demonstrate the parental origin of the deletion in PWS and AS. Since it is well established that PWS occurs as a result of an error on the paternally derived chromosome and AS as a result of an error on the maternally derived chromosome [Butler et al., 1983; Knoll et al., 1989;

Magenis et al., 1990], use of these methods in conjunction allows diagnosis of a chromosome deletion and establishment of origin to predict the clinical outcome.

Four *de novo* cases of abnormal appearing chromosomes 15 have been discovered in AF samples referred to our laboratory for advanced maternal age (AMA) or a history of a previous child with a chromosome abnormality. Of these four cases, three had what appeared to be a deletion at the chromosome level and one had a structural rearrangement. These have been further characterized using G-, R-, and Q-banding as well as isotopic and fluorescent in situ hybridization of DNA probes specific for the 15q11.2-q13 region [Harnden and Klinger, 1985].

**CLINICAL REPORTS**

Case 1

A 36-year-old woman, JJ, gravida 4, para 1, sab 2, was referred for AMA and family history of Down syndrome (maternal cousin once removed). An initial maternal serum alpha-fetoprotein appeared slightly elevated, but within the normal range in a subsequent assay. G-band analysis of the AF chromosomes indicated a deletion of 15q11.2-q13 (fig 2a). Parental chromosomes were normal.

Case 2

A 30-year-old woman, NL, gravida 2, para 1, presented for amniocentesis at 18 weeks of gestation with the indication of a previous child with a partial deletion of the chromosome 7 short arm. Ultrasound at the time of the procedure was normal. G-band analysis of the AF chromosomes documented a deletion of 15q11.2-q13. A subsequent fetal

blood sample also showed the deletion (Fig. 2b). The karyotypes of both parents were normal.

Case 3

A 37-year-old patient, GG, gravida 3, para 2, presented at 16.5 weeks gestation for amniocentesis due to AMA. Ultrasound evaluation indicated a normal fetal anatomy and good fetal movement. In all AF cells there appeared to be a small proximal deletion of 15q11.2-q11.2 (Fig. 2c). Fetal umbilical blood sampling was performed at 19 weeks for clarification of the fetal karyotype (Fig. 2c). A deletion was also noted in this analysis. Parental chromosomes were normal.

Case 4

Patient CK, who was referred for AMA at age 35 years was gravida 2, para 1. AF chromosomes demonstrated a chromosome 15 long arm inversion. Comparison of AF, cord blood and parental blood chromosomes indicated that the parents' chromosomes were normal and that the inversion was *de novo* with breakpoints that appeared to involve the PWS/AS region and the most distal band of the long arm (Fig. 2d). Multiple staining techniques were used to examine this structurally rearranged chromosome and assign breakpoints of 46,XY, inv(15)(pter->q12::q26->q12::q26->qter).

**METHODS**

Cultures and Chromosome Preparations

Peripheral blood lymphocytes from parents and fetal umbilical blood samples were grown using routine techniques, then synchronized with amethopterin to obtain an adequate number of cells in early metaphase with high band number [Yunis and Chandler, 1977]. Chromosome analyses were accomplished using trypsin G-banding [Seabright, 1971], R-banding [Schweizer, 1980] and Q-banding [Caspersson et al., 1970]. The chromosome 15 origin was established by comparing parental and fetal R- and Q-banded chromosome 15 heteromorphisms [Olson et al., 1986; Magenis et al., 1990]. Amniocyte cultures were grown to 75% confluency in a closed tissue culture flask system with 50:50 Chang:MEM medium. To a 5 ml culture, 0.1 ml of 10 μg/ml colcemid was added 2.5-3.0 hours before harvesting the cells in order to increase the length of the chromosomes without severely reducing the mitotic index. Cells were then

enzymatically released from the flask surface with trypsin and chromosomes prepared according to a variation of the above protocol.

Probes

The Prader-Willi/Angelman syndrome cosmids -regions A (D15S11) and B (GABRB3)(with and without a chromosome 15 identifier marker cosmid) and chromosome 15 classical satellite (D15Z1) probes were procured from ONCOR. The PWS/AS cosmids A and B hybridize to specific sequences in bands 15q11-15q13. These bands include the PWS/AS critical region [Kuwano et al., 1992]. The satellite probe hybridizes to short DNA repeats related to AATGG in "classical" satellites located in the pericentromeric heterochromatin of chromosome 15.

The PWS/AS syndrome cosmids-region A and B were used in all case studies. In addition, the PWS/AS syndrome cosmids-region A and B including the identifier marker cosmid were used in Case 2. The chromosome 15 classical satellite probe was used in Cases 2, 3 and 4 for identification of the chromosome 15.

Four other DNA sequences specific to 15q11-q13 were used in *in situ* hybridization procedures. The probes used in isotopic *in situ* hybridization were pIR10-1 (D15S12)[received from Marc Lalande][Donlon et al., 1986; Nicholls et al., 1989] and p189-1 (D15S13) [from ATCC]. These probes had insert sizes of 0.8kb and 0.9 kb respectively. The 6.2kb clone pbsML34 (D15S9)[received from ATCC and subcloned into bluescript (Stratagene)] and the 37kb clone cRN189-1(D15S13) (received from Robert Nicholls) were use in FISH. The latter clone includes the p189-1 sequence used in the isotopic *in situ* procedure. The SNRPN P1 clone, with an

insert of approximately 70-80 kb was isolated and provided by Stuart Leff [ Özçelik et al., 1992, Sanjines and Leff, unpublished material]. All probes were grown and isolated using standard protocol [Maniatis, 1989]. The compound IPTG was added to the inoculum of the large scale prep of the SNRPN P1 clone at an OD of 0.2-0.35 for a final concentration of 1 mM in order to increase the yield. Large scale preps were purified by a CSCl gradient.

The proposed order of these probes on chromosome 15 is: p arm telomere-D15Z1-cen-D15S9-D15S11-D15S13-SNRPN-GABRB3-D15S12-q arm telomere [Kuwano et al., 1992].

Fluorescent *in situ* hybridization (FISH)

Slides containing high quality metaphase spreads were treated with RNase for 1 hour at 37°C then washed in 2XSSC and dehydrated in a 70%, 80% and 95% ethanol series. The chromosomes were denatured by immersing each slide into 70% formamide/2XSSC at 71°C for 3 min then dehydrated in ice cold 70%, 80%, and 95% ethanols.

Hybridizations using ONCOR probes were carried out as per the manufacturer's protocol. For chromosome identification, the classical satellite probe was sequentially hybridized in a separate in situ hybridization procedure.

Plasmid DNA was nick translated (BRL) incorporating biotin-11-dUTP. A probe cocktail was made containing 50% formamide/2XSSC, 0.1 mg/ml cot-1 DNA with probe concentrations ranging from 30 ng/µl to 60 ng/µl. This cocktail was denatured at 75°C for 10 min and then allowed to prehybridize for 2 hours at 37°C before being applied to the chromosome

preparations. After 16 hours of hybridization at 37°C, slides were washed in three changes of 50% formamide/2XSSC at 43°C. The remaining washes and method of detection are as per Trask and Pinkel, [1990].

The chromosomes were visualized on a Zeiss Axiophot equipped with Zeiss FITC/PI and DAPI/FITC/TEXAS RED filter sets. Between 25-50 cells were counted for each FISH procedure. Data was scored as the number of chromotids with a signal per cell.

Isotopic *in situ* hybridization

Slides were treated as described in the FISH protocol except that probes were nick translated with tritiated nucleotides. After the overnight hybridization and washes, the slides were dipped in Kodak autoradiographic NTB-2 liquid emulsion. The slides were then placed in the dark at 4°C for 10-15 days. Slides were developed, and then the chromosomes were R-banded for identification. Silver grains were analyzed over fluorescent R-banded preparations by a double-illumination system. For illustrative purposes these slides were destained in methanol and stained with Wright stain. Two normal male control patients were used in each *in situ* procedure. Between 110-145 cells were examined for each of the probes, pIR10-1 and p189-1 [Harper and Saunders, 1981; Magenis et al., 1985].

## RESULTS

In Case 1, in which there appeared to be a deletion at the chromosome level, FISH of ONCOR probes PWS/AS region A and B was performed; a deletion in the region of 15q11.2-13 was verified. FISH of the SNRPN probe indicated only one copy of this sequence to be present (Fig. 3a). The chromosome 15 homologs were distinguished both by the deletion and the heteromorphic variable regions. All 3 probes showed hybridization only to the normal homolog. Chromosome origin studies indicated that the deleted chromosome 15 in the fetus was of paternal origin, predicting the phenotype of PWS in the fetus.

In all cells from the AF and a fetal blood sample from Case 2, in which there appeared to be a small proximal deletion of chromosome 15q11.2-q13, FISH of probes PWS/AS region A and B confirmed the cytogenetic analysis. In all cells examined both probes showed hybridization to only the normal homolog (Fig. 3c). Origin studies were not completed in this family. The initial chromosome heteromorphism study was uninformative and no further material was available for better quality preparations or for molecular analysis.

Case 3, GG, appeared cytogenetically deleted for the 15q11.2-q11.2 region. However, hybridization of probes pbsML34, ONCOR probes PWS/AS region A and B and the chromosome 15 classical satellite probe did not demonstrate a molecular deletion. These probes hybridized to both homologs in all cells examined (Fig. 3d). The isotopically labeled probes, pIR10-1 and p189-1 hybridized to both the normal and abnormal chromosomes 15 as well. A compilation of the silver grain distribution (which represents hybridization of the probe) over all chromosomes

indicated that the probes pIR10-1 and p189-1 showed a distribution of 10 % and 11.7 %, respectively, of the total grains over 15q11-q13. Silver grains were present on both the normal and abnormal chromosome. There was no significant accumulation of silver grains on any other chromosome pair (Fig. 4a and b). Chromosome origin studies indicated that the abnormal appearing chromosome was paternally derived as is indicated by R-band heteromorphisms (fig. 5). However, the paternal chromosome 15, appeared to be cytologically normal.

Case 4 was a *de novo* paracentric inversion of a chromosome 15 long arm. Breakpoints appeared to be in q11.2 and q26. *In situ* hybridization was undertaken for further breakpoint analysis and to detect any loss of genetic material. Material detected by DNA probes pbsML34, cRN189-1, PWS/AS region A and B and the SNRPN gene was present on both homologs (Fig. 3b). However, all of the probes were rearranged to the very distal breakpoint region of the inverted chromosome. The centromeric satellite material, detected by the classical satellite probe remained at the centromere of both homologs. The proximal breakpoint of the inversion is therefore between the centromere and the D15S9 locus. The chromosome heteromorphism study determined that the *de novo* rearranged chromosome 15 was maternal in origin.

In Case 1, both chromosome and probe hybridization results were available to the parents at the time of counseling. In cases 2,3, and 4, parents were counseled based on the results of the chromosome studies alone. In all four cases, the parents chose to terminate the pregnancy. Therefore pregnancy outcome data are unavailable. Autopsies were not performed.

## DISCUSSION

The proximal long arm of chromosome 15 (q11-q13) is a structurally unstable region of the genome, prone to breakage and rearrangement. Reports in the literature include *de novo* microdeletions, *de novo* microduplications, small additional pseudodicentric chromosomes, translocations--to any number of chromosomes, often to the telomeric region [Ledbetter et al, 1982; Donlon, 1988; Freeman et al, 1993; Reeve et al., 1993].

This region is as complex as it is unstable. The PWS and AS which have been localized to this region are quite distinct in their phenotypic manifestation, most patients have an observable chromosomal deletion of 15(q11-q13) [Ledbetter et al., 1981; Knoll et al., 1989; Magenis et al., 1990]. What determines whether a patient will be affected with PWS or AS depends on the parental origin of the chromosome 15 deletion. Not all patients with PWS and AS have a deletion of chromosome 15. In approximately 30 % of PWS [Ledbetter and Cassidy, 1988] and 50 % of AS patients [Prembrey et al., 1989; Knoll et al., 1989] the chromosome 15 pair appears normal both at the cytogenetic and molecular level. A portion of these patients is affected due to uniparental disomy of chromosome 15, a situation in which the patient has both chromosome 15 homologs contributed from one parent. Uniparental disomy is thought to occur at a frequency of 20-24 % in PWS [Mascari et al., 1992] and 3-4 % in AS [Nicholls et al., 1989]. The clinical presentation of PWS or AS, depending on whether the structural or functional loss of genetic material is paternal or maternal in origin, has led to the conclusion that the PWS/AS region is an imprinted region [Hall, 1990]. In other words, there is differential

expression of a gene or set of genes dependent of whether the genetic material is inherited from a male or female [Solter 1987; Sapienza, 1990].

Attempts are in process to map the PWS/AS critical regions at the molecular level [Knoll et al., 1990; Kuwano et al., 1992], but large gaps in the map exist in which there are stretches of DNA of unknown function. The critical gene or genes that, when defective, result in PWS or AS are yet unknown. The SNRPN gene has been proposed as a candidate gene for PWS [Leff et al., 1992; Özçelik et al., 1992; Reed and Leff,1994], but the relationship to PWS or to other potentially involved genes in this contiguous gene syndrome is yet to be defined. The smallest region of overlap (SRO) for the PWS, put forth by Buiting et al. [1993] includes the SNRPN gene but is large enough to encompass other genes as well.

Likewise, the gene or genes responsible for AS are unknown. Loss of the ∂-aminobutyric acid receptor ß3 subunit gene (GABRB3) has been implicated in AS. A family in which 3 sibs, all affected with AS, are deleted for the GABRB3 gene currently define the minimal AS critical region [Hamabe et al., 1991; Saitoh et al., 1992]. Sinnet et al. [ 1993] has shown that in this family, as well as in another AS patient with a 45,XY,-13,-15,+der (13),t(13;15)(p13;q13)mat karyotype, the distal breakpoint is within a 130kb intron in the GABRB3 gene. These data suggest that the AS critical region does not include genetic material distal to the GABRB3 gene but does not exclude the possibility of abnormal regulation of genes more distally located due to the structural rearrangement of genetic material.

Therefore, it is the case that the proximal 15 long arm probes chosen for *in situ* hybridization are used to define a deletion rather than assign a diagnosis of PWS or AS. This is possible only after the origin of the

chromosome 15 with the deletion is determined. Cases 1 and 2 illustrate this, in that in both cases a deletion was clearly established, but only in Case 1 was an origin study informative. Therefore, only in Case 1 was it possible to make a diagnosis of PWS.

Case 3, which clearly appears deleted at the cytologic level, was not deleted for any of the probes used in the *in situ* hybridization experiments in this study. It is possible that this fetus is hemizygous for other DNA sequences not detected by these probes or has some other unusual underlying chromatin structure. It is unlikely that the apparent deletion represents a familial structural variation. This abnormal appearing chromosome in the fetus appears structurally normal in the father and a subsequent fetus--both of which are phenotypically normal .

Case 4, with an inversion of almost the entire long arm of chromosome 15, presented as an unusual counseling challenge. The risk for abnormality in all *de novo* inversions combined may be as high as 20 % [Warburton, 1984]. This being a particularly unstable region and regulated in an imprinted fashion contributes two factors which may not be adequately reflected in a risk assessment across all chromosome regions. *In situ* hybridization demonstrated that all of the probes used in the study were present on the inverted homolog, but rearranged to the distal region of the chromosome. It was not possible to determine with absolute assuredness that all the genetic material in the critical PWS/AS region was present or that all the genes would function properly in their rearranged position.

It is clear from our experience and that of others that this unstable region of the genome warrants close scrutiny in all AF samples. This is initially achieved with high resolution banding and secondarily with the

use of multiple staining techniques for further breakpoint analysis. A comparison of the chromosomes from the AF sample with those of the parents is essential for establishing any familial chromosome structural variation. When a case warrants further investigation at the molecular level, DNA probe analysis may be used to confirm or more precisely define a suspected chromosome abnormality. Given the technical skills necessary to accurately examine the PWS/AS region, it is likely that the percentage of microdeletions associated with PWS and AS is in actuality greater than 70% for PWS and 50% for AS. An origin study in which the chromosome heteromorphisms or DNA polymorphisms of parent and fetal samples are compared may allow one to detect uniparental disomy as well as to predict a clinical outcome.

Clearly, establishing the responsible gene or genes involved in PWS and AS, with an understanding of how the expression of these genes is regulated, will substantially increase the ability to diagnose and accurately assess recurrence risks and risks for other family members.

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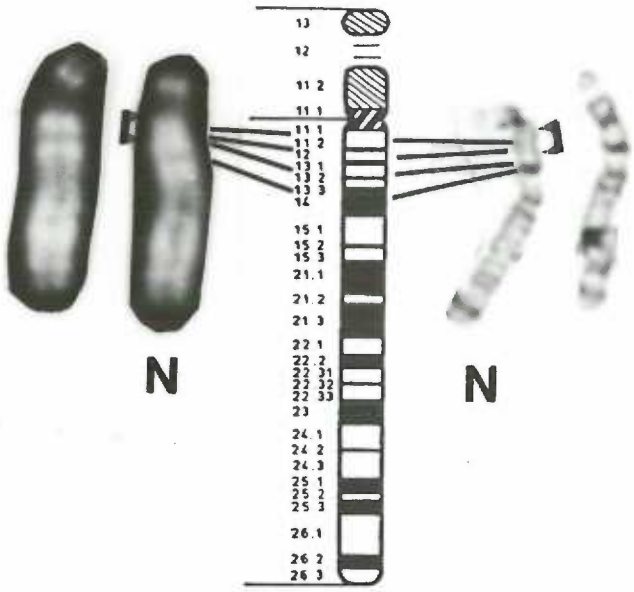


Fig. 1 Ideogram of chromosome 15 at the 850 band stage. Two pairs of chromosome 15 at the 850 band stage are illustrated, one stained with fluorescent R-banding (left) and one with high-resolution G-banding (right). Lines from the normal homolog of each pair, labeled "N", indicate landmark bands. Brackets on the normal chromosomes indicate the commonly deleted region of this chromosome seen in Prader-Willi and Angelman syndrome. Note that band q12 at this stage splits to form two smaller gray sub-bands separated by a pale band, as is seen in the normal prophase G-banded chromosome. The ISCN ideogram [Harnden and Klinger, 1985] has been altered to depict this consistent finding [Magenis and Toth-Fejel, 1992]

Fig. 2. **a-b** Two pairs of G-banded chromosomes 15 from patient JJ, case 1 (a) and NL, case 2 (b). The abnormal chromosome is on the right of each pair. Arrows indicate deletion breakpoints in q11.2q13 (JJ and NL). **c** Two pairs of G-banded chromosomes 15 and one pair of R-banded chromosomes 15 from patient GG, case 3. Left pair of G-banded chromosomes are from AF sample, right-hand pair from the fetal blood sample. The abnormal chromosomes is on the right side of each pair. Arrows indicate the apparent deletion in the band q11.2q11.2. Note the loss of fluorescent material in band q11.2 in the abnormal R-banded chromosome 15. The centromere of chromosome 15 does not stain using this technique allowing a sharp demarcation between the centromeric region and band q11.2. **d** G-banded (left) and R-banded (right) chromosome 15 pairs of patient CK, case 4. The abnormal chromosome with the long arm inversion is on the right of each pair. Arrows indicate the breakpoint in bands q11.2 and q26.

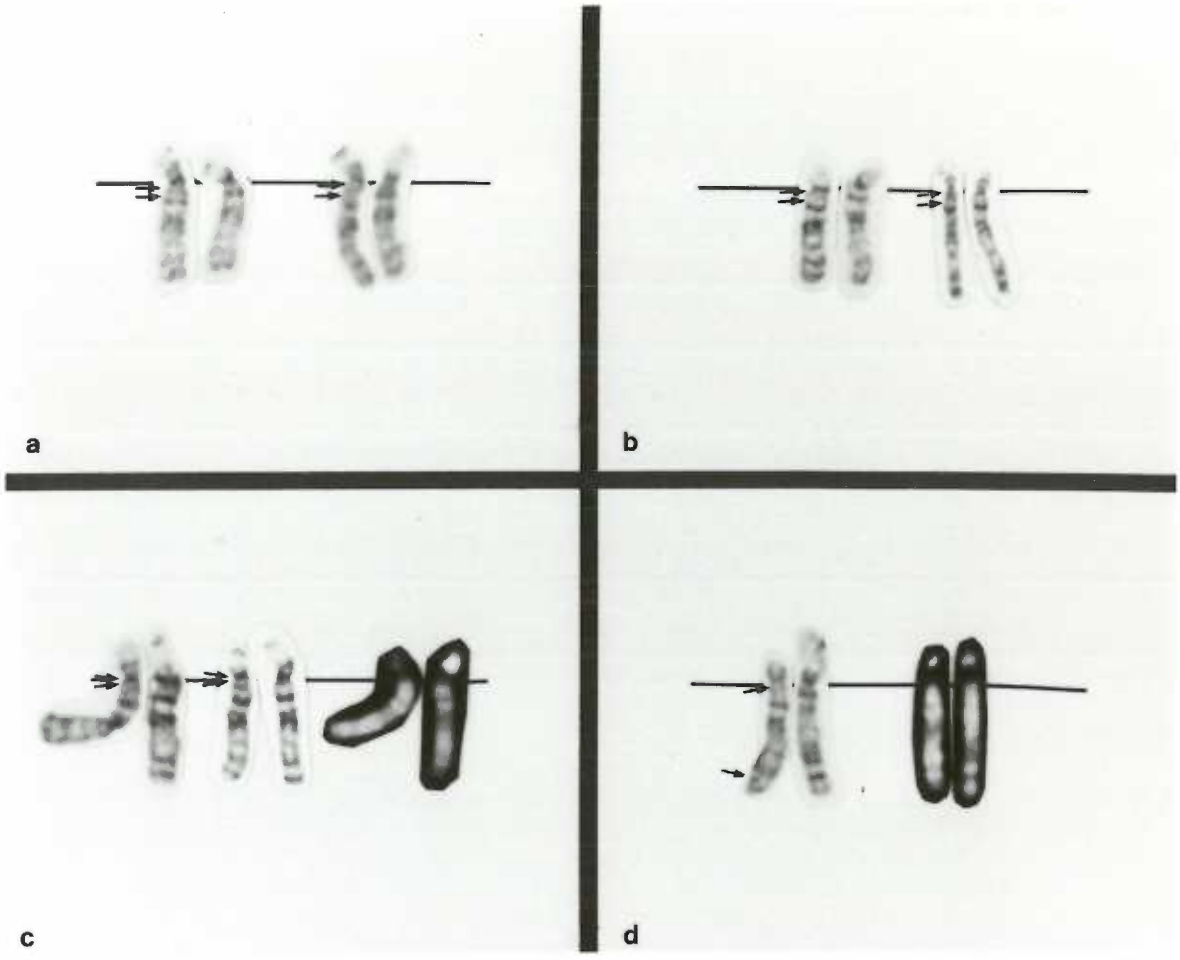
a
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Fig. 3a. Chromosomes of AF sample from patient JJ, case l, with a karyotype of 46,XX,del(15)(q11.2-q13) . Large arrows indicate the normal chromosome 15; small arrows point to the abnormal chromosome 15. **1** G-banded chromosome spread. **2** Identical cell with sequential FISH of the ONCOR PWS/AS region A probe. Note that hybridization is with only the normal chromosome 15 homolog. **3-4** G-banded metaphase spread (left) and sequential FISH of ONCOR PWS/AS region B. **5** Chromosome spread showing FISH of SNRPN probe. **6** Identical cell with sequential FISH of chromosome 15 classical satellite probe used for chromosome 15 identification. Fig. 3b. Chromosomes of AF sample from patient CK, case 4, with a karyotype of 46, XY, inv (15)(pter->q12::q26->qter). Large arrows indicate the normal chromosome 15; small arrows point to the abnormal chromosome 15. **1** FISH of the probe pbsML34. **2** FISH of the probe cRN189-1. **3-4** FISH of the probe SNRPN (left) and the sequential FISH of ONCOR chromosome 15 classical satellite probe (right). **5-6** FISH of the ONCOR probe PWS/AS region A (left) and sequential FISH of ONCOR chromosome 15 classical satellite probe (right). **7-8** FISH of the ONCOR probe PWS/AS region B (left) and sequential FISH of ONCOR chromosome 15 classical satellite probe (right). In all hybridization studies, the unique sequence copy probes are present in band q11.2 on the normal chromosome 15. These probes hybridize to the distal region of the long arm of the inverted chromosome 15. Classical satellite genomic material is not included in the inversion. Fig. 3c. Chromosomes from the AF sample of patient NL, case 2 with a karyotype of 46, XX ,del (15)(q11.2-q13). Large arrows indicate the normal chromosome 15; small arrows point to the abnormal chromosome 15. **1** FISH of the ONCOR probe

PWS/AS region A with ID marker. **2** FISH of the ONCOR probe PWS/AS region B. Hybridization of each of the two PWS/AS region probes is present on only the normal homolog. Homolog identification was confirmed by sequential FISH with the chromosome 15 classical satellite (not shown).

Fig. 3d. Chromosomes from the AF sample of patient GG, case 3 with a karyotype of 46,XX,del(15)(q11.2q11.2). Large arrows indicate the normal chromosome 15; small arrows point to the abnormal chromosome 15. **1-2** FISH with ONCOR probe PWS/AS region A (top) and sequential FISH with the chromosome 15 classical satellite probe (bottom). **3-4** FISH with ONCOR probe PWS/AS region B (top) and sequential FISH with the chromosome 15 classical satellite probe (bottom). **5** FISH with the probe pbsML34. **6** Sequential R-banding for chromosome identification. All probes are present in two copies, indicating normal hybridization to both homologs.

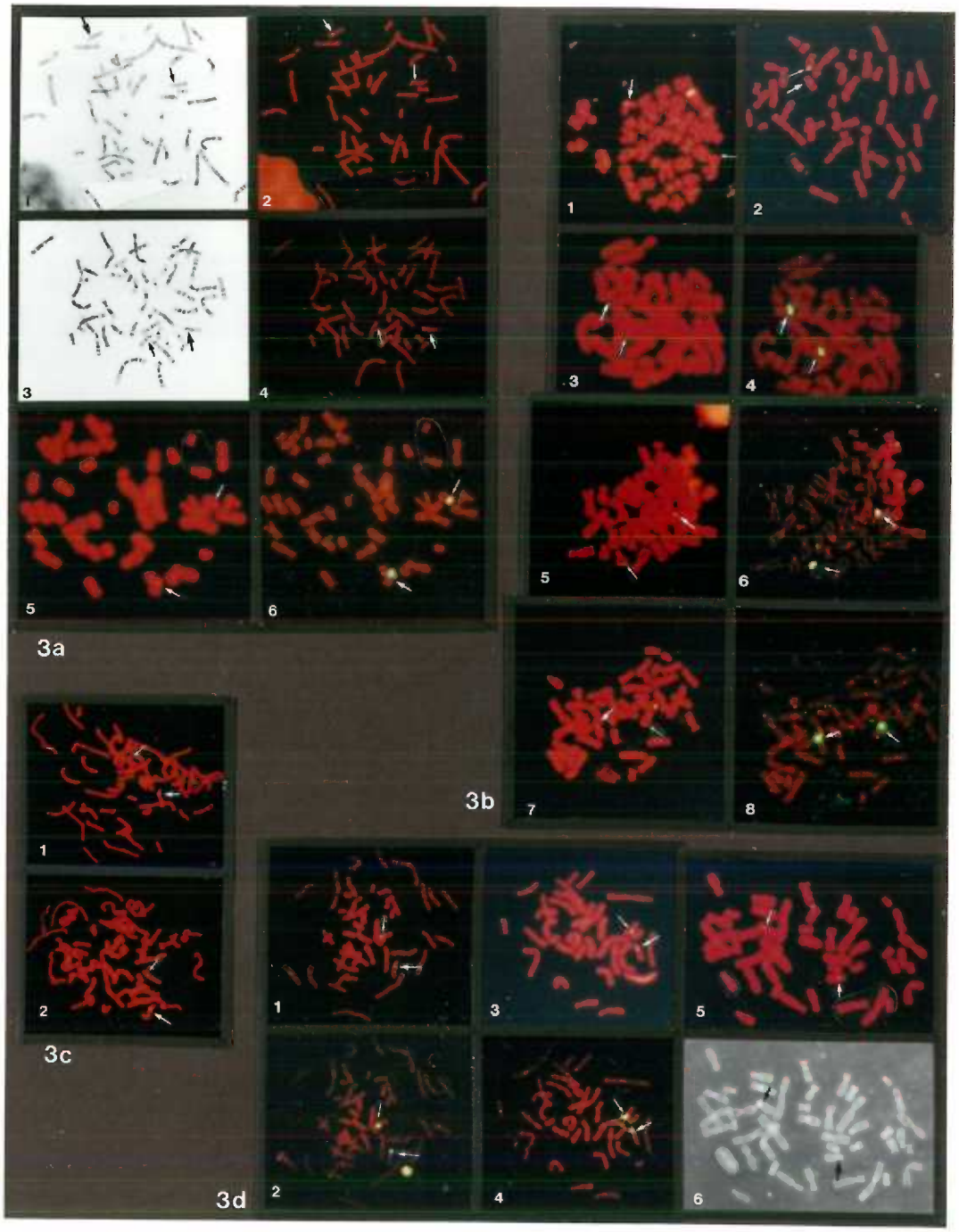
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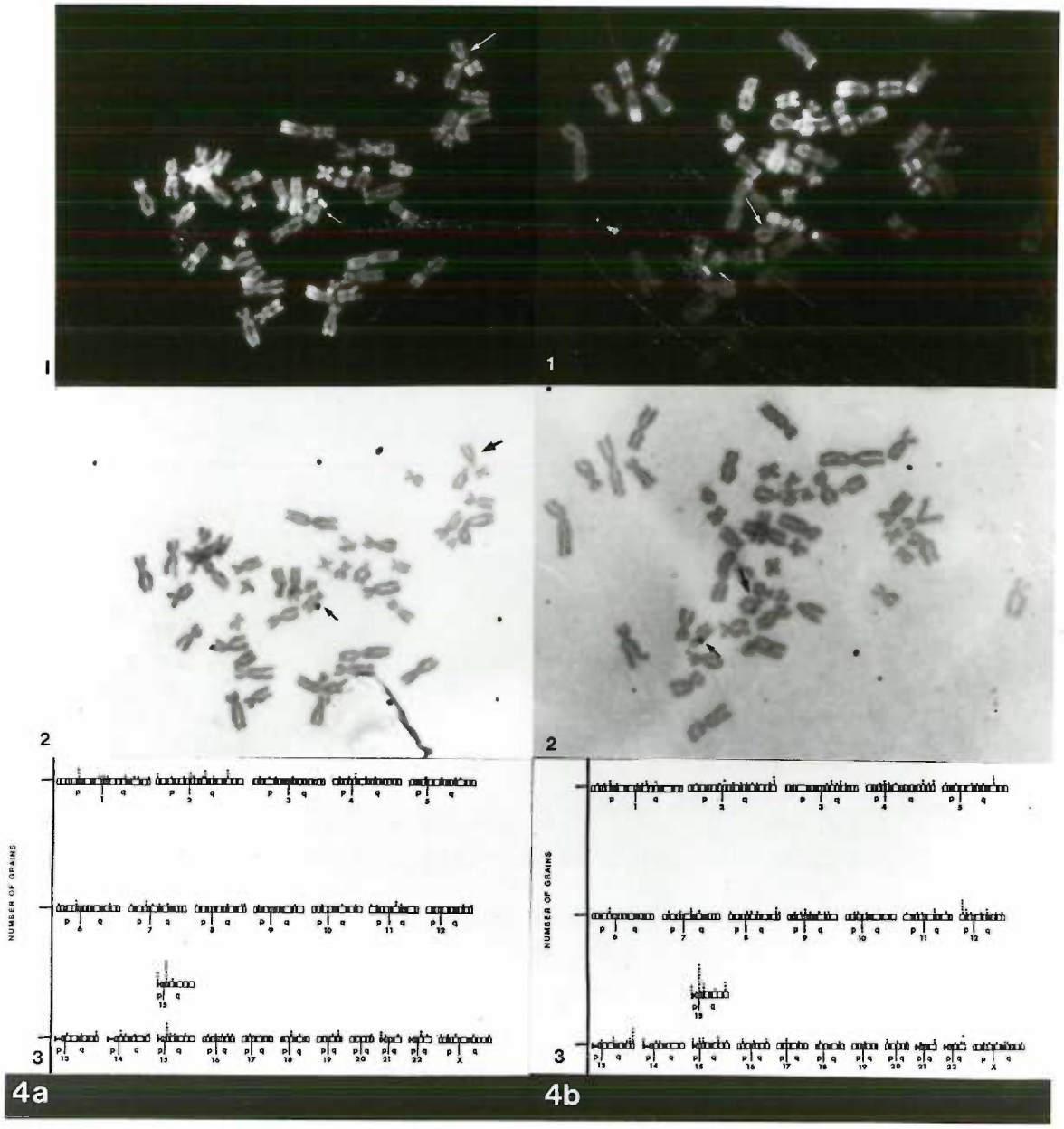
Fig. 4a. Isotopic hybridization of the pIR10-1 probe with AF chromosomes from patient GG, case 3. Large arrows indicate the normal chromosome 15 homolog; small arrows point to the abnormal chromosome 15. **1** R-banding of a metaphase spread for chromosome identification. **2** Identical cell after hybridization of probe, pIR10-1, Wright stained to visualize chromosomes and silver grains. Abnormal chromosome shows hybridization, indicated by the presence of a silver grain. Normal homolog did not show hybridization of probe in this cell. **3** Ideogram of grain distribution results from in situ hybridization. Note that both chromosome 15 homologs (abnormal placed above normal) show a peak of grains above background. Fig. 4b. Isotopic hybridization of the p189-1 probe with AF chromosomes from patient GG, case 3. Arrows indicate chromosome 15 homologs as above. **1** R-banding of a metaphase spread. **2** Identical cell after hybridization of the p189-1 probe, Wright stained for visualization of chromosomes and silver grains. Both normal and abnormal chromosomes show hybridization, indicated by the silver grain present on the proximal long arm of chromosome 15. **3** Ideogram of grain distribution results from in situ hybridization. Hybridization of the probe to the normal and abnormal chromosome 15 is above background hybridization levels of any other chromosome pair.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4a
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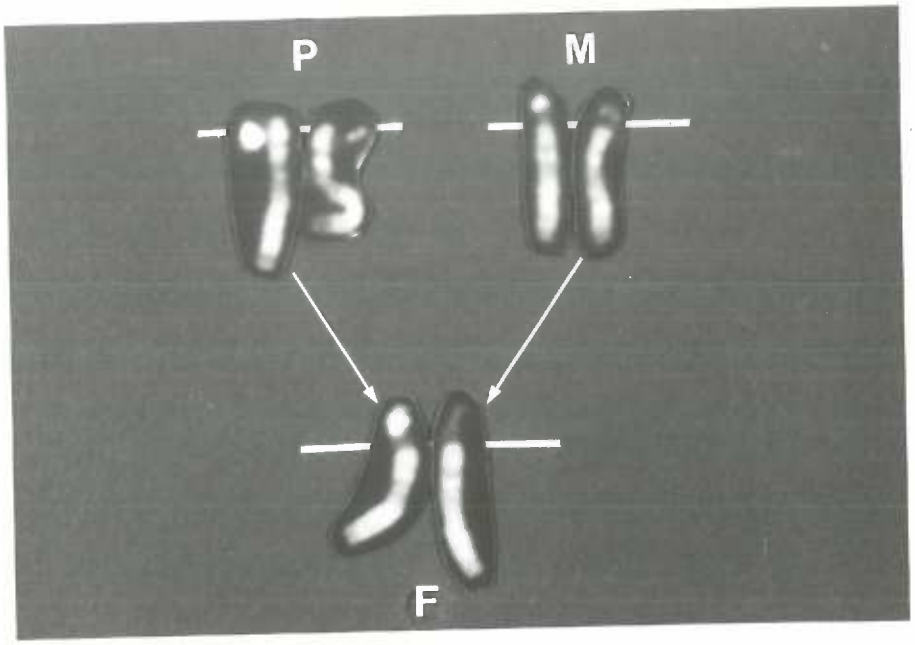


Fig. 5. R-banded chromosomes from Case 3 (P, father; M, mother; F, fetus). The arrows indicate the parental origin of each of the chromosome 15 homologs inherited in the fetus. In the fetus, the origin of the homolog with the very bright stalk is paternal, while the homolog with the dull staining stalk is maternal in origin. The R-banding patterns shows that both parents have band 15q11.2 present, seen as the first of three bright bands on the long arm using this technique. The fetus has reduced brightness in this band indicating that there is loss of genetic material (see fig. 2c).

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The Impact of Imprinting: Prader-Willi Syndrome Resulting From Chromosome Translocation, Recombination and Nondisjunction

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**Summary**

Prader-Willi syndrome (PWS) is most often the result of a deletion of bands q11.2-q13 of the paternally derived chromosome 15, but also occurs due to maternal uniparental disomy (UPD) of this region, or rarely from a methylation imprinting defect. A significant number of cases are due to structural rearrangements of the pericentromeric region of chromosome 15. We report two cases of PWS with UPD in which there was a meiosis I nondisjunction error involving an altered chromosome 15 produced by both a translocation event between the heteromorphic satellite regions of chromosomes 14 and 15 and recombination. In both cases, high resolution banding of the long arm was normal and FISH of probes D15S11, SNRPN, D15S10 and GABRB3 indicated no loss of this material. Chromosome heteromorphism analysis showed that each patient had maternal heterodisomy of the chromosome 15 short arm, while PCR of microsatellites demonstrated allele specific maternal isodisomy and heterodisomy of the long arm. SNRPN gene methylation analysis revealed only a maternal imprint in both patients. We suggest that the chromosome structural rearrangements, combined with recombination in these patients disrupted normal segregation of an imprinted region, resulting in uniparental disomy and PWS.

**Introduction**

Prader-Willi syndrome (PWS) is an autosomal disorder characterized by neonatal/infantile hypotonia, mental retardation, short stature, small hands and feet, hypogonadism, obesity, and hyperphagia (Prader et al. 1956).

Several genetic mechanisms are responsible for the development of PWS. The majority of patients (75%) carry a deletion of the paternally derived chromosome 15q11-q13 (Ledbetter et al. 1981; Butler and Palmer 1983) with most non-deletion PWS patients having maternal uniparental disomy (UPD) of chromosome 15 (Nicholls 1994). A small number of chromosomally normal patients carry an imprinting defect (Reis et al. 1994). PWS may be the clinical outcome from any chromosome 15 structural change in which there has been a physical or functional loss of genetic material in the imprinted PWS critical region.

The PWS critical region is located in a region of approximately 3-5 Mb (Donlon et al. 1986; Mutirangura et al. 1993) of proximal chromosome 15. By virtue of the fact that only the paternal allele of the small nuclear ribonucleoprotein-associated polypeptide SmN (SNRPN) gene is expressed, its map location of 15q11-12 within the smallest region of deletion overlap for PWS (Özçelik et al. 1992), parent-specific differential DNA methylation (Glenn et al. 1993; Glenn et al. 1994; Sutcliff et al. 1994) and the predominant expression of this gene in neuronal tissue (McAllister 1988), the SNRPN gene is considered a primary candidate gene for PWS.

Although a maternal age effect has been known since 1933 (Penrose), the sequence of events that interfere with proper segregation, that presumably result in trisomy initially and then UPD, are unknown.

Nondisjunction is mostly attributable to maternal errors at the first meiotic division (MI) (Polani 1981; Magenis and Chamberlin 1981), and associated with advanced maternal age. An increase of nondisjunction is associated, as well, with chromosome structural rearrangements including Robertsonian translocations (Cattanach and Moseley 1973; Miller 1981).

If proper meiotic segregation is dependent on timely resolution of chiasmata and/or the proper binding of chromosome-specific spindle fibers to centromeres and their associated kinetochore-related proteins, then rearrangements of pericentromeric or unstable regions of the genome may disrupt normal disjunction and lead to uniparental disomy.

To further explore the mechanisms of recombination and segregation in chromosome structural rearrangements that lead to UPD, we have studied in depth, at the cytogenetic and molecular levels, two PWS patients with complex rearrangements of chromosome 14 and 15. FISH and special staining techniques were used to determine the presence or absence of chromosome material, translocation breakpoints, and chromosomal origin. Molecular studies including microsatellite polymorphism analysis, and VNTR analysis, were used to determine recombination and paternity, respectively. Parent-specific patterns of methylation in the SNRPN gene were examined to assess the imprinted nature of the region 15q11-q13.

**Subjects, Materials and Methods**

*Case one*

Patient ME, was an 11 year old female with the diagnosis of Prader-Willi syndrome who had essentially all of the diagnostic features. Consistent with the major and minor criteria (Holm et al. 1993), she had neonatal/infantile hypotonia, feeding problems and failure to thrive in infancy followed by rapid weight gain before the age of 5 years. Her facial features included narrow bitemporal diameter, almond shaped eyes and down-turned mouth. Notable were her dark eyes and hair color (fig. 1a). She had short stature ( fifth percentile). Her hands and feet were small for height and age, the hands being narrow with straight ulnar borders. She showed moderate developmental delay as well as hyperphagia and aggressive episodes.

The parental ages at the time of ME's birth were both 34 years.

*Case two*

Patient DK was a 39 year old male with PWS. His neonatal and early infancy history included hypotonia, hyporeflexia, feeding difficulty, and no crying. Esotropia, bilateral cryptorchidism and clubfeet were noted. At birth DK's mother's age was 24 years and father's age was 27 years. While DK's weight is now fairly well controlled, he has had a history of accelerating weight gain after age one year. PWS characteristics were evident; DK had narrow, upslanted palpebral fissures and a downturned mouth with a thin upper lip. His eye and hair color were dark; hands and feet were small even for his short stature (fig. 1b). Trilafon, prescribed to reduce DK's disruptive outbursts, was presumably responsible for this patient's dystonia.

*High Resolution Chromosome Banding, Heteromorphism Analysis and Parental Origin*

High resolution chromosomes were prepared from peripheral blood lymphocytes, G-banded (Yunis 1977), R-banded (Schweitzer 1980) and Q-banded (Caspersson 1970). The chromosome 15 origin was established by comparing parental and patient Q-banded chromosome 15 heteromorphisms (Olson et al. 1986; Magenis et al. 1990).

*FISH*

FISH studies were performed as per the ONCOR protocol using the following chromosome 15 probes: D15Z, which is specific for highly repeated centromeric alphoid DNA; D15Z1, a probe which recognizes short repeats related to AATGG in "classical" satellite DNA located in pericentromeric heterochromatin; and the 15q11-q13 specific probes D15S11, SNRPN, D15S10, and GABRB3. Probes were labeled with digoxigenin or biotin and detected with FITC or rhodamine. Chromosomes were counterstained with DAPI or propidium iodide and visualized on a Zeiss Axiophot equipped with Zeiss FITC/PI and DAPI/FITC/TEXAS RED filter sets. The number of chromatids with a signal were scored in approximately 20 cells for each FISH procedure.

*Methylation Studies*

Two micrograms of peripheral lymphocyte DNA were digested with *Xba*I and the methylation sensitive enzyme *Not*I, electrophoresed through 1.0% agarose gels, and transferred to Biodyne B membranes. Hybridizations were performed with a 600 bp *Not*I-*Eco*RI fragment that

included the -1 exon of the SNRPN gene which detects a 4.3 kb *Xba*I band from the methylated maternal allele and a 0.9 kb *Not*I band from the unmethylated paternal allele ( probe graciously provided by R. D. Nicholls, Case Western Reserve University, Cleveland, and D. J. Driscoll, University of Florida, Gainesville).

*PCR Determination of Chromosome Origin*

Origin studies were carried out by examining chromosome 15 microsatellite repeats by PCR analysis. Primers for the amplification of D15S101, D15S102, D15S113, D15S87, D15S11, GABRA5, GABRB3, and FES were obtained from Research Genetics, Inc. (Huntsville, AL) and used as per the manufacturer. mts-2 and mts-4 are $(CA)_n$ repeats located in the fibrillin gene (FBN1) (Pereira et al. 1994).

PCR products were mixed with an equal volume of formamide loading dye (90% formamide, 0.1 % bromophenol blue, 0.1% xylene cyanol), heated at 80°C for 4 min, and electrophoresed through 6% denaturing polyacrylamide gels containing 8.3 M urea and 32% formamide. Amplification products were transferred to Biodyne B membranes by capillary action and visualized by autoradiography after hybridization to a $^{32}$P-labelled oligonucleotide, $[CA]_{15}$ or $[ATTT]_7$, specific for the repeat sequence. Hybridizations were performed at 43°C in 500 mM $Na_2HPO_4$, 1 mM EDTA pH 8.0, 7% (w/v) sodium dodecyl sulfate (SDS). Membranes were washed in 3xSSC (1xSSC=0.15 M NaCl, 0.015 M trisodium citrate), 0.5% SDS at 50°C. In the cases of the $(CA)_n$ repeats, membranes were also washed in 1xSSC, 1%SDS at 55°C.

**Results**

*Case one, patient ME*

Cytogenetics

High resolution G- and R-banding of chromosomes from ME were normal (fig. 2). Parental origin studies, using chromosome heteromorphisms, revealed one chromosome 14 and one 15 with satellites not found on the chromosomes 14 or 15 of either parent (fig. 3a). Since chromosome heteromorphisms are generally inherited in a Mendelian fashion and are stable markers used to follow the parental origin of a chromosome (McKenzie and Lubs 1975; Olson et al. 1986), this rare finding prompted parentage testing using the VNTRs TBQ7 (D10S28), EFD52 (D17S26), YNH24 (D1S44) and CMM101 (D14S13). Results of this testing demonstrated a 99.99% probability of paternity (data not shown). A chromosome 15 satellite of one homolog of ME was similar to a chromosome 14 of her mother's, but was reduced in size and fluorescence. Correspondingly a chromosome 14 homolog in ME had a small, dull satellite similar to the chromosome 15 satellite of ME's mother. These data suggested a *de novo* translocation of chromosome 14 short arm satellite (maternal chromosome c in figure 3a) to the short arm satellite region of chromosome 15 (maternal chromosome h in 3a) with some apparent loss of chromosome 14 stalk and satellite. FISH analysis showed no loss of any of the single copy probes, D15S11, SNRPN, D15S10, and GABRB3. Approximately twenty cells were examined for each probe. All cells showed a probe signal on each chromatid of each homolog (as well as the identification probe signals) (fig 4a). In all cases the single copy probe annealed, as expected, to the translocation chromosome indicating that the

translocation breakpoints were more proximal. Probes D15Z1 and D15Z demonstrated that classical and alphoid satellite material was present on both chromosome 15 short arms with some cross hybridization to short arm regions of a single chromosome 14 and 22. This same cross hybridization was a consistent finding in the mother of ME but not her father. It is unclear whether this cross hybridization was an artifact of the hybridization conditions (Higgins et al. 1985) or indicated actual additional sites of chromosome 15 satellite-related DNA. In either case, however, it served as an origin marker showing maternal transmission of the chromosome 14 homolog (chromosome c in figure 3a) to ME. Taken together, the molecular and cytogenetic data indicated that ME had a karyotype of 46, XX,-15pat, +15mat, t(14;15) (15pter->p13::14p12->14qter;14pter->p12::15p12->15qter).

DNA Analysis

Microsatellite polymorphism analysis indicated loss of the paternal allele and homozygosity for the 15q11-q13 loci GABRB3, GABRA5 and D15S102, indicating maternal isodisomy. The D15S11, D15S113, D15S87 and FES loci were uninformative (Table 1). The FBN1 (mts-2, mts-4) and D15S101 loci showed loss of the paternal alleles and heterozygosity for the maternal alleles, indicating maternal heterodisomy (fig. 5).

The parent-specific methylation imprint of the SNRPN gene was examined in ME to determine if epigenetic modification had taken place. A solely maternal methylation pattern was observed at the -1 exon of the SNRPN gene; i.e., only the 4.3 kb *Xba*I maternal fragment was visible (fig 6).

*Case two, patient DK*

Cytogenetics

Chromosome studies, using the techniques of high resolution G-, R-, and Q-banding, of DK and his parents revealed that he had a karyotype of 45, XY,-15pat,+15mat, t(14;15)(p13p13) mat (fig. 2b). The Robertsonian translocation of chromosomes 14 and 15 was maternal in origin (chromosome d in fig. 3b) as was the other chromosome 15 homolog in DK (chromosome c in fig. 3b). Accordingly, he had maternal heterodisomy of chromosome 15. A comparision of chromosome heteromorphisms of DK, his sister BH, and his parents revealed that DK did not have a paternal chromosome 15 contribution, unlike BH, who also carried the translocation and was normal (fig. 3b).

FISH analysis showed no loss of any of the sequences revealed by single copy probes, D15S11, SNRPN, D15S10, and GABRB3. Of the approximately twenty cells examined for each probe, all showed a probe signal on each of the chromatids of each homolog (as well as the identification probe signals) (fig 4b). No cross hybridization of chromosome 15-specific satellite III DNA was found; a-satellite DNA remained intact on the translocation chromosome as well as the normal chromosome 15 homolog.

DNA Analysis

Microsatellite $(CA)_n$ repeat polymorphisms at loci D15S101, FBN1 (mts-4), and D15S87 showed a loss of the paternal allele and heterozygosity of maternal alleles, indicating maternal heterodisomy in DK (Table 1). The PCR amplification results of FBN1 and FES are shown in figure 5. The FES alleles exhibited maternal homozygosity and loss of the paternal

allele indicating maternal isodisomy due to a meiosis I recombination. The alleles were not informative at the D15S113 locus, and not independently informative at the D15S11 and GABRB3 loci. Knowing that DK and BH share the translocation chromosome and thus certain alleles, it was possible to infer that the origin results at the D15S11 and GABRB3 loci were consistent with an inheritance of heterozygous maternal alleles, indicative of maternal heterodisomy. The parent-specific methylation pattern of the SNRPN gene at the -1 exon was solely maternal, exhibiting only a 4.3 kb *Xba*I fragment (fig. 6).

**Discussion**

Structural rearrangements represent a notable number of cases of PWS. In a study, conducted in our laboratory, that examined the chromosome results in a cohort of 50 clinically defined PWS patients, three patients (6.0%) had a translocation involving chromosome 15 (unpublished data). Molecularly defining the chromosome structural changes in such patients will determine the genetic material, that when altered in location or physically lost, contributes to the segregation abnormalities that cause this syndrome. With this goal, the chromosome structural changes in patients ME and DK were determined with high resolution molecular cytogenetic analysis using special staining techniques and FISH. ME was shown to have a *de novo* translocation between the short arm satellite heteromorphic regions of maternally derived chromosomes 14 and 15. A Robertsonian translocation between the short arm/satellite regions of chromosome 14 and 15 was found in DK and shown to be maternally derived.

A translocation, as described above in ME, involving the chromosome satellite region is a very rare event. For this reason, chromosome heteromorphic marker regions were exceptionally useful in discerning in patient ME that normal chromosome 15 segregation had not occurred and that the meiotic error in the mother of patient ME had occured in meiosis I. Thus, the chromosome data showed that both ME and DK inherited both chromosome 15 homologs from their mother due to a MI error and consequently had *primary* maternal heterodisomy.

The malsegregation of meiosis I, observed as UPD in these patients, does not appear to have occured due to a failure of pairing. The

microsatellite results demonstrated that each patient had both maternal isodisomy and heterodisomy at various chromosome 15 loci. The recombination on chromosome 15 in ME and DK was consistent with a previously observed chiasma frequency of 0.14 in the short arm and 1.91 in the long arm (Hultén 1974). Thus, the results obtained from studying patients ME and DK suggest that disturbances in chromosome orientation and spindle alignment, rather than a failure in pairing and crossing-over between homologous chromosome arms, was the predominant cause of nondisjunction. These results have been previously demonstrated in oocytes of mice, heterozygous for multiple Robertsonian translocations (Eichenlaub-Ritter et al. 1990).

Both patients showed normal placement of chromosome 15 centromeric a-satellite material, although the Robertsonian chromosome in DK also had an intact chromosome 14 centromere. If chromosome specific a-satellite sequences are among the genetic materials responsible for proper kinetochore formation and chromosome segregation (Willard 1990; Brown et al. 1994), a Robertsonian chromosome or any chromosome with a pericentromeric rearrangement, as in the case of these two patients, could pose problems during meiotic segregation.

The results from special staining techniques, combined with the molecular data, suggested the following sequence of events for these two patients. Prior to meiosis in the mother, a translocation event took place between the chromosome satellite regions of chromosomes 14 and 15 such that a derivative 15 was formed. During the normal pairing of chromosomes, crossing over occurred and was then followed by a meiosis I nondisjunction event. Had there been instead a meiosis II nondisjunction error in ME, the heterochromatic regions of the two

homologs would have been identical. This fact is irrespective of the translocation event. At meiosis II, segregation of chromatids was such that the egg destined to be fertilized received two maternal chromosomes 15 (heterodisomy), having isodisomic regions due to crossing over and in ME having short arm heteromorphic regions differing from the original maternal homologs.

In conclusion, we suggest that the structural changes in the chromosomes found in patients DK and ME contributed to the nondisjunction and uniparental disomy responsible for their PWS phenotype. It is clear that the structural changes seen in these patients were not obligatory for nondisjunction. Not all patients with UPD have a chromosome structural rearrangement, or as was the case in DK's family, UPD was not the outcome in all individuals who received a translocation chromosome. This was illustrated in BH, sister of DK. If nondisjunction initially occurred in BH, it was unknown. The loss of one maternal chromosome 15 would have rescued a trisomic zygote and given rise to a normal fetus. However, chromosome structural rearrangements may in some way predispose the cell to malsegregation either by inhibiting the ability of the cell to properly complete homolog pairing, recombination, or metaphase I chromosome alignment, and have been shown to occur at a notable frequency in this patient population. It is, therefore, deemed prudent to further investigate all cases of structural rearrangement that include an imprinted region for uniparental disomy.

**Acknowledgments**

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a. b.

**Figure 1a.** Patient, ME, at age 11 years. She has the PWS characteristic facial features of slight bitemporal narrowing, almond shaped eyes, and a down turned mouth. Note that her eye and hair color are dark, consistent with UPD.

**b**. Patient, DK, at age 39 years. This patient, too, has almond shaped eyes, a down turned mouth and brown eyes and hair. He has short stature, as well as, small hands and feet.

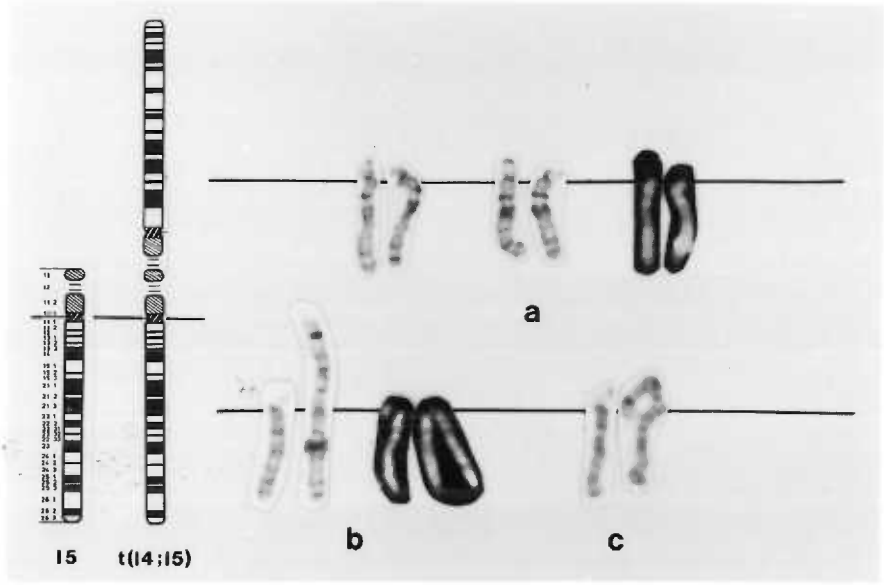
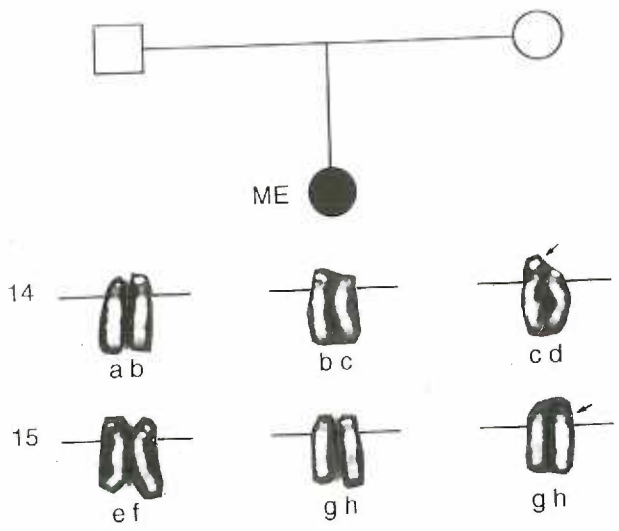



**Figure 2.** High resolution chromosomes 15 from patients ME, DK and BH. 850 band stage ISCN ideograms of normal 15 and t(14;15) chromosomes are at the far left. **a.** G-banded (left two pairs) and R-banded (right pair) chromosomes of ME. **b.** G-banded (left) and R-banded chromosomes of DK **c.** G-banded chromosomes from BH. Chromosomes from each patient appear intact with no visible deletion of the proximal 15 long arm.

**Figure 3a.** Origin of chromosomes 14 and 15 inherited in ME. Q-banded chromosome heteromorphisms indicate that chromosome 14 b, with a dull short arm, stalk and bright satellite was paternally inherited. The dull satellite and bright short arm tip of homolog c in ME does not match either parent's chromosomes 14 but is similar to chromosome 15, homolog h, in the mother. Chromosome 15 g with its stalkless short arm and small slightly bright satellite, is identical to the maternal chromosome 15 homolog g in ME but the satellite region of homolog h in ME does not match either parent. (Arrows indicate translocation sites.) **b.** Origin of normal chromosome 15 and rob t(14;15)mat chromosome in DK and sister, BH. Both DK and BH inherited the translocation chromosome from their mother (homolog d). BH inherited the paternal homolog b, while DK inherited homolog c.

**a**




**b**

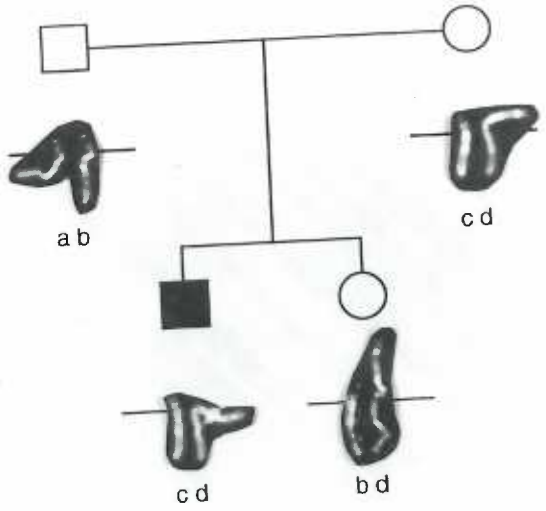
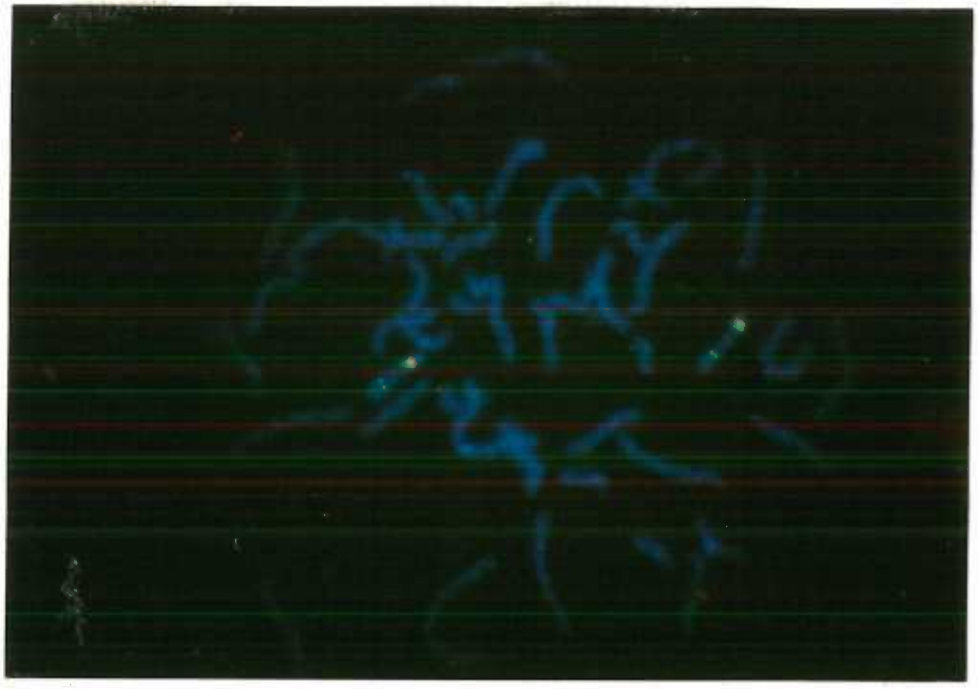
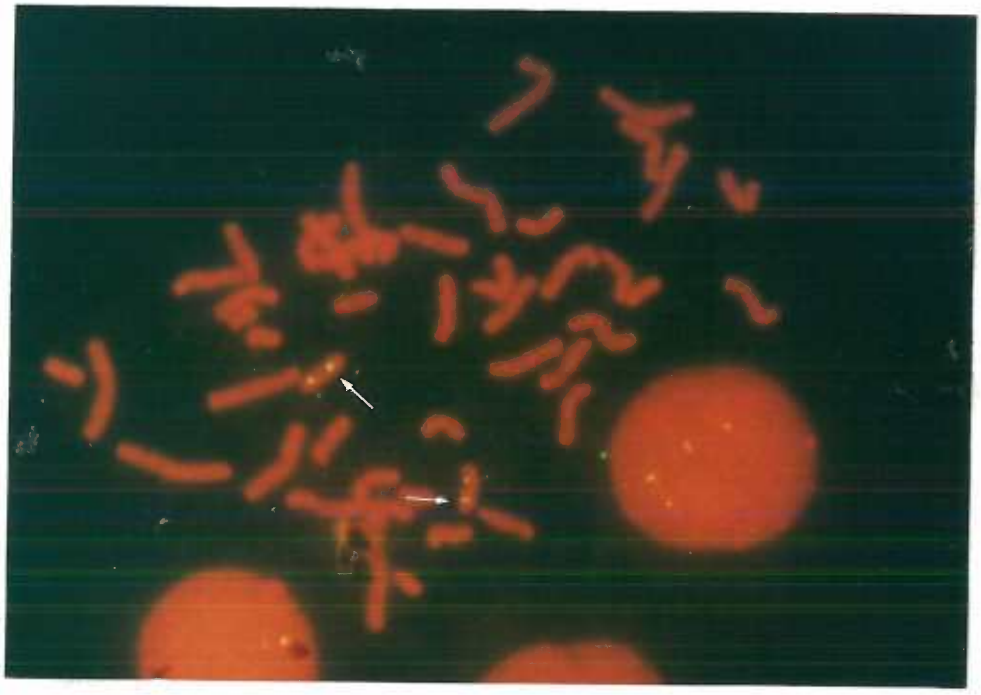


**Figure 4.** FISH results of ME, her mother and DK. Arrows indicate the probe signal. The other signal present is the identifier probe. Chromosomes appearing orange were counterstained with propidium iodide, blue with DAPI **a.** Patient ME (1) a-satellite (rhodamine-labeled/pink) and D15S11 (FITC-labeled/green), (2) SNRPN (FITC-labeled/yellow), (3) and (4) chromosome 15 classical satellite of ME (3) and her mother (4) show several sites of hybridization. **b**. Patient DK (1) D15S11 (rhodamine-labeled/pink) and classical satellite (FITC-labeled/green) (2) SNRPN (rhodamine-labeled/pink) and classical satellite (FITC-labeled/green). In both patients, chromosome 15 homologs showed no loss of material detected by these probes. The identifier probe indicated that the chromosome 15-specific loci remained intact on the chromosome 15 portion of the translocation chromosome in DK.

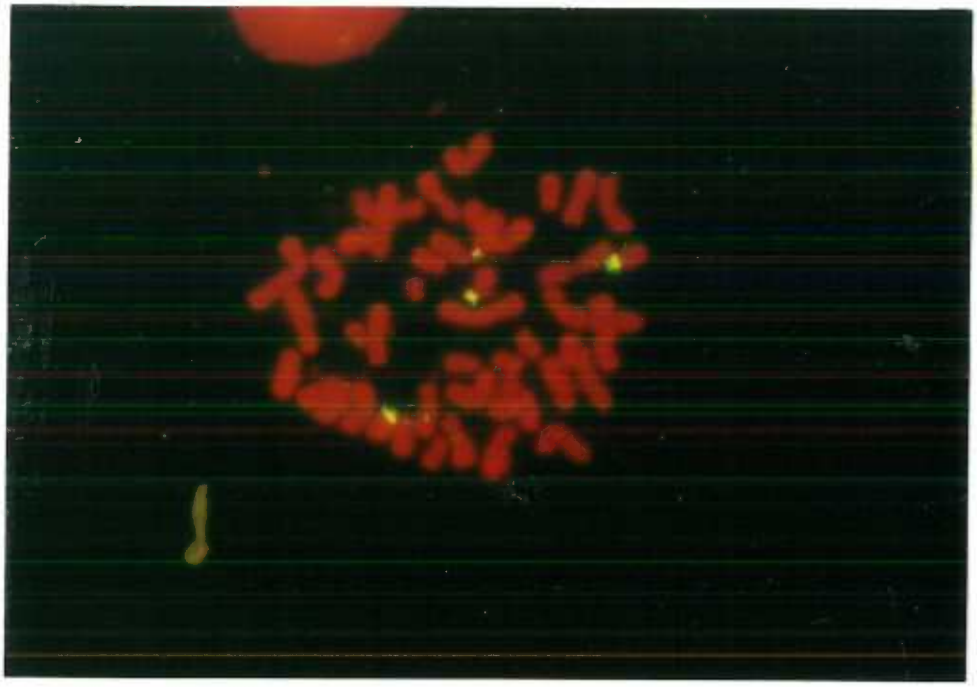
a (1).



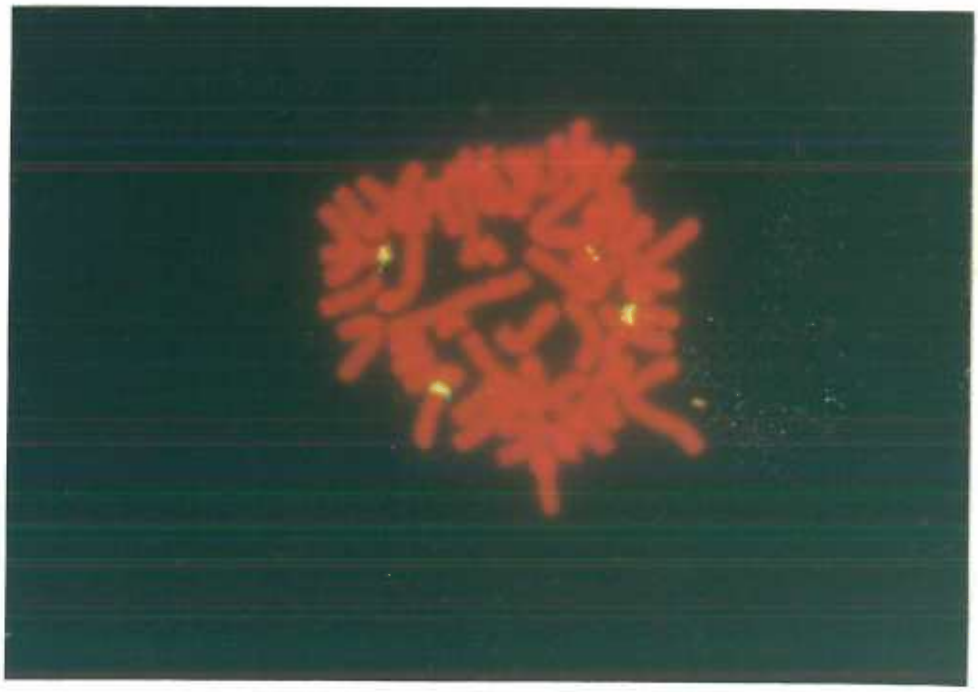
(2).



(3).



(4).



b (1).

(2).

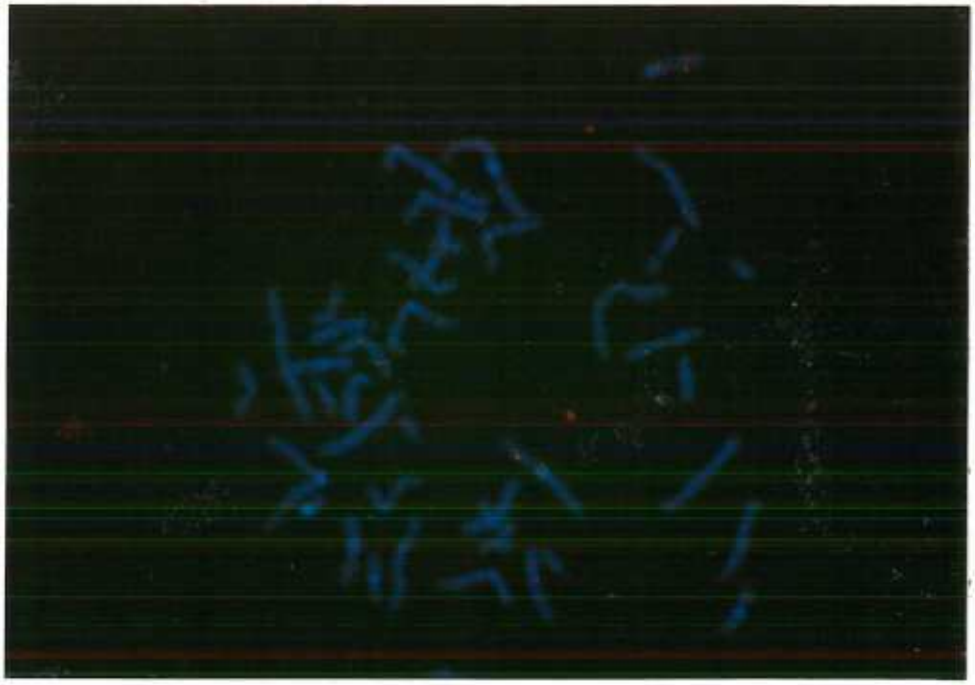


**Figure 5.** PCR analysis of chromosome 15-specific microsatellite polymorphisms in the PWS patients and their families. **left.** (bottom) Patient ME, amplification results at the GABRB3 locus indicating maternal isodisomy. The FBN1 locus (mts-2) (top) shows maternal heterodisomy suggesting that a meiotic recombination has occured. **right.** Amplification results in patient, DK, and his family at loci FBN 1 (mts-4) and FES. The maternal heterodisomy at the FBN 1 locus in DK was seen at all informative sites examined except locus FES, which indicated maternal isodisomy and an obligatory crossing over event. BH shows normal allelic inheritance at all loci tested. See Table 1 for specific allele data for all loci examined. F= father, M= mother

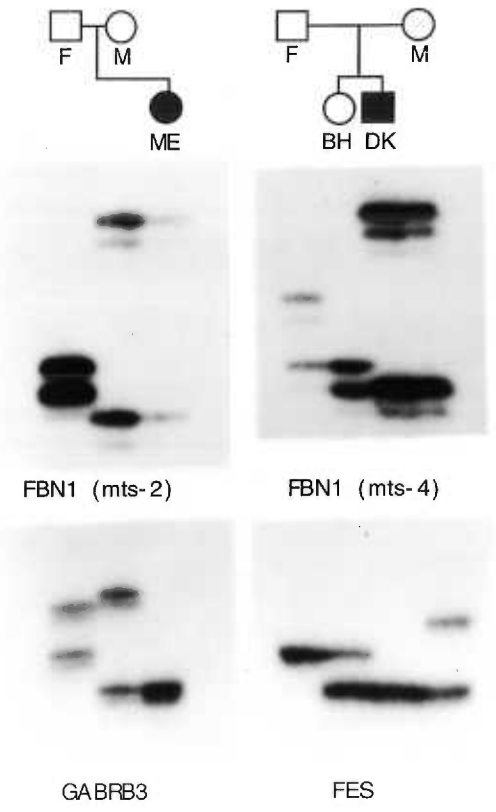

F
M
ME
F
M
BH DK
FBN1 (mts-2)
FBN1 (mts-4)
GABRB3
FES

**Figure 6.** DNA methylation pattern in the 5' region of the human SNRPN gene. DNA from peripheral blood lymphocytes was digested with *Xba* I and *Not* I and probed with a 600 bp *Not* I-*Eco*RI fragment from the SNRPN gene (-1 exon--¯1 intron). **a.** Lane 1 contains DNA analysis markers (BRL); lane 2 is a normal male control; lane 3 is patient ME. **b.** Lane 1 is a control PWS patient; lane 2 is DK's mother; lane 3 is DK, lane 4 is a normal female control, and lane 5 contains DNA analysis markers (BRL). DNA from the controls and DK's mother show the expected normal 4.3 kb *Xba* I maternal and 0.9 kb *Not* I paternal fragments. ME and DK, however, are lacking the paternal 0.9 kb fragment, consistent with the control PWS result.

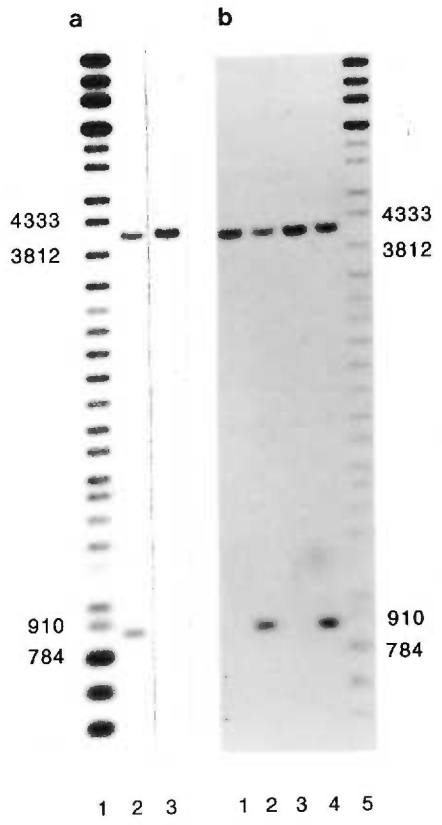

a
b
4333
3812
910
784
4333
3812
910
784
1 2 3
1 2 3 4 5

**Table 1 Chromosome 15-specific microsatellite results**

| | Case 1 | | | | Case 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | F | ME | M | I/U | F | BH | DK | M | I/U |
| Locus | | | | | | | | | |
| D15S11 | 1,2 | 2,2 | 2,2 | U | 1,3 | 1,3 | 1,2 | 1,2 | U |
| D15S113 | 2,2 | 2,2 | 1,2 | U | 1,2 | 2,3 | 2,3 | 2,3 | U |
| GABRB3 | 2,3 | 4,4 | 1,4 | I | 1,4 | 1,4 | 2,4 | 2,4 | U |
| GABRA5 | 3,3 | 1,1 | 2,1 | I | - | - | - | - | - |
| FBN-1 (mts-2) | 2,2 | 1,3 | 1,3 | I | 1,2 | 2,2 | 2,3 | 2,3 | U |
| FBN-1 (mts-4) | 2,3 | 1,4 | 1,4 | I | 2,3 | 3,4 | 1,4 | 1,4 | I |
| FES | 2,2 | 1,1 | 1,1 | U | 2,2 | 2,3 | 3,3 | 1,3 | I |
| D15S87 | - | - | - | - | 3,3 | 1,3 | 1,2 | 1,2 | I |
| D15S101 | 3,4 | 1,2 | 1,2 | I | 1,4 | 3,4 | 2,3 | 2,3 | I |
| D15S102 | 1,2 | 3,3 | 3,4 | I | - | - | - | - | - |

F: father, M: mother; I: informative, U: uninformative;

ME and DK: PWS patients, BH: normal patient

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Cytogenetic and Molecular Findings in a Case of Familial Angelman Syndrome: Reexamination of the D15S113 Locus.

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**Abstract**

Familial Angelman syndrome (AS), as in sporadic cases, is characterized by mental retardation, no speech, ataxic gait, characteristic facies and can be associated with either a structural rearrangement of chromosome 15 or normal appearing chromosomes. We present here a family with two maternal half-siblings, each apparently affected with AS. High resolution chromosome banding, followed by FISH analysis using probes from the proximal 15q11.2-q13 region, revealed that this genomic material was present and intact in both patients. PCR amplification of the D15S113 $(CA)_n$ repeat (obtained through Research Genetics; Mutirangura et al. 1992 ) suggested that both patients and their mother carried a deletion of this region of chromosome 15. In order to confirm that this result was not due to the failure of an allele to amplify, alternative primers for the amplification of the D15S113 $(CA)_n$ repeat were obtained. In addition, an $(ATTT)_n$ repeat at the D15S113 and a $(CTTT)_n$ repeat at the D15S1234 loci were amplified. Biparental amplification of alleles at the D15S113 and D15S1234 loci was achieved by the alternative and additional primers, suggesting that the initial amplification of a single allele in these patients was not a reflection of a deletion. Consistent with the microsatellite results, examination of the methylation pattern at the 5' end of the SNRPN gene showed biparental disomy of chromosome 15 homologs in these patients, as well as ruling out an imprinting defect at this locus. These data indicate that, as in this family, an apparent deletion at the D15S113 locus may merely represent a failure to amplify an allele and must be confirmed by using alternative primers from this locus.

**Keywords:** Angelman syndrome, imprinting, D15S113

## Introduction

Angelman syndrome (AS) is a disorder characterized by severe mental retardation, seizures, ataxic gait and/or tremulous movement of limbs (Angelman 1965). The distinguishing behaviors of frequent laughter/smiling with a happy demeanor and an easily excitable personality are often accompanied by hand flapping movements. Abnormal facies include microcephaly with a flat occiput, prominent chin, broad mouth, and wide spaced teeth with accompanying excessive drooling (Williams et al. 1995).

Previous studies have indicated that approximately 75 percent of Angelman syndrome (AS) patients have a maternal chromosome deletion of 15q11.2-q13 (Magenis et al. 1987; Donlon 1988; Nicholls et al. 1989; Knoll et al. 1989; Magenis et al. 1990). Molecular refinement of the breakpoints indicates that the majority of AS patients have a molecular deletion that includes all the loci from ZNF127 to D15S12 (Buiting et al. 1993). Unique cases of AS have demonstrated smaller deletions between D15S10 and D15S113 (Saitoh et al. 1992; Reis et al. 1993) and D15S122 and D15S113 (Greger et al. 1994). In addition, AS patients have been reported with a submicroscopic deletion located proximal to D15S113, within what may be an "imprinting center" (IC) (Buiting et al. 1995). It may be within this domain that imprinting, or parent-specific methylation and gene expression for the 15q11.2-13 region, are regulated. Sites of parent- specific methylation of alleles have been reported at the ZNF127 (Driscoll et al. 1992; Glenn et al. 1993a), PW71 (Dittrich et al. 1992) and SNRPN (small nuclear ribonucleoprotein polypeptide N) (Glenn et al. 1993b; 1994; Sutcliffe et al. 1994; Glenn et al. 1996) loci. The SNRPN gene has been

shown to be functionally imprinted (Leff et al. 1992; Glenn et al. 1993b; Reed and Leff, 1994),with the paternal 5'-SNRPN allele being unmethylated and exclusively expressed. In addition, the SNRPN gene maps to the smallest region of deletion overlap for PWS (Özçelik et al. 1992) and is therefore considered the candidate gene for this syndrome. An imprinted gene responsible for AS, however, has yet to be identified.

The imprinted nature of this region of the genome lends itself to unique consequences of abnormalities of epigenetic regulation of gene expression and segregation. Approximately 2-4 percent of AS cases are due to uniparental disomy (UPD), a situation in which both chromosome 15 homologs are paternal in origin (Malcolm et al. 1991). Of the remaining AS patients, having normal appearing chromosomes and biparental inheritance, 2 percent may be due to an imprinting defect affecting the normal parent-specific methylation of loci within the 15q11-q13 region (Reis et al 1994).

We present a family with two maternal half siblings, each apparently affected with AS. The purpose of this study was to investigate the loss or rearrangement of genetic material in these patients and their parents, as well as examine the epigenetic regulation within the proximal region of chromosome 15. This study provides comparative data for future mapping of the AS gene in familial and sporadic cases of AS.

**Materials and methods**

PATIENTS

Patient CD, a 5 year old female was a maternal half-sibling to her brother JT, who was age 12 years at the time of this study. Facies of both patients were consistent with Angelman syndrome and included a prominent chin, wide mouth and flat occiput; JT was microcephalic (fig. 1). Both patients showed developmental delay, and virtually absent speech. Patient CD had a wide based gait, limb hypertonia and truncal hypotonia with a mild ataxia and tremor. Patient JT had a milder ataxia which was more evident when he ran. He was somewhat tremulous. Both patients had abnormal EEGs. CD, affected with myoclonic seizures, was being treated with Phenobarbital.

KARYOTYPING and FLUORESCENT IN SITU HYBRIDIZATION (FISH)

High resolution chromosome G-banding (Yunis and Chandler 1977) was performed using prometaphase chromosomes prepared from PHA stimulated peripheral blood lymphocytes.

FISH studies were performed as per the ONCOR protocol using the following chromosome 15q11-q13 specific probes: D15S11, SNRPN, D15S10, and GABRB3. Probes were labeled with digoxigenin or biotin and detected with FITC or rhodamine. Chromosomes were counterstained with DAPI or propidium iodide. The number of chromatids with a signal were scored in approximately 20 cells for each FISH procedure.

PCR ANALYSIS AND NUCLEOTIDE SEQUENCING

*Patient studies*

Origin and deletion studies were carried out by examining chromosome 15 microsatellite repeats by PCR analysis. Primers for the amplification of $(CA)_n$ repeats at D15S11, GABRB3, GABRA5, D15S113 (113F-113R) were obtained from Research Genetics, Inc. and used as per the manufacturer, with the addition of an elongation step at 72°C for 40 seconds at each cycle. $(CA)_n$ repeats at the D15S113 locus were also amplified as above using the following primer sequences: forward, 5'-CTGGGCAACCAGAGTGAGAC-3' (MUT1F) and reverse, 5'-CATGTACTGTTTTATCCCTGTGGC-3' (MUT1R) (sequence graciously provided by A. Mutirangura). $(CTTT)_n$ repeats were amplified at D15S1234 (just distal to D15S113) (Trent et al. 1995) using the following primer sequences: forward, 5'-GCGGGGCAGCCACTATAAGACTTC-3' (TNT1F) and reverse, 5'-GCCTGGCAACAGAGTGAGACACTC-3' (TNT1R). Final primer concentration was 0.5μM. PCR conditions were 27 cycles of 1 min. at 94°C, 2 min at 55°C and 2 min at 72°C. The amplification product from a normal D15S113 allele was subcloned into the vector pCRII using the TA Cloning Kit (Invitrogen). Nucleotide sequencing was performed using fluorescently labeled primers specific for the vector (M13 forward and reverse primers) (Applied Biosystems) and fluorescent signals from sequencing gels read by an Applied Biosystems 373A automated sequencer. From these sequence data, a new primer was designed to use in conjunction with MUT1F to amplify an $(ATTT)_n$ repeat located proximal to the D15S113 $(CA)_n$. This primer set is as follows: forward primer sequence , MUT1F, and reverse, 5'-GAATACAGAAAGAGTATAAGCAGCAG-3' (QT1R). Final primer concentration was 0.5μM. PCR conditions were 35 cycles of 40 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C.

In order to assess the validity of the commercially obtained primers (113F-113R) at the D15S113 locus, 50 random DNA samples were amplified by these primers. The PCR conditions that were used were as per the manufacturer, with the addition of an elongation step at 72°C for 40 seconds at each cycle. The number of heterozygous and homozygous results were tabulated in order to assess the heterozygosit;y value achieved in this population.

DNA METHYLATION STUDIES

Two micrograms of peripheral lymphocyte DNA were digested with *Xba*I and the methylation sensitive enzyme *Not*I, electrophoresed through 1.0% agarose gels, and transferred to Biodyne B membranes. Hybridizations were performed with a 600 bp *Not*I-*Eco*RI fragment that included the -1 exon of the SNRPN gene. This fragment detects a 4.3 kb *Xba*I band from the methylated maternal allele and a 0.9 kb *Not*I band from the unmethylated paternal allele (probe graciously provided by R. D. Nicholls, Case Western Reserve University, Cleveland, and D. J. Driscoll, University of Florida, Gainesville).

**Results**

The clinical phenotype of both patient CD and JT was not inconsistent with AS. They both had all the clinical characteristics that are universally found in AS such as developmental delay, speech impairment, a movement or balance disorder and behavioral uniqueness which included frequent laughter/smiling and an apparent happy demeanor. These patients also showed a majority of physical and behavioral findings frequently observed or associated with AS (Table 1).

Both patient CD and JT had normal karyotypes by high resolution chromosome G-banding (Fig. 2). FISH of chromosome 15 centromere and long arm probes: D15S11, SNRPN, D15S10 and GABRB3 showed signals on both homologs, indicating that genomic material, homologous to these probes, was present and not abnormally rearranged (Fig. 3).

The results of amplification of microsatellite repeats in the patients and family members are shown in Table 2. Biparental inheritance of alleles for patients CD and JT was observed at all informative loci examined except at the D15S113 locus where discrepant results were obtained. The result of the $(CA)_n$ amplification at D15S113 (113R and 113F primers) indicated a loss of a maternal allele, with only the paternal allele present in both CD and JT (fig. 4). The mother and two aunts of CD and JT (SB and KR) also showed loss of an allele, but paternal in origin. Their father, who is the maternal grandfather of CD and JT, was heterozygous at this locus. In contrast, when this same $(CA)_n$ repeat was amplified with alternative primers, MUT1F-MUT1R, the patients and all family members did not show a deletion and biparental inheritance of alleles was observed (fig. 5). CD was either hemizygous or homozygous at the D15S113 locus. JT, and all other family members were heterozygous at this locus. At approximately 50 kb distal to the $(CA)_n$ repeat at D15S113, patients CD and JT, as well as all family members were heterozygous for alleles containing a $(CTTT)_n$ repeat at D15S1234. Moving proximal from the $(CA)_n$ repeat at D15S113, patients JT, and family members were each heterozygous for alleles containing an $(ATTT)_n$ tetranucleotide repeat, again having biparental inheritance of alleles. Patient CD was hemizygous or homozygous at this locus. Haplotype analysis of the microsatellites examined demonstrated that CD and JT may share the same maternal

alleles at the D15S11 and D15S113 loci and but have different alleles at the D15S1234 locus (see Table 2).

The methylation pattern of the 5' region of the -1 exon of the SNRPN gene was normal in patients CD and JT (Fig. 7). The methylation pattern for their mother and maternal grandfather was also normal (data not shown). In all cases, a maternal 4.3 kb and paternal 0.9 kb fragment was present. These results indicated that in all these individuals normal imprinting of the chromosome 15 proximal region had occurred. These results also confirmed the biparental inheritance of chromosome 15 homologs in patients CD, JT and their mother.

Of the 50 random DNA samples that were amplified by the commericially obtained primers 113F-113R three samples initially failed to amplify, but by repeat PCR, amplification of DNA was achieved in two samples. Thirty-two of 50 patients were heterozygous, meaning that two different alleles were observed. Seventeen were homozygous, showing only one allele (fig. 6). At least six different sized alleles were observed in this study. The amplification by these primers demonstrated notable shadow banding, making it often difficult to discern homozygous results from heterozygous results.

**Discussion**

The clinical phenotype of both patient CD and JT was not inconsistent with AS. They both had all the clinical characteristics that are universally found in AS, as well as a majority of physical and behavioral findings frequently observed or associated with AS. Although JT was less affected, both in his physical features and development. Without the clear establishment of a genetic defect, a differential diagnosis for these patients, however, cannot be completely discounted.

The high resolution chromosome banding analysis and molecular cytogenetics using dual color FISH did not demonstrate a deletion or an inversion of material within the 15q11.2-q13 region. The findings of normal karyotypes and FISH results in these patients are consistent with previous studies which have indicated that approximately 25% of AS patients do not have a visible chromosome deletion. The molecular cytogenetic results of patients CD and JT, reported in this study, are limited by the number and choice of probes D15S11, SNRPN, D15S10, GABRB3 (ONCOR) that were used in the FISH experiments, however.

There are reports of unique patients and families which have molecular deletions that have putatively placed the AS critical region distal to SNRPN and proximal to GABRB3 (Wagstaff et al. 1992, 1993; Reis et al. 1993; Greger et al. 1994). Most recently, a report by Buxton et al (1994) indicated loss of genetic material at the D15S113 locus in a patient with AS. The AS patients we presented here appeared to have the same loss of the maternally inherited genetic material at this locus using the published primers for D15S113 (Mutirangura et al. 1993). This, however, actually represented a failure of an allele to amplify. This conclusion is supported by the successful amplification of both parental alleles of this same $(CA)_n$ repeat at the D15S113 locus, using alternative primers, that gave a heterozygous result in patient JT, his mother, one maternal aunt and maternal grandfather. Patient CD appeared to be homozygous at this locus, but since PCR amplification is not reliably dosage sensitive, she may be hemizygous. One maternal aunt was homozygous. Genotypes inconsistent with a deletion for patient JT and his mother were observed with the amplification of flanking microsatellite repeats. While PCR microsatellite amplification at the D15S113 locus suggests that patient CD

is homozygous it is inconclusive. Amplification of flanking microsatellite repeats demonstrate that she is heterozygous at these loci. The apparent failure to consistently amplify an allele with the 113F-113R primers in this family is presumably due to a polymorphism at a primer annealing site.

From the 50 random DNA samples that were amplified by the commericially obtained primers 113F-113R, 32 of 50 patients were heterozygous, 17 were homozygous demonstrating a heterozygosity value of .64. The heterozygosity of these primers is reported by Research Genetics to be .73. The heterozygosity value of this study did not significantly differ from that reported by Research Genetics. However, because of large amount of shadow banding it was hard to discern one allele (a homozygous result) from two alleles (a heterozygous result). Since these were random samples, it was unclear how often an apparently homozygous result was actually a failure of an allele to amplify. Therefore while the heterozygousity value that was obtained was consistent with the value reported by the vendor, the use of alternative primers may be necessary to amplify DNA at the D15S113 locus or to confirm results..

A portion of AS patients, including siblings with AS show biparental inheritance of chromosome 15 homologs (Saitoh et al. 1992; Wagstaff et al. 1993) and normal imprinting in the 15q11-q13 region (Reis et al. 1994), just as do patients CD and JT. The possibility of an imprinting defect was studied in this family by examining the parent-specific methylation pattern at the 5' SNRPN gene. Examination of the differential methyation by Southern blotting will show a maternal 4.3 kb fragment and a paternal 0.9 kb fragment in a normal control. The result of this assay in AS, in the majority of cases, will be the presence of a single paternal 0.9 kb fragment. However, the presence of both the maternal and

paternal fragment by 5'-SNRPN Southern blotting analysis does not rule out a clinical diagnosis of AS. This test is an excellent diagnostic test of the Prader-Willi syndrome, detecting virtually 100 percent of cases, regardless of whether the etiology is due to a deletion, UPD or an imprinting defect. With regard to AS, however, approximately 30 percent of clinically well defined AS patients will not demonstrate loss of the maternal 4.3 kb fragment. Of the approximately 60-80 percent of patients who do show loss of the maternal 4.3 kb fragment by Southern blotting analysis, the etiology of their syndrome is due to a deletion, UPD, or an imprinting abnormality (Glenn et al. 1996; Toth-Fejel et al. unpublished data). Since the location and mechanism of the imprinting domain of the gene(s) responsible for AS is as yet unknown, it may be prudent not to rely solely on Southern blotting analysis of the imprinting of 5'-SNRPN as a diagnostic test for AS.

The haplotype analysis demonstrated that CD and JT shared the same maternal alleles at the D15S11 and D15S113 loci but did not have the same alleles at the D15S1234 locus, indicating that the mutation shared by these siblings must be proximal to the D15S1234 locus. The molecular analyses employed in this study did not rule out the possibility of a mutation in this family that is undetectable, thus far, by the choice of loci examined. Further haplotype analysis of microsatellites between the D15S11 and D15S113 loci is necessary to define the critical region that includes the mutation in this family.

The data obtained from this study suggest that CD and JT have AS syndrome, for which the molecular defect is yet unknown. While this study scrutinized various loci within the region of 15q11.2-q13, it is limited in its scope. This work does not rule out a deletion or deleterious

rearrangement of genetic material. Future studies, with regard to these patients and other similar patients, will include further deletion analysis as well as the investigation of an abnormal trinucleotide expansion as an alternative genetic mechanisms responsible for this syndrome.

**Acknowledgments**

The authors thank R.D. Nicholls and D.J. Driscoll for the SNRPN probe used this study, as well as Ronald J. Trent and Apiwat Mutirangura for the sequence data of the primers at D15S113 and D15S1234. We also thank the family included in this research study for their participation.

**Figure 1** **A.** patient CD at age 5 years **(left)** and half sibling JT at age 12 years **(right)**. Facies in both patients are consistent with AS. **B.** Profile of patient JT. The darker features in JT are due to his Hispanic ethnic background. **C.** Frontal view of CD. Note the wide open mouth and fair complexion.

A.

B.

C.

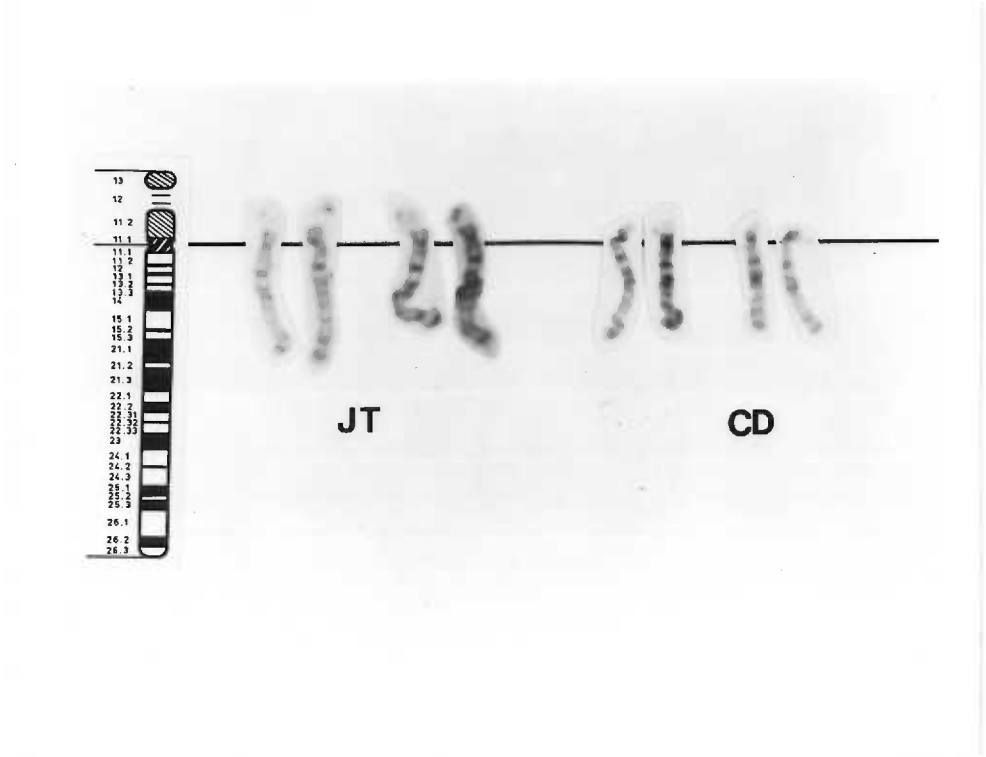
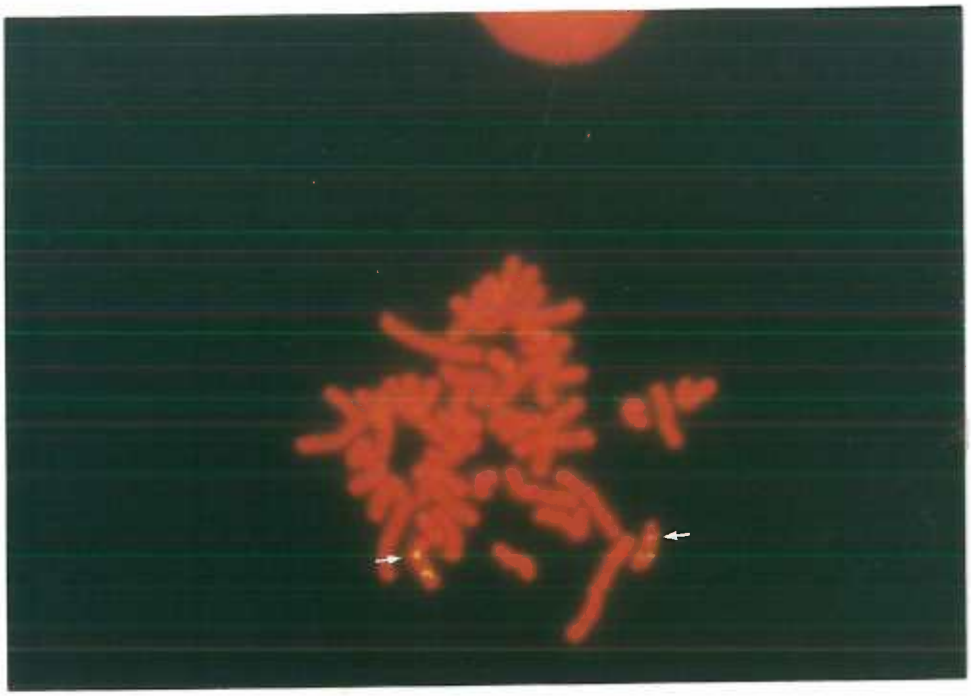


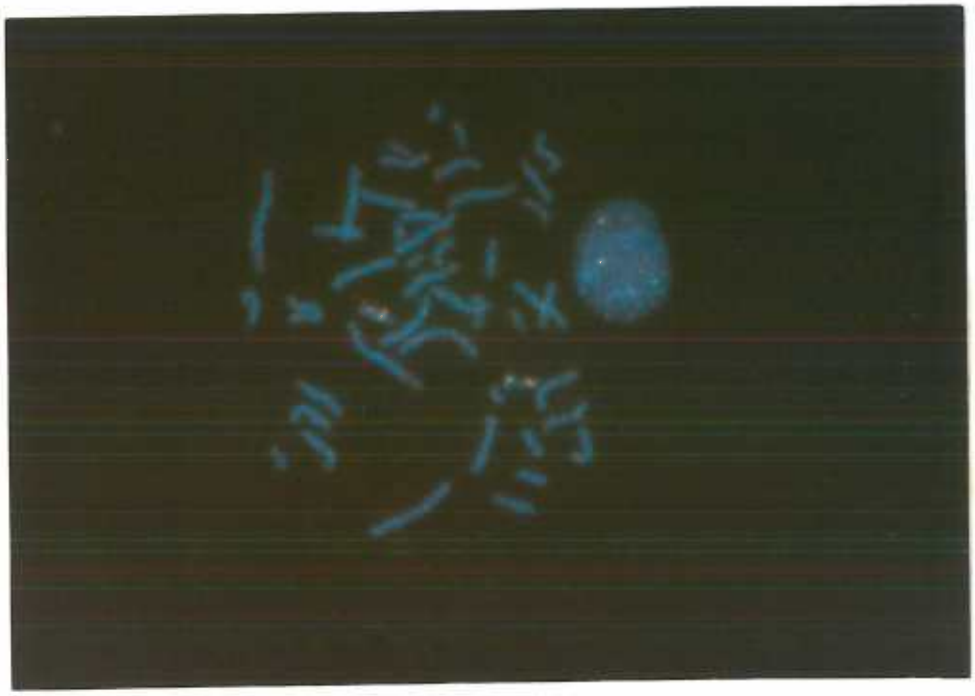

**Figure 2** ISCN chromosome 15 ideogram at 850 band stage and high resolution chromosome G-banding of chromosomes 15 from patients JT **(left)** and CD **(right)**. Chromosomes appear normal.

**Figure 3**. Results of FISH analysis of the chromosome 15 proximal probes with chromosomes from patients CD(**A, B**) and JT (**C, D**). Probes are labeled with rhodamine (red) or FITC (green/yellow) (see arrows). Chromosome 15 I.D. probe appears yellow (FITC) or red (rhodamine). **A.** SNRPN (arrow) **B**. D15S10 (red) and GABRB3 (green). **C.** D15S10 (yellow) **D.** GABRB3 (arrow)

A.

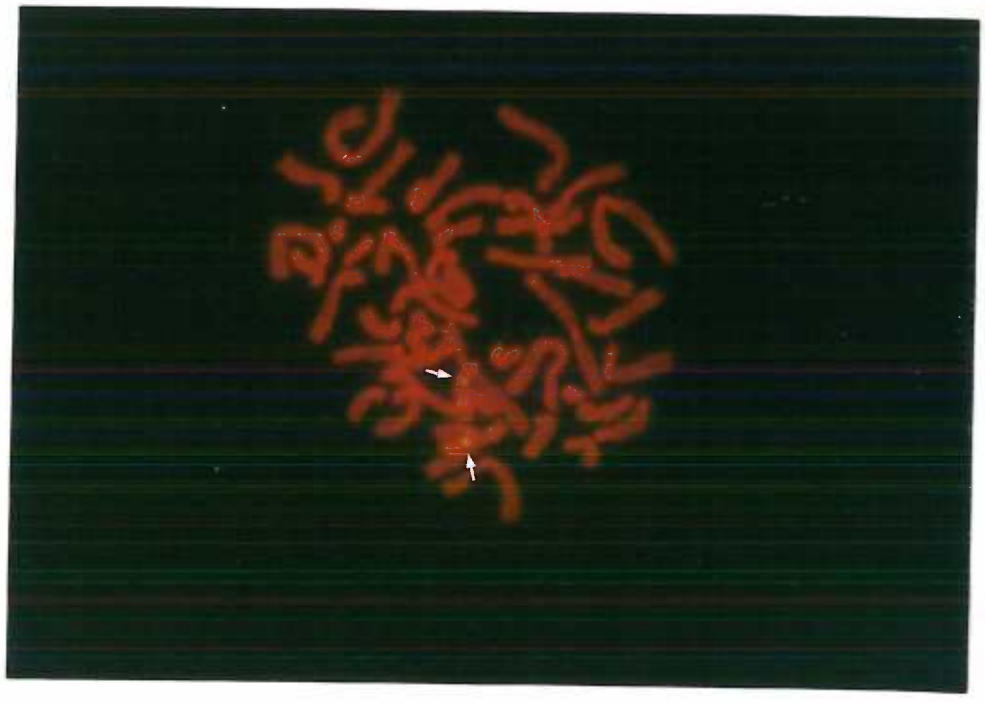


B.



C.

D.

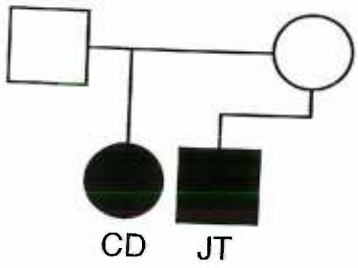


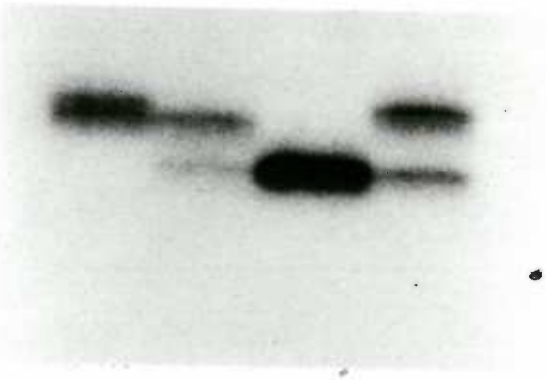
1 2 3 4 5 6 7

**Figure 4** PCR amplification results of primers 113F-113R at D15S113 (Research Genetics) Lane1: father of CD; Lane 2 sibling of CD; Lane 3: patient CD; Lane 4: patient JT; Lane 5: mother of CD and JT; Lane 6: the father of JT; Lane 7: water control Note that patients CD and JT do not appear to have a maternal allele at this locus.

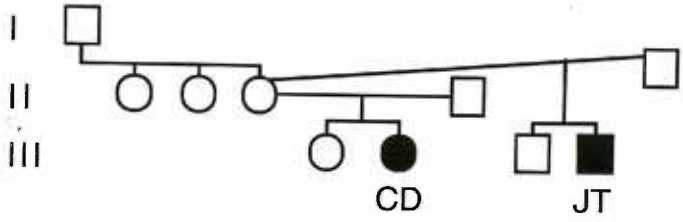
**Figure 5** Chromosome 15-specific microsatellite analysis of patients CD, JT and family members. The maternal grandmother is deceased and could not be tested. (**A**) $(CA)_n$ repeat at the D15S11 locus. (**B**), $(CA)_n$ repeat amplified with MUT1F-MUT1R primers at D15S113 locus. (**C**) (ATTT)n repeat amplified with MUT1F-QT1R primers at the D15S113 locus. (**D**) (CTTT)n repeat amplified with TNT1F-TNT1R primers. Results of amplification of three unrelated control samples (C1, C2, C3) are shown as well.

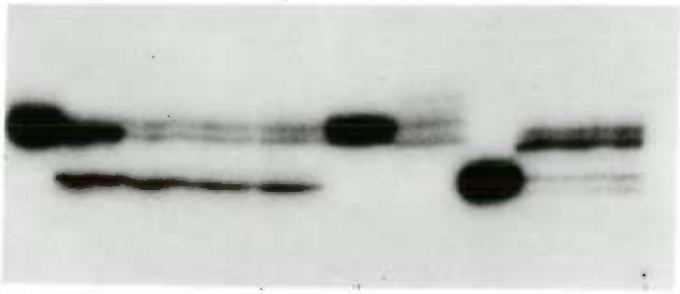
A



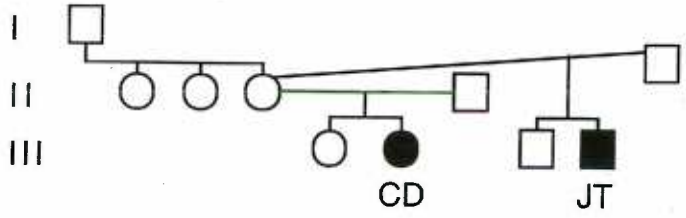



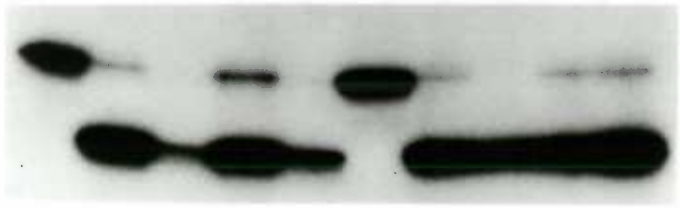
B



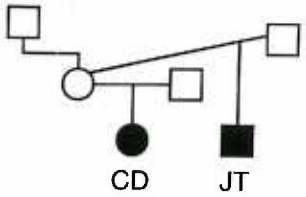



C



D




C1 C2 C3

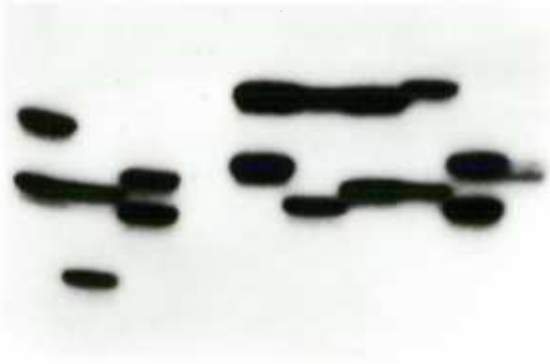



**Figure 6** The results of PCR amplication of nine of 50 random DNA samples with 113F-113R primers at the D15S113 (Research Genetics) locus. Note that there was a great deal of shadow banding which made the interpretation of the results difficult.

**Figure 7** DNA methylation pattern in the 5' region of the human SNRPN gene. Genomic DNA was digested with *Xba* I and *Not* I and probed with a 600 bp *Not* I-*EcoR*I fragment from the SNRPN gene (-1 exon--1 intron). This probe detects a 4.3kb *Xba* I band from the methylated maternal allele and a 0.9 kb *Not* I band from the unmethylated paternal allele. Lane 1: PWS patient with UPD. Lane 2: PWS patient with a chromosome deletion. Lanes 3 and 4: AS patients with apparently normal chromosomes. Lane 5: patient CD. Lane 6: patient JT. Lane 7: normal control. Lane 8: BRL Analytical Markers

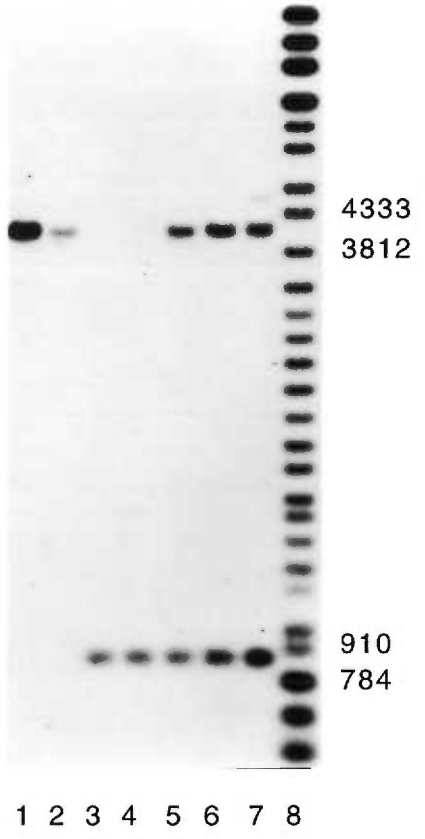

4333
3812
910
784
1 2 3 4 5 6 7 8

Table 1 Clinical findings in patients CD and JT*

| Patient | CD | JT |
|---|---|---|
| Developmental delay | + | + |
| None or minimal use of words | + | + |
| Ataxia of gait and/or tremulous movement of limbs | + | + |
| Frequent laughter/smiling; apparent happy demeanor; easily excitable, often showing hand flapping movements | + | + |
| Delayed disproportionate growth in head circumference, resulting in microcephaly | - | + |
| Seizures | + | - |
| Abnormal EEG | + | + |
| Flat occiput | + | + |
| Protruding tongue | - | - |
| Tongue thrusting; suck/swallowing disorders | + | + |
| Prognathia | - | + |
| Wide mouth, wide-spaced teeth | + | + |
| Frequent drooling | + | + |
| Excessive chewing/mouthing behaviors | + | + |
| Hypopigmented skin, light hair and eye color (compared to family) | - | - |
| Hyperactive lower limp deep tendon reflexes | + | + |
| Uplifted, flexed arm position | + | + |
| Sleep disturbance | + | + |
| Attraction to/fascination with water | + | + |

*Based on AS clinical criteria from Williams et al. 1995

Table 2 Chromosome 15-specific Microsatellite Results

| | PATIENTS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $F_{(CD)}$ | CD | JT | M | $F_{(JT)}$ | SR | SB | KR |
| LOCUS | | | | | | | | |
| D15S11 | 1,2 | 2,3 | 3,3 | 1,3 | 3,3 | 3,3 | 2,3 | 2,3 |
| GABRB3 | 3,3 | 2,3 | 1,3 | 1,2 | 1,3 | 2,2 | 1,2 | 1,2 |
| GABRA5 | 1,1 | 1,1 | 1,1 | 1,1 | --- | --- | --- | --- |
| D15S113 [Res. Gen] | 1,2 | 2,- | 4,- | 5,- | 4,5 | 2,3 | 5,- | 5,- |
| D15S113 [MUT-$(CA)_n$] | 1,2 | 2,2 | 2,3 | 2,3 | 2,3 | 2,2 | 2,3 | 2,3 |
| D15S1234 [$(CTTT)_n$] | 1,4 | 2,4 | 3,5 | 2,5 | 3,3 | 2,3 | --- | --- |
| D15S113 [$(ATTT)_n$] | 1,2 | 1,1 | 1,2 | 1,2 | 1,2 | 1,1 | 1,2 | 2,2 |

$F_{(CD)}$ =father of CD; $F_{(JT)}$ =father of JT; M =mother of CD and JT;
SR = maternal grandfather of CD and JT; --- =locus not tested.

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# A SYSTEMATIC APPROACH TO THE DIAGNOSIS OF PRADER-WILLI AND ANGELMAN SYNDROMES

The diagnoses of Prader-Willi (PWS) and Angelman (AS) syndromes, when first characterized in 1956 and 1965 respectively, were made on the basis of fulfilling clinical criteria (Prader et al 1956; Angelman 1965). With the advent of high resolution chromosome banding (HRCB), it was established that in the majority of patients with both syndromes, there was a deletion of band 15q11.2-q13. HRCB has remained the primary method for detecting the deletion and making the diagnosis in the majority of PWS and AS patients. With the development of a variety of scientific technologies, concomitant with a broader understanding of the genetic abnormalities and mechanisms responsible for these two syndromes, laboratories now have multiple methods at their disposal for diagnosing PWS and AS. Testing procedures, which include HRCB, molecular cytogenetics, quantitative Southern blotting and PCR assays, provide laboratories with different diagnostic approaches. The results obtained from each of these various procedures serve to answer different genetic questions and are necessary to pinpoint the particular etiologic mechanism responsible in each case of PWS or AS.

The reality of health care costs make it necessary to design a diagnostically proficient and cost effective PWS and AS testing stratagem. Limited resources demand that not every diagnostic test can be performed in every case. Preferential usage of tests should be made on the bases that (1) the test can detect multiple defects responsible for PWS and AS, (2) the test will detect the genetic defect that is most often responsible for PWS

and AS, (3) the test will provide the family with the most accurate recurrence risks. In order to best design a diagnostic testing strategy, each cytogenetic and molecular test must be assessed for the benefits and limitations of the information it can provide. Studies in which laboratories have reported their experiences with these techniques provide a basis for comparing the results obtained by utilizing the same test techniques in a study of PWS and AS patients at OHSU.

In the following sections, a false positive result refers to cases of PWS or AS for which the HRCB result was reported out as a chromosome 15 deletion, but for which various other laboratory techniques failed to substantiate the finding. A false negative result refers to HRCB cases for which no abnormality was reported, but was found by other laboratory techniques.

**Assessing The Benefits and Limitations of Cytogenetic and Molecular Testing: Reports from the Literature**

<u>High Resolution Chromosome G-banding (HRCB) and FISH</u>

Approximately 70% of PWS and AS patients will show a deletion of 15q11.2-q13 by HRCB (Ledbetter et al. 1981; Magenis et al. 1990; Nicholls 1993). One study of 14 PWS and 5 AS patients, comparatively studied by HRCB and FISH, resulted in 3 false positive cases by HRCB within the PWS group and 1 false negative case within the AS group (Delach et al. 1994). Butler (1995) has shown in his study comparing HRCB and FISH in 21 PWS and 5 AS patients that two false negative cases occurred in the PWS group and two false positive cases occurred within the AS group.

HRCB and karyotype analysis provide a global overview for the detection of chromosomal structural rearrangements. The intricacies and high reproducibility of the G-banding pattern provide an appropriate technique for microdeletion detection and breakpoint analysis. However technical skills between laboratories vary. Not all laboratories appreciate that HRCB implies that chromosome are at the 700-850 band level, nor can they achieve this. Laboratories may imply that they are performing HRCB but are actually only achieving a band level of about 550. This chromosome length is too short to detect a microdeletion. Also problematic is that not all chromosome preparations lend themselves to HRCB and microdeletion analysis due to poor quality of the sample. Microdeletions of 3-4 Mb, which are at the limit of cytogenetic resolution, may not be visible when analyzing less than optimal preparations. FISH may be utilized to substantiate the cytogenetic finding in these cases. FISH, used in conjunction with chromosome analysis, provides a molecular cytogenetic technique to confirm a suspected microdeletion, structural rearrangement or cryptic translocation (Ledbetter 1992). FISH, however, cannot discriminate between the parental origin of the chromosome homologs. Therefore this technique cannot be used to detect UPD.

Q-banding to detect chromosome 15 parental origin

Chromosome heteromorphisms have long been used as tools for establishing chromosome parental origin (McKenzie and Lubs 1975). They are stable and inherited in a Mendelian fashion. In a study of 39 unrelated persons, which were analyzed by means of quinacrine fluorescent staining in order to assess the amount of variation and the discriminatory power of Q-band heteromorphisms, the chance of finding two randomly selected

persons with an identical set of quinacrine variants was calculated to be 0.0003 (Olson et al. 1986) Twenty different chromosome 15 variants were demonstrated in the group of 39 subjects. It was, thus, shown that the heteromorphic regions of the chromosome 15 short arm are powerful markers for distinguishing between individuals, and that this type of analysis is a highly reliable method for the identification of the parental origin of a chromosome 15. Parental blood samples are required for this technique, which in some cases, may prohibit performing this assay.

PCR of Microsatellites to Detect UPD

The parental origin of a chromosome or allele can be traced by the PCR amplification of polymorphic sites from the DNA of a patient and parents. Normal biparental inheritance or UPD of chromosome 15 can be established in this fashion. In all cases of typical PWS in which there is a deletion of 15q11.2-q13, or in which there is maternal UPD of chromosome 15, PCR microsatellite analysis will show the amplification of only the maternal allele(s). In cases of typical AS, in which there is a 15q11.2-q13 deletion or in which there is paternal UPD, PCR analysis will show the amplification of only the paternal allele (Mutirangura et al. 1993b; Ledbetter and Engel 1995).

In a comparison study of 27 PWS patients, 13 of whom had a chromosome 15 deletion and 14 who were normal by karyotype analysis, PCR of chromosome 15 microsatellites showed the deletion to be of paternal origin in all deletion cases and demonstrated maternal uniparental disomy in all of the 14 karyotypically normal cases (Butler 1996).

This type of analysis may be impractical in some cases, in that in addition to requiring a DNA sample from the proband, DNA from the proband's parents, which may not be obtainable, is required as well. Also of note, is the fact that dosage analysis by PCR of microsatellites is not a reliable method to distinguish deletion cases from UPD cases (Mutirangura et al. 1993b). Deletions can be substantiated by HRCB or by FISH.

Parent-Specific Methylation analysis

Quantitative Southern blotting analysis detects parent-specific sites of methylation at the PW71 locus and at the 5' end of the SNRPN gene. These assays which involve the digestion of genomic DNA with methylation-sensitive and methylation-insensitive enzymes produce highly reproducible maternal and paternal specific fragments.

At the PW71 locus, genomic DNA is digested with *Bgl*II and *Cfo*I and probed with PW71 which produces a *Bgl* II maternal band of 8.0 kb and a *Cfo* paternal band of 6.4 kb in the normal individual. Southern blotting analysis of the parent-specific methylation differences at the PW71 locus was shown (Gillessen-Kaesbach et al. 1995), to accurately detect 28 of 28 clinically diagnosed cases of PWS. Lerer et al. (1994), however, showed that in 22 cases of PWS, 21 of whom had normal karyotypes, methylation analysis showed loss of the paternal fragment in 6 cases, maternal UPD in 4 cases, and 12 cases of biparental inheritance of alleles in which both the maternal and paternal fragments were present. The one deletion case in this study showed loss of the paternal fragment by Southern analysis. The assessment, made by these authors, of the discrepant results between the cytogenetic and molecular data was that, in their hands, Southern blotting assays were much more reliable than their karyotype analysis. In another

study of 27 cases of PWS, 13 chromosome deletion cases all showed loss of the paternal fragment. The remaining 14 normal cases indicated maternal UPD in 6 cases, with the remaining 8 cases showing the presence of both the maternal and paternal fragments (Butler 1996). Finally, a study of 27 PWS patients, involving 23 deletion cases and 4 UPD cases, determined by chromosome and PCR microsatellite analysis, showed loss of the paternal fragment in all 27 cases (Kokkonen et al. 1995).

Parent-specific methylation differences at the 5' end of the SNRPN gene are detected by digesting DNA with *Not*I and *Xba*I to give an *Xba* I maternal 4.3 kb fragment and a *Not* I 0.9 kb paternal fragment. PWS patients demonstrate loss of the paternal allele, while AS patients show loss of the maternal allele (Sutcliffe et al. 1995). At present, there are no large studies comparing cytogenetic and 5' SNRPN methylation results within or between the PWS, AS and normal populations. The parent-specific methylation pattern at the SNRPN locus has been shown to be stable and reproducible in multiple tissue types such as lymphocytes, fibroblasts, lymphoblasts and amniocytes (Glenn et al. 1996).

The case studies presented above indicate that Southern blotting analysis of the parent-specific differences at the PW71 and SNRPN loci can detect and distinguish between cases that are karyotypically normal, as well as those involving deletions and UPD. This type of analysis will not, however, detect structural rearrangements involving chromosome 15 in PWS or AS patients. Nor will it distinguish cases of UPD from those that are due to an imprinting abnormality. An imprinting abnormality will demonstrate biparental origin of chromosome 15 homologs by PCR or chromosome heteromorphism analysis, but the methylation pattern of the homologs will be uniparental in its pattern. In these cases, the etiology

of a positive result by Southern blotting analysis can be determined by alternative techniques. HRCB and FISH can be used to detect a structural rearrangement, and PCR of microsatellites may be used to substantiate UPD or an imprinting abnormality.

Recurrence Risks

An understanding of the underlying defects responsible for PWS or AS is important since each defect carries a particular recurrence risk. When a single individual in a family has PWS due to a deletion or UPD, the recurrence risk is approximately 1% (Bray and Wilson 1986; American Society of Human Genetics/American College of Medical Genetics Test and Technology Committee 1996). In cases of PWS or AS that are associated with a translocation present in one parent, the recurrence risk is not actually known but is thought to be substantially higher (Ledbetter and Engel 1995). An increased recurrence risk for abnormal outcome in carriers of chromosome rearrangements is consistent with other reported studies (Daniel et al. 1989; Stene and Stengel-Rutkowski 1988). The recurrence risk in cases of PWS or AS due to an imprinting abnormality or those without an identifiable cause may be as high as a 50% (Reis et al. 1994).

## Assessing The Benefits and Limitations of Cytogenetic and Molecular Testing: Findings From the OHSU PWS and AS Cohort

The standard laboratory diagnostic analysis at OHSU, similar to many other laboratories, has initially relied on HRCB and then included FISH analysis to diagnose cases of PWS and AS. Chromosome 15 parental

origin has been primarily established by chromosome heteromorphism analysis. In order to establish additional testing methods to detect and determine the genetic defects responsible for cases of PWS and AS, PCR of chromosome 15-specific microsatellites and 5' SNRPN parent-specific methylation testing was performed on DNA samples from a cohort of PWS and AS patients. The data obtained from these tests were evaluated for consistency with patient cytogenetic results. Since there have been no large studies comparing cytogenetic results with 5' SNRPN parent-specific methylation results, within or between the PWS, AS and normal populations, I specifically sought to determine the validity of this test. The ultimate goal was to design and evaluate a cytogenetic and molecular testing approach for the diagnosis of PWS and AS.

OHSU PWS and AS Study Cohort

The OHSU study included a cohort of 51 PWS and 37 AS patients which were clinically assessed by geneticists from the OHSU genetics clinic, including Dr. R. Ellen Magenis, as per the protocols set forth by Holm et al. (1993) and Williams et al. (1995). See Appendices A and B for a complete listing of clinical criteria for each syndrome.

Cytogenetic and Molecular Techniques Utilized in this Study

The PWS and AS cohort was studied by multiple cytogenetic and molecular techniques. A tabulation of PWS and AS patients, specifying which cytogenetic and molecular techniques were used to evaluate these patients, is presented in Table 1 (the numbers in bold are studies I personally completed). All patients were evaluated by high resolution chromosome banding. Within this group, chromosomes from 19 PWS

and 24 AS patients were also R-banded in order to better evaluate the integrity of the 15q11.2-q13 region. FISH studies were completed on 28 PWS and 25 AS patients using a combination of the following chromosome 15-specific probes: D15S11, SNRPN, D15S10 and GABRB3 (ONCOR). Chromosome 15 parental origin was assigned by Q-banding in 12 PWS and 23 AS patients. PCR of chromosome 15-specific microsatellites were used to evaluate chromosome parental origin in 5 PWS and 3 AS cases. These results were used to demonstrate the parental origin of the chromosome carrying a deletion, as well as to demonstrate disomy status (i.e. to detect uniparental disomy). The methylation pattern of the 5' end of the SNRPN gene was evaluated in 19 PWS and 16 AS patients in order to assess this assay as a method for detecting PWS and AS cases that were due to a deletion, UPD, structural rearrangements or an imprinting abnormality.

Not all tests were performed on all patients. From the PWS group, a total of 8 patients had all tests performed. From the AS group, a total of 11 patients had all tests performed. Figure 1 represents the sequence of multiple tests that were performed on this latter group of PWS and AS patients.

High Resolution Chromosome Banding Results

In the cohort of PWS and AS patients studied at OHSU by HRCB, 34 of 51 (67%) PWS patients showed a deletion of 15q11.2-q13. Thirty of 37 (81%) AS patients showed a deletion of this same region (see Table 2). Three PWS patients had structural rearrangements involving chromosome 15 producing the following karyotypes:

PWS16: 45,XY,t(14;15)(p13;p13)

PWS18: 46,XX,t(14;15)(p13;p13)

PWS19: 46,XX,-15,-17,+t(15;17)(q13;p13.3),
+r(15)(p11.2q11.2)/45,XX,-15-17,
+t(15;17)(q13;p13.3)

The structural rearrangement in the patient designated as PWS19 was unbalanced and deleted for 15q11.2-q13.

FISH results

In this study, 28 PWS and 25 AS patients were studied by FISH using at least 2 of proximal chromosome 15-specific probes (see Tables 3 and 4). Within the PWS group, 27 of 28 FISH study results were consistent with the HRCB results. Within the AS group, 24 of 25 FISH study results were consistent with the HRCB results. In each of the PWS and AS group studies, there was one false positive result by HRCB (patients are designated by bold type in Tables 3 and 4). The HRCB studies, in both cases indicated a deletion of 15q11.2-q11.2. The FISH studies, however, demonstrated that the genomic material represented by these probes was present on both homologs, giving a nondeletion result.

Q-band chromosome heteromorphism analysis

Chromosome 15 parental origin was established by Q-banding in a total of 12 PWS patients (see Table 5). Of the 4 deletion cases that were examined all were paternal in origin. Of the 6 karyotypically normal cases, 4 demonstrated maternal UPD and 2 patients showed biparental inheritance of their chromosome 15 homologs. Two of the three rearrangement cases showed maternal UPD. The remaining rearrangement case, which included a deletion of 15q11.2-q13, indicated biparental origin of the chromosome 15 homologs, with the deletion being

paternal in origin. Within the AS group of 23 patients that were studied for chromosome parental origin, all 17 deletion cases were maternal in origin. The 5 cytogenetically normal cases were shown to have biparental chromosome 15 origin (see Table 5).

PCR of microsatellite analysis

Within the OHSU cohort, chromosome parental origin studies were also carried out by examining chromosome 15 microsatellite repeats by PCR analysis at the following loci: D15S11, D15S113, GABRA5, GABRB3, all of which are located in 15q11.2-q13.

The three karyotypically normal, and two structural rearrangement PWS patients that were examined by PCR all showed loss of the paternal allele at all loci tested, indicating maternal UPD. Four of the 5 cases were heterodisomic (both maternal homologs represented) in nature and one case demonstrated isodisomy (only one maternal homolog represented). The 3 karyotypically normal AS patients all showed biparental inheritance of alleles (see Table 5).

Parent-Specific Methylation analysis

The 5' SNRPN methylation pattern was examined in 19 PWS patients (see Table 6). All 19 cases, regardless of whether they involved a chromosome deletion, structural rearrangement or were normal, demonstrated the presence of only the maternal fragment. Within the AS group, 11 of 11 chromosome deletion patients demonstrated loss of the maternal fragment. Of the 5 AS patients with normal appearing chromosomes, 4 patients showed both a paternal and maternal fragment and 1 patient showed loss of the maternal fragment

Comparison Study of multiple techniques

As specified by the Thesis Advisory committee, a subset of PWS and AS patients were chosen to directly compare the results obtained from the

various cytogenetic and molecular tests within the same patient. Two groups consisted of either 11 PWS or 11 AS chromosome deletion patients. Two other groups consisted of either 8 PWS or 5 AS non-deletion cases (Tables 7 and 8). Comparison of test data provided the following results.

Within the PWS group of 11 patients with chromosome deletions, FISH studies substantiated the deletion. Similarly, within the PWS group of nondeletion cases, no deletion was found by FISH. Of the 11 PWS deletion cases, the 2 that were examined for parental origin, had a deletion that was paternally derived. All 6 non-deletion PWS cases examined demonstrated maternal UPD by PCR or Q-band analysis. 5' SNRPN methylation results of the 11 deletion patients demonstrated only a maternal fragment as did the 6 non-deletion patients with maternal UPD.

In summary, in all PWS cases the cytogenetic result was consistent with the FISH result. In addition, the chromosome parental origin results were consistent between Q-band and PCR data, and served to substantiate UPD in 6 of 6 non-deletion cases. Finally, 8 of 19 PWS cases that were not detected by HRCB, were detected by methylation analysis of the 5' SNRPN gene.

Within the AS deletion group of 11 chromosome deletion patients, FISH studies substantiated the deletion. The 6 cases within this deletion group that were examined for parental origin were shown to all have maternally derived deletions. Of the 5 chromosome normal patients, all showed biparental chromosome origin. Methylation analysis of the 5' SNRPN gene, within the 11 AS deletion cases, showed only a paternal fragment in each case. Within the 5 chromosome nondeletion AS cases, 4 showed both a maternal and paternal fragment. One non-deletion AS case showed only the paternal fragment.

In summary, in 15 of 16 AS cases, the cytogenetic result was consistent with the FISH result. The chromosome origin results were consistent between Q-band and PCR data. No substantiated cases of UPD were observed. In 4 of the 5 chromosome normal cases, methylation analysis of the 5' SNRPN gene results were consistent, giving a normal result as well. One of 5 chromosome normal cases that was not detected by HRCB or FISH was detected by methylation analysis.

## Discussion of Results

### Prader-Willi Syndrome Patient Cohort

Within the cohort of 51 PWS patients, by HRCB, 67% had a deletion within the 15q11.2-13 region, 27% had a normal karyotype and 6% had a structural rearrangement. These values do not significantly differ from the reported values in the literature ($p>0.05$). The actual incidence of structural rearrangements within the PWS population is unknown, however, the 6% incidence of structural rearrangements in this group represents a notable number of cases.

Of the 28 PWS cases studied with FISH, one false positive result, by HRCB, was obtained. The FISH result is thought to be correct rather than the HRCB result since biparental inheritance was established by PCR microsatellite analysis, and the DNA methylation study demonstrated both a maternal and paternal fragment. This number of false positive results is not beyond the number observed in the study by Delach et al. 1994.

The frequency of UPD in this population was not directly assessed in this study, since not all 14 cytogenetically normal patients received chromosome parental origin studies. However, of the 5 patients tested, all

showed maternal UPD, consistent with the reported data that all UPD in PWS is maternal in origin. Four of 5 cases had heterodisomy. One case showed isodisomy. Two of three of the structural rearrangement cases had maternal UPD, consistent with the idea that structural rearrangements are associated with UPD (Ledbetter and Engel 1995). The third case involving a structural rearrangement had normal biparental disomy. The karyotype was mosaic, with one cell line having a deletion of 15q11.2-q13.

All 19 cases studied for 5' SNRPN methylation showed loss of the paternal 0.9 kb fragment. This was regardless of whether the cytogenetic result was normal or indicated a deletion or structural rearrangement. Two patients had normal HRCB studies, all FISH probes present and biparental inheritance of chromosome 15 homologs. Methylation assays were not performed on these two samples, so an imprinting abnormality cannot be ruled out.

In summary, the results obtained in this study indicated that HRCB provided a global view of the full karyotype, as well as an examination of chromosome 15 integrity. The majority of PWS cases (67%) were detected by this method. FISH was necessary to substantiate deletions particularly in the cases involving deletions less than the length of band 15q11.2. In the remaining cases with normal karyotypes, chromosome parental origin studies performed in conjunction with HRCB studies, were essential for the diagnosis of UPD. The 5' SNRPN methylation assay was able to diagnosis all cases of PWS studied, regardless of whether the genetic abnormality was due to a deletion or UPD. Making a distinction between a deletion and UPD, however, requires highly accurate quantitation and handling of DNA samples. This assay was not capable of detecting

structural rearrangements. Therefore, while the Southern blotting method offered the best diagnostic method, it did not provide information with regard to all of the specific genetic lesions responsible for the syndrome. Without this information, providing recurrence risks is not possible.

Many of the features of PWS, such as obesity, hypogonadism, cryptorchidism and short stature can occur in other syndromes as separate findings, and may be due to different genetic abnormalities (Smith 1982). By limiting an initial genetic test to the 5' SNRPN methylation assay, one assesses a single syndrome at a single locus. The patients that were studied in this cohort were a select group of clinically diagnosed PWS patients. Within the confines of this select group, the 5' SNRPN gene methylation assay was capable of providing a diagnosis of PWS in all of the cases. However, patients normally referred for PWS are not as rigorously clinically evaluated. Many may not actually have PWS. Results from a study of 450 patients referred for PWS indicated that only 28 patients were diagnosed positive for PWS by methylation analysis (Gillessen-Kaesbach et al. 1995). This study did not provide the karyotype data for the study group subjects, so it is unknown if chromosome abnormalities were found in the positive group or in the remaining 432 patients. These data was not tabulated for the OHSU PWS and AS cohort study, but will be done in the future, since it may show preferential differential diagnoses for these patients and provide clinicians with valuable diagnostic information.

Approximately 40% of moderate to severe cases of mental retardation are due to a chromosome abnormality (Connor and Ferguson-Smith 1984). Specific chromosome abnormalities are responsible for at least 60 identifiable syndromes, which collectively are more common than

all the Mendelian single-gene disorders (Borgaonkar 1989). It is, therefore, prudent and standard practice of care that cases referred for PWS, be evaluated by karyotype analysis, particularly in cases which have been evaluated as negative (i.e. normal) by methylation analysis.

**Angelman Patient Cohort**

Within the cohort of 37 AS patients, it was shown by HRCB that 30 patients (81%) had a deletion within the 15q11.2-q13 region, and that 7 (19%) had a normal karyotype result. These values do not significantly differ from the reported values in the literature (Nicholls 1993) ($p>0.05$).

Of the 16 AS cases studied by multiple techniques, 1 case of a false positive result occurred. By HRCB, this deletion was thought to include only a portion of band q11.2. A deletion this small requires substantiation by FISH. At the initial time of the study, however, FISH technology had not yet been developed. Parent blood samples were not available for this patient, and thus disomy status analysis was not possible. Subsequent to these findings, another blood sample for this patient was obtained. At this time, Southern blotting analysis of 5′ SNRPN methylation was shown to be abnormal in this patient, demonstrating loss of the maternal 4.3 kb allele. Since FISH analysis demonstrated that there was no deletion detected by the D15S11, SNRPN, D15S10 and GABRB3 probes within this region, this Southern blotting result substantiates that the HRCB result was in error. Since parent DNA samples were unavailable, it was not possible to confirm whether this patient had biparental inheritance of the chromosome 15 homologs. Therefore, it is not possible to determine whether this patient is affected with AS due to an imprinting abnormality or UPD.

Also within the 16 AS cases studied by multiple techniques, there was one case of an apparently false negative result. In this case, both the HRCB and FISH results indicated a normal result. A Q-band chromosome parental origin study demonstrated that the chromosomes were biparental in inheritance. Southern blotting analysis at the 5' SNRPN gene, however, demonstrated loss of the maternal allele. This result is consistent with an imprinting abnormality.

Thus, within the cohort of AS patients included in this study, there was one possible case of UPD, and 1 or possibly two cases of AS due to an imprinting abnormality. Two of the 37 AS patients were not examined for chromosome origin, so disomy status is unknown.

In total, 16 cases of AS were examined for parent-specific methylation at the 5' SNRPN gene. All 11 deletion cases showed loss of the maternal 4.3kb fragment. Within the group of 5 nondeletion patients tested, 4 patients showed both the 4.3 kb maternal and 0.9 paternal fragments. The one patient (mentioned above) who did not have a cytogenetic deletion, and had biparental chromosome 15 inheritance by Q-band analysis, showed loss of the maternal 4.3 allele.

The results of these studies indicate that HRCB, as an initial test will detect the majority of AS patients (approximately 70%). This technique will also detect any unbalanced structural rearrangements that would contribute to the AS syndrome. Given that this syndrome is due to a microdeletion, it would be wise, in addition to HRCB, to perform FISH analysis using the chromosome 15 proximal probes: D15S11, SNRPN, GABRB3 and D15S10. Since the exact location of the gene responsible AS is not precisely mapped within the 4Mb PWS/AS critical region, it is

prudent to use all four probes to span this region when attempting to detect a deletion.

Southern blotting analysis of 5′ SNRPN methylation accurately diagnosed all deletion cases of AS, including one case missed by HRCB and one case missed by FISH. This technique demonstrated that it could be used to detect AS cases due to UPD and imprinting abnormalities. Both of these genetic abnormalities are not detectable by HRCB.

Chromosome parental origin studies accomplished by Q-banding or PCR were shown to be equally effective in demonstrating chromosome parental origin and disomy status. Southern blotting analysis of the 5' SNRPN gene was shown to be more efficient in determining disomy status, since no parent blood samples were required for this assay. However, the Southern blotting assay cannot distinguish between UPD and an imprinting abnormality. In the case of an imprinting abnormality, disomy status must be ascertained by Q-band or PCR analysis in combination with a demonstrated loss of the normal parent-specific methylation pattern.

Within the AS cohort, 5 (13.5%) patients failed to demonstrate a cytogenetic or molecular defect. Estimates from the literature suggest that approximately 15-30% of AS patients will not demonstrate a genetic abnormality by any of the above testing methods (White and Knoll 1995). At present, there are no large studies which provide data with regard to what percentage of AS patients are diagnosed positive for this syndrome from the clinically referred group. The diagnosis of this syndrome is difficult, and some of the features overlap with other syndromes such as Rett syndrome. It is therefore probable that many of the referred cases of suspected AS will not have AS and will require additional karyotype

analysis when a methylation test is performed initially. Cases for which a clinical diagnosis of AS is still suspected, but for which the tests included in this study did not detect a genetic abnormality, may be referred to a research laboratory for further molecular investigation.

**Providing a Diagnostic Approach For PWS and AS**

Based on a review of the reported PWS and AS diagnostic studies and combined with the tabulation of data obtained from the OHSU PWS and AS study, the following approach to the diagnosis of PWS and AS is provided.

An Approach to Diagnostic Testing for PWS/AS

A. Request that blood samples for PWS and AS be obtained in suitable tubes for both chromosome and DNA analysis.

B. Perform HRCB analysis and FISH of chromosome 15 proximal probes, including SNRPN, to span the PWS/AS region.

C. Perform a methyation analysis on all cases which:
   1. are normal by HRCB and FISH, in order to detect UPD or an imprinting abnormality
   2. have a structural rearrangement involving chromosome 15, in order to detect UPD.

D. If methylation analysis is abnormal, loss of the paternal allele confirms a diagnosis of PWS. Loss of the maternal allele confirms a diagnosis of AS.

E. If HRCB, FISH and methylation studies are normal, PWS can be ruled out. While most of the identifiable genetic causes are ruled

out, the clinical evaluation of AS may take precedence over the laboratory test results.

F. If HRCB and FISH are normal, but methylation studies are abnormal, UPD or an imprinting abnormality is likely.

1. Perform PCR of microsatellite markers or Q-band chromosome parental origin studies to demonstrate UPD or an imprinting abnormality.

a. Biparental inheritance of chromosome 15 homologs will rule out UPD and demonstrate an imprinting abnormality.

b. Maternal UPD will confirm a diagnosis of PWS.

c. Paternal UPD will confirm a diagnosis of AS.

If diagnostic testing is initiated by methylation analysis of the 5' SNRPN gene, and results in a positive test for PWS (i.e. loss of the paternal fragment) or AS (i.e. loss of the maternal fragment), it is advisable to request karyotype analysis to identify the presence of a structural rearrangement. Quantitative Southern blotting analysis may not discriminate between deletion and UPD cases, and these results should be confirmed by PCR analysis if parental samples are available. The data obtained from these additional tests are essential for providing recurrence risks and genetic counseling.

If all of the above testing strategies fail to demonstrate a genetic abnormality, then the referring clinician is advised to consult with a clinician experienced with the particular disorder. Alternative syndromes may be considered. In the neonatal period differential diagnosis of PWS should include syndromes with severe hypotonia (Gorlin et al. 1990).

Other syndromes that may be considered include Bardet-Biedel, Cohen, Fragile X, and Albright hereditary osteodystrophy. With reference to AS, different syndromes that may mimic AS, particularly in the infant period include Rett syndrome, and nonspecific cerebral palsy (Williams et al. 1995). Alternatively, exploration of neurodegenerative disorders, may provide the correct differential diagnosis in unresolved cases of apparent AS (Müller et al. 1994) .

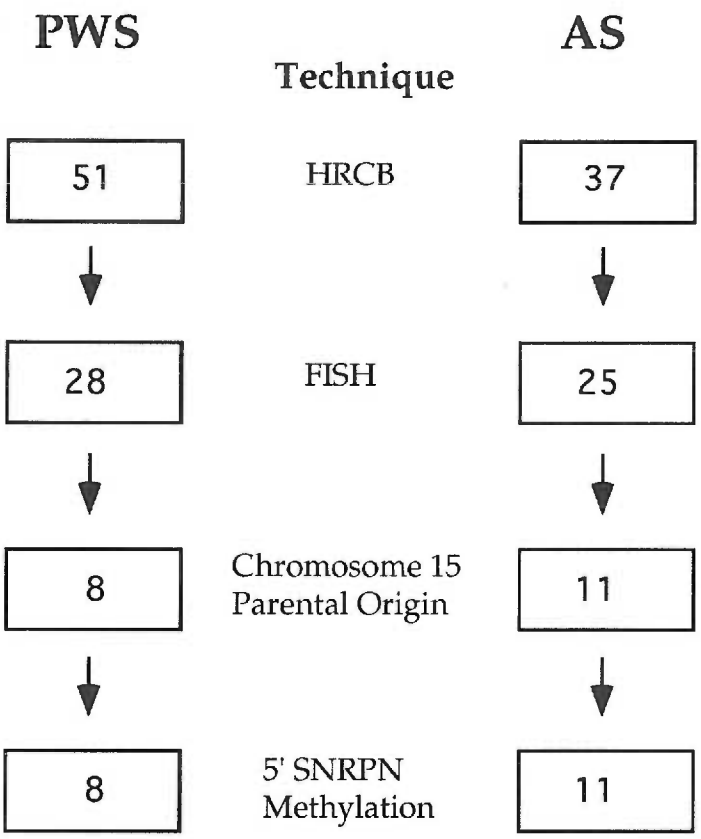


Fig. 1 Flow chart demonstrating the number of PWS and AS patients studied in succession by multiple techniques.

**Table 1** Number of PWS and AS patients evaluated with cytogenetic and molecular techniques.

| | no. | G | R | Q | FISH | PCR | Methylation |
|---|---|---|---|---|---|---|---|
| PWS | 51 | 51 | 19 **(17)** | 12 **(11)** | 28 **(7)** | 5 **(5)** | 19 **(19)** |
| AS | 37 | 37 **(3)** | 24 **(24)** | 23 **(12)** | 25 **(8)** | 3 **(3)** | 16 **(16)** |

G=G-banding. R=R-banding. Q=Q-banding.

**Table 2** Number of PWS and AS patients with chromosome 15 karyotype results

| | no. | del of 15q | normal | rearrangement |
|---|---|---|---|---|
| PWS | 51 | 34* | 14 | 3 |
| AS | 37 | 30* | 7 | 0 |

*One patient in this group showed all tested FISH probes present.

**Table 3** Total number of AS patients tested by various cytogenetic and molecular techniques*

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR origin results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| AS1 | F | 13 | 46,XX,del(15)(q11.2q11.2) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| **AS2** | **M** | **43** | **46,XY,del(15)(q11.2q11.2)** | **Not deleted** | **Not available** | **Not available** | **0.9 kb fragment** |
| AS3 | F | 11 | 46,XX,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS4 | F | 8 | 46,XX,del(15)(q11.2q11.2) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS5 | M | 13 | 46,XY,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS6 | M | 20 | 46,XY,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS7 | M | 16 | 46,XY,del(15)(q11.2q13) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS8 | F | 13 | 46,XX,del(15)(q11q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS9 | F | 2 | 46,XX,del(15)(q11q13) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS10 | M | 3 | 46,XY,del(15)(q11.2q13) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS11 | F | 27 | 46,XX,del(15)(q11.2q13) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS12 | F | 5 | 46,XX | Not deleted | Biparental | Biparental | 4.3/0.9 kb fragments |
| AS13 | M | 15 | 46,XY | Not deleted | Biparental | Biparental | 4.3/0.9 kb fragments |
| AS14 | F | 6 | 46,XX | Not deleted | Biparental | Biparental | 4.3/0.9 kb fragments |
| AS15 | M | 41 | 46,XY | Not deleted | Biparental | Not available | 4.3/0.9 kb fragments |
| **AS16** | **M** | **25** | **46,XY** | **Not deleted** | **Biparental** | **Not available** | **0.9 kb fragment** |
| AS17 | F | 7 | 46,XX,del(15)(q11q13) | Deletion | Maternal deletion | Not available | Not available |
| AS18 | M | 14 | 46,XY,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| AS19 | F | 18 | 46,XX,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | Not available |
| AS20 | F | 42 | 46,XX,del(15)(q11.2q12) | Not available | Not available | Not available | Not available |
| AS21 | F | 9 | 46,XX,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |
| AS22 | F | 26 | 46,XX,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |
| AS23 | F | 8 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS24 | F | 10 | 46,XX,del(15)(q11.2q12) | Not available | Maternal deletion | Not available | Not available |
| AS25 | F | 19 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS26 | M | 10 | 46,XY,del(15)(q11.2q12) | Deletion | Not available | Not available | Not available |
| AS27 | M | 25 | 46,XY,del(15)(q11.2q11.2) | Deletion | Not available | Not available | Not available |
| AS28 | F | 8 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS29 | F | 41 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| AS30 | F | 4 | 46,XX,del(15)(q11.2q11.2) | Deletion | Maternal deletion | Not available | Not available |
| AS31 | F | 14 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS32 | F | 25 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS33 | F | 12 | 46,XX,del(15)(q11.2q13) | Not available | Maternal deletion | Not available | Not available |
| AS34 | F | 7 | 46,XX,del(15)(q11.2q11.2) | Deletion | Not available | Not available | Not available |
| AS35 | F | 11 | 46,XX,del(15)(q11q13) | Deletion | Maternal deletion | Not available | Not available |
| AS36 | M | 13 | 46,XY | Not deleted | Not available | Not available | Not available |
| AS37 | F | 23 | 46,XX | Not deleted | Not available | Not available | Not available |

* The patient(s) with discrepant results when studied by multiple cytogenetic and molecular techniques are highlighted in bold.

**Table 4** Total number of PWS patients tested with various cytogenetic and molecular techniques*

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR origin results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| PWS1 | M | 4 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS2 | M | 5 | 46,XY,del(15)(q11.2q12) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS3 | F | | 46,XX,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS4 | F | 2 | 46,XX,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS5 | M | 33 | 46,XY,del(15)(q11.2q11.2) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS6 | F | 17 | 46,XX,del(15)(q11.2q11.2) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS7 | M | 22 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS8 | M | 28 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS9 | F | 4 | 46,XX,del(15)(q11q13) | Deletion | Paternal deletion | Not available | 4.3 kb fragment |
| PWS10 | M | 1 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS11 | M | 23 | 46,XY,inv(4) | Not deleted | Not available | Not available | 4.3 kb fragment |
| PWS12 | M | | 46,XY | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS13 | F | | 46,XX | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS14 | F | 47 | 46,XX | Not deleted | Maternal UPD | Not available | 4.3 kb fragment |
| PWS15 | F | 9 | 46,XX | Not deleted | Not available | Not available | 4.3 kb fragment |
| PWS16 | M | | 45,XY,t(14;15)(p13;p13) | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS17 | M | 29 | 46,XY | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS18 | F | | 46,XX,t(14;15)(p13;p13) | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS19 | F | | 46,XX,-15,-17, +t(15;17)(q13;p13.3), +r(15)(p11.2q11.2)/45,XX, -15,-17,+t(15;17)(q13;p13.3) | Deletion | Paternal deletion | Not available | 4.3 kb fragment |
| **PWS20** | **M** | **35** | **46,XY,del(15)(q11.2q11.2)** | **Not deleted** | **Not available** | **Not available** | **Not available** |
| PWS21 | F | 37 | 46,XX,del(15)(q11.2q12) | Deletion | Not available | Not available | Not available |
| PWS22 | F | 4 | 46,XX,del(15)(q11.2q13) | Deletion | Paternal deletion | Not available | Not available |
| PWS23 | F | 51 | 46,XX,del(15)(q11.2q12) | Not available | Not available | Not available | Not available |
| PWS24 | F | 4 | 46,XX,del(15)(q11.2q13) | Deletion | Not available | Not available | Not available |
| PWS25 | F | 8 | 46,XX,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |
| PWS26 | M | 8 | 46,XY,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| PWS27 | M | 51 | 46,XY,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |
| PWS28 | M | 14 | 46,XY,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| PWS29 | F | | 46,XX,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| PWS30 | M | 13 | 46,XY,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| PWS31 | M | 16 | 46,XY,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| PWS32 | F | 12 | 46,XX,del(15)(q11.2q13) | Not available | Not available | Not available | Not available |
| PWS33 | M | | 46,XY,del(15)(q11q13) | Not available | Not available | Not available | Not available |
| PWS34 | M | | 46,XY,del(15)(q11q12) | Not available | Not available | Not available | Not available |
| PWS35 | M | 13 | 46,XY,del(15)(q11q13) | Not available | Not available | Not available | Not available |
| PWS36 | M | 11 | 46,XY,del(15)(q11q13) | Not available | Paternal deletion | Not available | Not available |
| PWS37 | F | 17 | 46,XX,del(15) | Not available | Not available | Not available | Not available |
| PWS38 | M | | 46,XY,del(15)(q11q13) | Not available | Not available | Not available | Not available |
| PWS39 | M | | 46,XY,del(15) | Not available | Not available | Not available | Not available |
| PWS40 | F | 20 | 46,XX,del(15)(q11q13) | Not available | Not available | Not available | Not available |
| PWS41 | M | 8 | 46,XY,del(15)(q11.2q12.2) | Not available | Not available | Not available | Not available |
| PWS42 | M | 8 | 46,XY,del(15)(q11.2q12) | Deletion | Not available | Not available | Not available |
| PWS43 | M | 14 | 46,XY | Not deleted | Not available | Not available | Not available |
| PWS44 | F | 8 | 46,XX | Not deleted | Biparental | Not available | Not available |
| PWS45 | M | 13 | 46,XY | Not deleted | Biparental | Not available | Not available |
| PWS46 | M | 11 | 46,XY | Not available | Not available | Not available | Not available |
| PWS47 | F | 6 | 46,XX,del(15)(q11.2q11.2) | Not available | Not available | Not available | Not available |
| PWS48 | F | 31 | 46,XX | Not available | Not available | Not available | Not available |
| PWS49 | M | 23 | 46,XY | Not available | Not available | Not available | Not available |
| PWS50 | F | 11 | 46,XX | Not available | Not available | Not available | Not available |
| PWS51 | M | 14 | 46,XY | Not deleted | Not available | Not available | Not available |

* The patient with a discrepant result when studied by multiple cytogenetic and molecular techniques is highlighted in bold.

Table 5 Results of patients tested for chromosome parental origin and UPD by microsatellite and chromosome heteromorphism analysis

| | | Parental origin of deletion or UPD | | |
|---|---|---|---|---|
| | | paternal | maternal | biparental |
| | number | | | |
| PWS PATIENTS | | | | |
| chromosome | 12 | | | |
| deletion | 3 | 3 | 0 | 0 |
| nondeletion | 6 | 0 | 4 | 2 |
| rearrangement | | | | |
| deletion | 1 | 1 | 0 | 0 |
| nondeletion | 2 | 0 | 2 | 0 |
| microsatellites | 5 | | | |
| deletion | 0 | - | - | - |
| nondeletion | 3 | 0 | 3 | 0 |
| rearrangement | | | | |
| deletion | 0 | - | - | - |
| nondeletion | 2 | 0 | 2 | 0 |
| AS PATIENTS | | | | |
| chromosome | 23 | | | |
| deletion | 17 | 0 | 17 | 0 |
| nondeletion | 5 | 0 | 0 | 5 |
| microsatellite | | | | |
| deletion | 0 | - | - | - |
| nondeletion | 3 | 0 | 0 | 3 |

**Table 6** Number of patients tested for the SNRPN methylation pattern and result.

| Karyotype | no. tested | maternal 4.3 kb fragment present | paternal 0.9 kb fragment present | biparental 4.3kb/0.9kb fragments present |
|---|---|---|---|---|
| **PWS** | | | | |
| Deletion | 10 | 10 | 0 | 0 |
| Normal | 6 | 6 | 0 | 0 |
| Rearrangement | 3 | 3 | 0 | 0 |
| **AS** | | | | |
| Deletion | 11 | 0 | 11 | 0 |
| Normal | 5 | 0 | 1 | 4 |

**Table 7** Selected PWS patients tested by specific cytogenetic and molecular techniques

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR origin results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| PWS1 | M | 4 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS2 | M | 5 | 46,XY,del(15)(q11.2q12) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS3 | F | | 46,XX,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS4 | F | 2 | 46,XX,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS5 | M | 33 | 46,XY,del(15)(q11.2q11.2) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS6 | F | 17 | 46,XX,del(15)(q11.2q11.2) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS7 | M | 22 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS8 | M | 28 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS9 | F | 4 | 46,XX,del(15)(q11q13) | Deletion | Paternal deletion | Not available | 4.3 kb fragment |
| PWS10 | M | 1 | 46,XY,del(15)(q11q13) | Deletion | Not available | Not available | 4.3 kb fragment |
| PWS11 | M | 23 | 46,XY,inv(4) | Not deleted | Not available | Not available | 4.3 kb fragment |
| PWS12 | M | | 46,XY | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS13 | F | | 46,XX | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS14 | F | 47 | 46,XX | Not deleted | Maternal UPD | Not available | 4.3 kb fragment |
| PWS15 | F | 9 | 46,XX | Not deleted | Not available | Not available | 4.3 kb fragment |
| PWS16 | M | | 45,XY,t(14;15)(p13;p13) | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS17 | M | 29 | 46,XY | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS18 | F | | 46,XX,t(14;15)(p13;p13) | Not deleted | Maternal UPD | Maternal UPD | 4.3 kb fragment |
| PWS19 | F | | 46,XX,-15,-17, +t(15;17)(q13;p13.3), +r(15)(p11.2q11.2)/45,XX, -15,-17,+t(15;17)(q13;p13.3) | Deletion | Paternal deletion | Not available | 4.3 kb fragment |

**Table 8** Selected AS patients tested by specific cytogenetic and molecular techniques*

| Subject number | Sex | Age (yr) | Karyotype | Summary of FISH results | Summary of Chromosome origin results | Summary of PCR origin results | Methylation DNA pattern using SNRPN |
|---|---|---|---|---|---|---|---|
| AS1 | F | 13 | 46,XX,del(15)(q11.2q11.2) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| **AS2** | **M** | **43** | **46,XY,del(15)(q11.2q11.2)** | **Not deleted** | **Not available** | **Not available** | **0.9 kb fragment** |
| AS3 | F | 11 | 46,XX,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS4 | F | 8 | 46,XX,del(15)(q11.2q11.2) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS5 | M | 13 | 46,XY,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS6 | M | 20 | 46,XY,del(15)(q11.2q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS7 | M | 16 | 46,XY,del(15)(q11.2q13) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS8 | F | 13 | 46,XX,del(15)(q11q13) | Deletion | Maternal deletion | Not available | 0.9 kb fragment |
| AS9 | F | 2 | 46,XX,del(15) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS10 | M | 3 | 46,XY,del(15) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS11 | F | 27 | 46,XX,del(15) | Deletion | Not available | Not available | 0.9 kb fragment |
| AS12 | F | 5 | 46,XX | Not deleted | Biparental | Paternal | 4.3/0.9 kb fragments |
| AS13 | M | 15 | 46,XY | Not deleted | Biparental | Biparental | 4.3/0.9 kb fragments |
| AS14 | F | 6 | 46,XX | Not deleted | Biparental | Biparental | 4.3/0.9 kb fragments |
| AS15 | M | 41 | 46,XY | Not deleted | Biparental | Not available | 4.3/0.9 kb fragments |
| **AS16** | **M** | **25** | **46,XY** | **Not deleted** | **Biparental** | **Not available** | **0.9 kb fragment** |

*The patients with discrepant results when studied by multiple cytogenetic and molecular techniques are highlighted in bold.

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## DISCUSSION

### PWS AND AS ARE COMPLEX CHROMOSOME CONDITIONS

The underlying genetic mechanisms responsible for PWS and AS are far more complex than those responsible for other chromosome conditions. There are multiple types of genetic defects that can lead to both of these syndromes. The 15q11.2-q13 chromosome region implicated in these two syndromes is imprinted. Consequently, normal development depends on inheritance of this region from both parents. The absence of a specific parental contribution results in the syndrome-specific phenotype. This absence may be the result of structural loss due to a deletion within the 15q11.2-q13 region or to functional loss, as in the case of UPD of chromosome 15 or an imprinting abnormality. The clinical phenotypes among PWS or AS patients do not broadly differ according to the underlying genetic mechanism. Therefore multiple laboratory testing techniques are required for specific diagnosis, prognosis and recurrence risks. In addition, the basis of the imprinting mechanism in these syndromes is not well understood. Therefore, while an imprinting defect may be observed by an abnormal methylation pattern in a patient, the full clinical relevance to the patient is not known.

### CLINICAL DIAGNOSIS OF PWS AND AS

The clinical descriptions of PWS and AS, first reported in 1956 and 1965 respectively (Prader et al. 1956; Angelman 1965), provided a clinical basis for detecting and diagnosing patients with these patterns of anomalies. The described pathognomonic developmental abnormalities and malformations set these disorders apart, and thus defined them as distinct syndromes. The development of standardized diagnostic clinical criteria for both PWS and AS in recent years has served to improve the

consistency of clinical findings from which patients are diagnosed for these disorders (Holm et al. 1993; Williams et al. 1995). Accurate diagnosis is crucial since the validity and value of research data that are obtained from studies of PWS and AS patients are completely dependent on the correctness of the clinical diagnosis of the patient population being studied. A clinical diagnosis of PWS is easier than that of AS. The developmental delay, facies, body habitus, neonatal history and adult behaviors, when viewed as a gestalt, are quite distinctive. AS, however, is much more difficult to diagnose, particularly in infancy. These patients are only subtly dysmorphic and body proportions are normal. The characteristics of developmental delay, ataxia with tremulous movements, lack of speech and facial features of AS are more difficult to confidently put together as a whole and designate as definitely AS. It may be, therefore, that not all of the patients for which AS research data are based actually have AS. While only speculative, it may be that part of the difficulty with finding the gene responsible for AS is that the data obtained from AS studies are confused by the contamination of data from non-Angelman patients within the study group.

CYTOGENETIC DIAGNOSIS OF PWS AND AS

Throughout my studies, the cytogenetic finding of a 15q11.2-q13 deletion overlap in both PWS and AS has remained constant. At one time, the cytogenetic studies in the Magenis laboratories appeared to indicate that the deletions in PWS patients might be smaller than those of AS patients. This finding was merely due to sampling size. In actuality, cytogenetic studies by the Magenis clinical cytogenetics laboratory, other laboratories and myself have found that deletion size varies among patients within both syndromes. The common region of overlap between

and within each syndrome is band q11.2 (Magenis and Toth-Fejel 1991). Therefore, I submit that while the critical region of PWS and AS is referred to in the literature as in 15q11.2-q13, my work, as well as others has demonstrated that it is more likely in 15q11.2.

**Detection of 15q11.2 Abnormalities by HRCB**

The smallest deletion visible by cytogenetic analysis is approximately 3-4 Mb, or roughly the length of band 15q11.2. Loss of a smaller band, such as band 15q12 may be detectable using high resolution G-banding in combination with special stains such as R-banding, which gives a fluorescent reverse pattern to G-banding. The contrast between the dark and light bands allows visual detection of the loss of chromatin. Deletions within a single light or dark band are visible by appearing reduced in size, with flanking bands appearing closer together. A difficulty, however, with assessing loss of chromatin within a band such as 15q11.2 is that it is juxtaposed to a region of pericentromeric heterochromatin. Experience I have gained by studying approximately four thousand chromosome 15 homologs indicates that 15q11.2 is a difficult chromosome region to analyze and particularly when only G-banding is used. By C-banding it can be shown that the amount of heterochromatin may vary between individuals but is inherited in a stable and Mendelian fashion from one generation to another (Arrighi and Hsu 1971). It is possible to C-band a chromosome 15 and then sequentially stain it with G-banding, but it is hard to assess with precision the boundary between the C-band positive heterochromatin and the G-band negative euchromatin. Chromosomes must be collected at a cell cycle stage which permits band length of chromosomes to be greater than or equal to 800. This length is more difficult to achieve in chromosomes from amniotic

fluid and fibroblast samples than blood samples. While comparing chromosome band length between homologs in the same cell, may be valid, band length comparisons between tissue types may not always be so. In other words, it may not be possible to accurately compare band 15q11.2 in an amniotic fluid sample with the same homolog from a blood sample of the parent from which it originated. in addition, small duplications of band q11.2, without phenotypic effect are not infrequent, farther compounding size assessment problems (Clayton-Smith et al. 1993). Therefore, due to the variability of pericentromeric heterochromatin juxtaposed to 15q11.2 and variations in band length, detection of intraband deletions in 15q11.2 may not always be possible.

**Detection of chromosome 15 abnormalities by FISH and HRCB**

The conjoint usage of fluorescent in situ hybridization (FISH) and HRCB is prudent when examining chromosomes for any suspected microdeletion syndrome abnormality or delineating a subband deletion. Multicolor FISH may be particularly useful for delineating inversions and duplications within the 15q11.2-q13 region. It was shown in chapter 4 of this thesis that multi-probe FISH was able to detect all 15q11.2-q13 cytogenetic deletions in PWS and AS. It was also possible to more accurately detect other changes in the integrity of the 15q11.2-13 region. In two cases, 1 PWS and 1 AS, FISH with probes from D15S11, SNRPN, D15S10, and GABRB3 (ONCOR) was able to demonstrate that a deletion reported by HRCB was most likely not a true result. The studies reported in chapter 4 do not suggest that the FISH technique replace HRCB, since no FISH studies were performed without HRCB. These techniques, HRCB and FISH, used in combination, provide more of information than either alone. For example, in one PWS patient with a complex mosaic karyotype

(involving a rearrangement of chromosome 15 and 17, a deletion of 15q11.2-q13, and a small ring chromosome), the identity of the component chromosomes of the rearrangement was dependent on cytogenetic analysis. However, accurate breakpoint analysis and documentation of the deletion was not made until FISH studies were performed.

## PRENATAL DIAGNOSIS OF CHROMOSOME 15

### Chapter 1

The first chapter of this thesis dealt with examining structural changes of the 15q11.2-q13 region in amniocyte chromosomes. The study included an investigation of four amniotic fluid samples, three of which were deletion cases and one an inversion case, all involving the 15q11.2-q13 region. The structural abnormalities were visible by standard cytogenetics. FISH [D15S11, SNRPN, D15S10, and GABRB3 (ONCOR)] was employed to substantiate the findings, refine breakpoints and predict the phenotypic outcome.

Case 1 and case 2 FISH results were in agreement with the cytogenetic results. In case 3, the FISH did not substantiate the cytogenetics results. In the fourth case, the FISH results helped define the breakpoints of the paracentric inversion, but did not help predict the clinical outcome. Clinical outcome of PWS was predicted in case 1 because of the findings of paternal origin of the deletion. With regard to case 3, in which the alleged deletion was not substantiated by FISH, several reasons are possible. Only five probes were used to screen for a deletion. They span the approximate 4 Mb PWS/AS critical region, but do not contiguously cover it. Two gaps of almost 1Mb each in length were left unprobed. It is possible that the chromosome was indeed deleted, but for genetic material not detected by

these probes. It seems unlikely, however, that the deletion was this small since the apparent deletion was visible and loss of genetic material from between these gaps would not be cytogenetically visible. The apparently deleted chromosome was determined to be paternal in origin. This same chromosome in the fathers' blood sample did not appear deleted, as did the chromosome in an additional umbilical cord blood sample obtained from the fetus, suggesting that the deletion was real and *de novo* in nature. Initially, I thought that the apparent difference might be due to the fact that I was comparing chromosomes from different tissues in a region that has subtle chromosome condensation differences between blood and amniocyte chromosomes. This was ruled out, however, by examining blood chromosomes from both parents and the fetus.

An alternative explanation is that this patient's chromosomes may have been normal, with all genetic material intact in the 15q11.2-q13 region. In this case, the apparent loss of genetic material could have represented a loss of pericentromeric heterochromatin in the paternally derived chromosome. In addition, if there were a large amount of pericentromeric heterochromatin in the normal maternal homolog, comparisons to the apparently abnormal homolog of the fetus it would have made the abnormal homolog appear short by comparison. This is an extremely important point to keep in mind since most cytogenetic analyses depend on comparison of homologs.

At the time of this study, the SNRPN gene had been cloned, but its role as a candidate gene for PWS was speculative. There were no gene(s) within this region that, when defective, were known to be responsible for PWS or AS. It was less clear then than it is now what material within this region was essential for normal development.

The fourth case had a paracentric inversion with the proximal breakpoint within 15q11.2-13 and a distal breakpoint near the very distal tip of the chromosome long arm. All FISH probes, including the SNRPN gene, were rearranged to the very distal breakpoint region of the inverted chromosome. While the SNRPN gene was cloned by the time this study was completed, an assay to detect normal imprinting of this gene was unknown.

The proximal breakpoint of the inversion in this case was in the common proximal breakpoint of deletions observed in PWS and AS (Christian et al. 1995). This breakpoint was centromeric to the most proximal gene mapped to this region. The distal breakpoint was telomeric. Given what is known now about this region, if: (1) normal chromosome 15 disomy were observed and (2) proper imprinting within 15q11.2-q13 at the D15S9 (ZNF174), D15S63 (PW71) and SNRPN loci could have been established, improved genetic counseling could have been given to this patient. This patient, with a *de novo* chromosome inversion, which are approximated to occur at a frequency of 1/10,000, would have had an empiric risk of serious congenital anomaly of 9.4% (Warburton 1991).

The FISH results of this study were not available when the cytogenetics was completed and conveyed to the parents. They subsequently chose to terminate the pregnancy, so clinical follow-up was unavailable. In all but case 1, a second tissue (umbilical cord blood) was obtained for confirmation of the abnormality. The cytogenetic results obtained in these tissues were consistent with amniotic fluid (AF) results. A skin sample was received from case 1, but failed to grow. These cases

demonstrated the difficulty of delineating a subband deletion of 15q11.2, particularly in AF samples, solely by cytogenetic means.

Prenatal diagnosis of this region is still difficult. The advent of FISH has greatly increased the ability to evaluate a suspected microdeletion, duplication or microinversion. However, there is still the disadvantage, a lack of known phenotype to help with diagnosis. Prenatal diagnosis, based on amniocentesis that is initiated at approximately 14 weeks, must rely solely on laboratory analysis. Until the genes responsible for AS and PWS are well established, the FISH results merely indicate loss or gain of genomic material. Chromosome 15 origin must be established in cases of a chromosome 15 abnormality in order to predict the syndromic outcome of PWS versus AS.

In case 3 and particularly in case 4, Southern blotting methylation analysis of the 5'SNRPN gene might have provided information about the functional status of the imprinted region in these apparently abnormal chromosomes 15. This assay has been demonstrated to be a valid test in this tissue (Glenn et al. 1996).

A complicating issue of prenatal diagnosis of chromosome 15 abnormalities is that the interpretation of the results from FISH analysis are not always well correlated with a phenotype. For example, duplications of subregions within 15q11.2-q13, are known to occur (Clayton-Smith et al. 1993). They may be familial or *de novo*, and result in an abnormal phenotype in only a subset of cases. It has yet to be defined, within this group of patients, precisely what genetic material is duplicated and what the correlations are with clinical outcome.

**Guidelines for Prenatal Diagnosis of PWS and AS**

Chromosome analysis of amniotic fluid samples has already been established as normal standard of care in cases of advanced maternal age and previous history of a chromosome abnormality, including a previous history of PWS and AS. Carriers of chromosome structural abnormalities, including Robertsonian translocations, are at an increased risk for nondisjunction (Hamerton 1971; Ledbetter and Engel 1995) and, thus, also warrant prenatal diagnostic testing. An approach to the prenatal diagnostic testing of PWS and AS is presented below. This approach combines cytogenetic, molecular cytogenetic and Southern blotting analysis. Cytogenetic and molecular cytogenetic analysis have been shown to be valid testing techniques given the provisos described above. The parent-specific 5'-SNRPN methylation analysis has been shown to be a reliable test for PWS and AS in studies performed for this thesis. The parent-specific methylation patterns have been demonstrated to be stable in multiple tissues, including AF (Glenn et al. 1996).

An Approach to Prenatal Diagnostic Testing of PWS and AS

A. Perform high quality cytogenetic analysis with attention to regions known to be associated with the microdeletion syndromes.

B. Maintain backup AF cultures in the event that DNA analysis is warranted.

C. Perform FISH analysis on AF and chorionic villus samples in which a deletion or structural rearrangement involving chromosome 15 is suspected by karyotype analysis or in fetuses who have a positive history, as stated above.

   I. Utilize FISH with probes from D15S11, SNRPN, GABRB3, and D15S10 (with control identifier probe with each test probe)

which span the PWS/AS critical region and provide a broad view of potential deletions throughout this region.

A. Examine for hybridization signals in 10 cells from two independent cultures.

B. Interpret loss of a test probe signal from a chromosome 15 homolog in every cell, with presence of control signal, as a deletion.

C. Interpret the presence of probe signal on both homologs which also show hybridization of the control signal as a normal result. A normal result by FISH analysis suggests that the apparent deletion does not include genetic material homologous to the probes, and may be due to normal variation of pericentromeric heterochromatin or duplication of euchromatin.

II. Perform 5′ SNRPN methylation analysis on AF samples referred for a previous history of PWS or AS due to UPD, as well as on samples with familial or *de novo* structural rearrangements, particularly those involving Robertsonian translocations.

A. A normal result by HRCB and 5′ SNRPN rules out the majority of causes of PWS and AS. However, approximately 20-30% of AS patients will not demonstrate any abnormality by HRCB, FISH, or 5′ SNRPN methylation analysis.

III. Perform 5′ SNRPN methylation analysis In cases of 15q11.2-q13 structural rearrangements such as duplications, insertions, or

inversions, that have been confirmed by FISH analysis, to evaluate the regional imprinting status by.

The association of chromosome structural rearrangement with an increase in nondisjunction and subsequent UPD (Ledbetter and Engel 1995) makes it imperative to establish the chromosome origin and disomy status of the chromosome homologs involved, particularly with regard to chromosome 14 and 15.

Although both PWS and AS occurs at a frequency of only approximately 1/15,000, laboratory technologist time may be used wisely when it is required to confirm that no PWS/AS microdeletion is present in an AF sample. AF chromosome culturing and preparation has reached a level of quality that within an AF sample there will be a number of cells with chromosomes at the length necessary to evaluate the 15q11.2-q13 region. Furthermore, requiring the visibility of the 15q11.2 band drives the quality of the study to a band level more likely to elucidate other microdeletion syndromes.

**Genetic Counseling Issues and Recurrence Risk**

The recurrence risk figures associated with PWS and AS are discussed in chapter 4 of this thesis. An empiric recurrence risk figure of 1% is estimated for PWS or AS cases due to a deletion or UPD and is thought to be notably higher in cases in which the parent is a carrier of a translocation (Bray and Wilson 1986; American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996). The 1% figure for cases, including those due to UPD, would appear to be reasonable. It is in agreement with the study by Kalousek et al. (1989) who demonstrated that 2% of specimens from chorionic villus samples showed confined placental mosaicism when

placentae were examined after delivery. Some of these were shown to involve chromosome 15 and might be expected to have UPD for chromosome 15.

## PARENTAL ORIGIN STUDIES OF CHROMOSOME 15 IN PWS AND AS

The parent-specific origin of PWS and AS has remained a consistent finding not only in the chromosome 15 parental origin studies of 4 PWS and 18 AS deletion patients that were presented in this thesis, but also in all reported deletion cases in the literature (Butler and Palmer 1983; Magenis et al. 1990; Butler 1995, 1996).

### Uniparental Disomy of chromosome 15 in PWS and AS

In the studies contained within this thesis, I demonstrated 6 cases (11.8%) of UPD in our PWS population of 51 patients. This value does not significantly differ from the reported frequency of approximately 20-30% ($p>0.05$). The frequency of UPD may be actually higher in the OHSU population however, since not all patients with normal cytogenetic findings were tested for chromosome parental origin. Two PWS patients did not show a deletion by cytogenetics and FISH, and had biparental inheritance of their chromosome 15 homologs. DNA samples had not yet been obtained on these patients at the closure of this study, so 5' SNRPN methylation studies were not performed. It is, therefore, uncertain whether these patients represent the approximate 2% of PWS patients who have normal biparental inheritance of chromosome 15 but who have an imprinting error demonstrable by an abnormal methylation pattern by Southern blotting analysis.

In the cohort of AS patients, 1 possible UPD case and 1 or possibly 2 cases of AS due to an imprinting abnormality were observed. It was not

possible to distinguish UPD from an imprinting error in one AS patient since parental DNA samples were unavailable. In an AS cohort of 37 patients, this number is reasonable since UPD and imprinting abnormalities are thought to represent less than 10% of AS patients. Due to the imprinted nature of the 15q11.2-q13, abnormalities of epigenetic gene regulation occur. The abnormal outcome of UPD is the evidence of the unusual, parent-specific regulation of this region. Chromosomes for which no imprinting has been shown, such as 13, 18, and 21, (Ledbetter and Engel 1995) do not produce an abnormal clinical phenotype when disomy status is uniparental.

**Nondisjunction, Trisomy 15 and UPD**

The proposed model of an initial nondisjunction event, followed by a trisomy 15 and then the subsequent "rescue" of the fetus by loss of one of the chromosome 15 homologs by Kalousek et al. (1989), has been discussed previously in this thesis. In keeping with this model, the higher frequency of UPD in the PWS versus AS group observed in my study ,as well as in others, most likely represents the fact that approximately 95% all nondisjunction is maternal in origin (Antonarakis 1991) and 77% are due to a meiosis I error (Antonarakis et al. 1992). The model of initial trisomy followed by chromosome loss implies that there is somatic loss, but that it is not parent-specific. The clinically normal outcome in individuals that occurs when the chromosome 15 lost leads to biparental origin of chromosome 15 are not ascertained. By bias of ascertainment, only the abnormal cases are obtained, showing loss of a specific parental homolog. This model, which evidence suggests is valid, does not address the question of what causes the initial nondisjunction event, that in some cases results in UPD.

## MECHANISMS OF NONDISJUNCTION

**Chapter 2**

The study I presented in chapter 2 sought to explore the mechanisms of recombination and segregation in chromosome structural rearrangements that lead to UPD. Mechanisms proposed as possible contributing factors to nondisjunction have been presented in the Introduction section of this thesis and include advanced maternal age attributed to maternal errors at the first meiotic division (Magenis and Chamberlin 1981; Polani 1981), chromosome asynapsis or desynapsis implying a relative deficiency of chiasmata, a reduction in the number and/or alteration in the distribution of chiasmata or the failure of these structures to resolve in a timely fashion (Darlington 1929; Carpenter 1994; Sherman et al. 1994). Alterations in number and distribution of recombination events have also been proposed as an underlying cause of nondisjunction (Antonarkis et al. 1986; Warren et al. 1987; Morton et al. 1990; Hassold et al. 1991; Sherman et al. 1991; Robinson et al. 1993a). Structural rearrangements, particularly Robertsonian translocations, have been associated with an increase in nondisjunction as well (Hamerton 1971; Miller et al. 1981). Whether the observed nondisjunction occurs due to a specific effect of the translocation on segregation or merely by chance as one of the possible segregation products is not clear. While it was substantiated by Brown et al. (1994), that the a-centromere sequences are essential for mitotic segregation, the effect of pericentromeric sequences on normal segregation and nondisjunction has not been well studied.

Therefore, two PWS patients with rearrangements of chromosome 14 and 15 plus UPD were studied in depth at the cytogenetic and molecular levels to determine the essential chromosome material or genetic

mechanism that might be responsible for their UPD. Specifically, the aspects of these two patients that were evaluated as possible contributing factors to nondisjunction were: changes in placement of pericentromeric chromatin due to chromosomal structural changes and recombination.

By HRCB, special staining with Q-and R-banding and distamycin/DAPI used in conjunction with FISH probes: D15S11, SNRPN, D15S10, GABRB3, I was able to show that, in both cases, changes in the placement of pericentromeric chromatin had occurred. Patient 1 had a *de novo* translocation also involving chromosomes 14 and 15 satellite regions. In addition this patient and her mother had a translocation of distamycin/DAPI (chromosome 15 classical satellite) positive chromosome 15-specific pericentromeric material to the chromosome 14 homolog involved in the translocation. Patient 2 had a Robertsonian translocation involving chromosomes 14 and 15 with fusion of the satellite region. This placed the centromeres, the proper identity of which is in some fashion essential for spindle attachment and proper meiotic segregation, of these two chromosomes in close proximity. These two patients were similar in that pericentromeric changes had occurred to alter chromatin near the area of spindle attachment.

Further examination of the centromeric chromatin by FISH of centromeric probes in these patients showed normal placement of chromosome 15 centromeric a-satellite material. The α-satellite sequences are thought to be among the most basic or critical genetic material responsible for proper kinetochore formation and chromosome segregation (Willard 1990; Brown et al. 1994). Of note, however, in the case of the Robertsonian chromosome, is that while normal chromosome

15 α-satellite material was present, the chromosome 14 centromere was also present.

The microsatellite results demonstrated that each patient in this study had both maternal isodisomy and heterodisomy at various chromosome 15 loci, the different allelic origins apparently being brought about by recombination. Whether patients 1 or 2 had a reduced or increased amount of recombination is difficult to surmise, since only a limited number of loci were examined. What these data were able to show, however, was that pairing had taken place and that the nondisjunction was not due to asynapsis.

More germane may be whether the observed recombination, in combination with the observed chromosome rearrangements, contributed to the nondisjunction event. It has been suggested for some time that an increase in nondisjunction is associated with chromosome structural rearrangements including Robertsonian translocations (Hamerton 1971; Cattanach and Moseley 1973; Miller 1981). The examination of oocytes from mice that were heterozygous for multiple Robertsonian translocations indicated that disturbances in chromosome orientation and spindle structure, rather than a failure in pairing and crossing-over between homologous chromosome arms, was the major cause of nondisjunction in those cells (Eichenlaub-Ritter et al. 1990) .

I suggest that a similar phenomenon occurred in these patients. The structural changes in the chromosomes found in patients 1 and 2 contributed to the nondisjunction and uniparental disomy responsible for their PWS phenotype. The proper orientation of structurally rearranged chromosomes and their centromeres at metaphase I may have been impossible due to abnormal pairing of these structurally altered

chromosomes. In addition, it may be that as meiotic spindle fibers attempted to attach to the kinetochores, nucleated by chromosome specific α–satellite sites, the pericentromeric rearrangements in these two cases posed problems with spindle site recognition or attachment due to physical hindrance. If proper meiotic segregation is dependent on timely resolution of chiasmata and/or the proper binding of chromosome-specific spindle fibers to centromeres, then the pericentromeric rearrangements observed in these two patients may have disrupted normal disjunction and led to uniparental disomy.

There is no direct evidence from this study that the karyotypes of these patients were initially trisomic for chromosome 15 material with subsequent loss of the normal paternal chromosome 15. PCR analysis and parent-specific DNA methylation studies failed to detect any paternal chromosome 15 contribution in ME's or DK's blood. However, these results would be expected if the trisomy were confined to the placenta. No placental tissue was available since chromosome analysis was initiated past the newborn age in both of these patients.

The interpretation of these results from this study are limited by the number of patients examined. Delineation of the essential chromosome regions necessary for proper chromosome segregation will require the study of a greater number of structurally rearranged chromosomes involved in cases of nondisjunction and/or UPD in order to determine the common sequences or structural changes of abnormal chromosomes.

**Robertsonian or reciprocal translocations and UPD**

Of relevance to note is that not all patients with UPD are associated with a structural rearrangement, nor is UPD the outcome in all individuals who receive a translocation. While the incidence of UPD in

Robertsonian and reciprocal translocation carriers is unknown, a case reported by myself and those reported by Ledbetter and Engel (1995) suggest that carriers of Robertsonian and reciprocal translocations are at a somewhat increased risk for nondisjunction.

## UPD AND IMPRINTING ABNORMALITIES IN AS

The high frequency of UPD that is observed in PWS, which is addressed and discussed in chapter 2, was not found in the OHSU cohort of AS patients. Paternal UPD in association with AS represents less than 5% of the total reported AS cases. This is presumably attributable to the origin of the error in this syndrome. Since most nondisjunction errors are maternal in origin and occur in meiosis I (as discussed above), maternal heterodisomy resulting in PWS is more likely to occur, and this is in fact the case. The majority of paternal disomy cases in AS, in contrast, show complete isodisomy, suggesting a monosomy 15 conception followed by duplication of a single paternal chromosome (Mutirangura et al. 1993b; Ledbetter and Engel 1995).

The discrepancy in the frequency of etiologic mechanisms responsible for PWS and AS is found in the group of patients with normal appearing chromosomes. In both syndromes, literature data suggest that approximately 20-40% of patients will not show a deletion of 15q11.2-q13. However, while within the PWS group without deletion the overriding majority of patients will demonstrate maternal UPD, but within the AS group less than 5% will demonstrate paternal UPD and in the remaining 15-35% no genetic defect is detectable. The figures ascertained in the OHSU study did not significantly differ from these values ($p>0.05$).

**Chapter 3**

Two AS patients were selected from this group of AS patients in the OHSU cohort that were shown to be normal by cytogenetic analysis. They were of particular interest since (1) they were maternal half-siblings with a maternally inherited syndrome and (2) haplotype analysis was possible since they were from a relatively large family that had two normal children as well.

Patients CD and her half-brother JT, both had many of the physical characteristics that are universally found in AS, as well as many of the behavioral findings frequently observed or associated with AS.

The HRCB analysis and molecular cytogenetics using dual color FISH of proximal 15q probes failed to demonstrate a deletion or an inversion of material within the 15q11.2-q13 region.

When I began this study, the reports of unique AS patients and families, which had molecular deletions, had putatively placed the AS critical region distal to SNRPN and proximal to GABRB3 (Wagstaff et al. 1992, 1993; Reis et al. 1993; Greger et al. 1994). Most recently, a report by Buxton et al (1994) had indicated loss of genetic material at the D15S113 locus in a patient with AS. The AS patients, CD and JT, presented in this study appeared to have the same loss of the maternally inherited genetic material at this locus. Their mother demonstrated loss of the paternal allele at this locus. This result was achieved by using commercial primers, 113F-113R for a (CA)n repeat at the D15S113 locus (Research Genetics). The apparent loss of a parental allele, however, actually represented a failure of an allele to amplify. This conclusion was supported by the successful amplification of both parental alleles of this same $(CA)_n$ repeat at the D15S113 locus, using alternative primers, that gave a heterozygous result in patient JT, his mother, one maternal aunt and maternal grandfather. One maternal aunt were

homozygous. CD appeared to be homozygous at this locus, but since PCR is not dosage sensitive, hemizygosity cannot be ruled out. Genotypes inconsistent with a deletion were observed with the amplification of a flanking (ATTT)n repeat at D15S113 and a (CTTT)n repeat at D15S1234. The apparent failure to consistently amplify an allele in this family was presumably due to a base pair change at the D15S113 (113F-113R) primer annealing site. Due to this change is it possible that the primers were prevented from annealing and, thus, could not amplify.

Upon personal communication with Dr. S. Malcolm, the director of the laboratory from which the Buxton et al. (1994) paper was reported that demonstrated loss of the maternal D15S113 allele, she indicated that she believes that the results obtained in their paper to be erroneous.

In order to assess the validity of the commercially obtained primers at the D15S113 locus, 50 random DNA samples were amplified by these primers. Three samples failed to amplify, but by repeat PCR, amplification of DNA was achieved in two of these. The heterozygosity of these primers is reported by Research Genetics to be .73, meaning 73 of 100 samples will demonstrate heterozygous alleles. The results of this study were as follows: 32 of 50 patients were heterozygous; 17 were homozygous. The results obtained did not significantly differ from those reported by Research Genetics. However, it should be noted that the amplification by these primers demonstrated notable shadow banding. It was not always easy to discern homozygous results from heterozygous results. Also, since these were random samples, it was unclear how often a homozygous result was actually a failure of an allele to amplify. This could not be substantiated because these were random samples, for which other family members were not tested. Therefore, while the heterozygosity value that I obtained was consistent with the value reported by

the vendor, my personal experience with these primers suggests that they are unreliable and alternative primers should be used to amplify DNA at the D15S113 locus.

## IMPRINTING ABNORMALITIES IN AS

Approximately 20-40% of AS patients show biparental inheritance of chromosome 15 homologs and normal imprinting in the 15q11.2-q13 region (Nicholls 1993), just as did patients CD and JT. Within this population, it had been observed, however, that 2-4% of patients have an imprinting defect. demonstrating an abnormal methylation pattern at the D15S9, D15S63 and SNRPN loci. The pattern is solely paternal in origin. In two reported AS patients, shown to have an abnormal paternal methylation pattern, a deletion between D15S63 and SNRPN has been demonstrated (Buiting et al. 1995) The deletions apparently prevent proper resetting of the methylation pattern in the next generation.

The possibility of an imprinting defect was studied in patients CD and JT, as well as in some family members, by examining the parent-specific methylation pattern at the 5' SNRPN gene. Examination of the differential methylation by quantitative Southern blotting will show a maternal 4.3 kb fragment and a paternal 0.9 kb fragment in a normal control. The result of this assay in AS, in cases due to a deletion, UPD or an imprinting defect, will show loss of the maternal 4.3 kb fragment and the presence of a single paternal 0.9 kb fragment. All family members that were studied, as well as patients CD and JT, demonstrated the presence of both the 4.3 kb maternal and 0.9 kb paternal fragment of the 5'-SNRPN gene.

It is my opinion that the normal result obtained by Southern blotting analysis did not rule out a clinical diagnosis of AS in these two patients. As was demonstrated by the OHSU PWS/AS study, this test is an excellent diagnostic test for the Prader-Willi syndrome, detecting virtually 100 percent of cases, regardless of whether the etiology is due to a deletion, UPD or an imprinting abnormality. In my study of 16 clinically diagnosed AS cases, the 5' SNRPN methylation assay was able to positively diagnose 12 patients. Given that the location and mechanism of the imprinted gene(s) responsible for AS is as yet unknown, it is not prudent to rely solely on Southern blotting analysis of the imprinting of 5' SNRPN as a diagnostic test for AS.

Microsatellite analysis demonstrated that CD and JT shared the same maternal alleles at the D15S11 locus and the proximal region of the D15S113 locus that included the $(CA)_n$ repeat. These patients did not share the same maternal alleles at the distal region of D15S113 locus containing the $(ATTT)_n$ repeat nor did they share the same maternal alleles at the D15S1234 locus, indicating that the mutation shared by these siblings must be proximal to the $(ATTT)_n$ repeat at the D15S113 locus. The molecular analyses employed in this study did not rule out the possibility of a genetic defect in this family. Further molecular investigation proximal to the (ATTT)n repeat at the D15S113 locus may reveal the molecular defect in this family.

In conclusion, the clinical and scientific data obtained from this study do not argue against CD and JT having AS syndrome due to a yet unidentified molecular defect. While this study scrutinized various loci within the region of 15q11.2-q13, it is limited in its scope. The cytogenetic and molecular results of patients CD and JT, reported in this study, were

limited by the number and choice of probes and primers (as outlined in the text) that were used in the FISH and PCR experiments. In the absence of a definitive genetic defect, specific for AS, a different diagnoses for these patients must be considered.

As discussed above, the clinical diagnosis of AS is difficult. Patient CD was independently examined by a clinical geneticist from the University of Florida, an institution where there is a lot of interest and expertise with AS. It was the opinion of this geneticist, as well, that patient CD had AS. Since the gene(s) that when defective are responsible for AS are not yet known, it is my opinion that the clinical diagnosis should take precedence over the normal laboratory findings and that efforts should continue to search for an AS related defect.

## REDEFINING THE AS CRITICAL REGION

Currently the smallest region of deletion overlap in AS has its proximal boundary at the SNRPN gene and distal boundary at the distal end of the D15S113 locus, as cited above. A recent report of an AS patient with an unbalanced translocation of chromosomes 14 and 15, with subsequent loss of the proximal 15q material proximal to the D15S10 locus (Burke et al. 1996) would suggest that the distal boundary is actually proximal to the D15S113 locus. The majority of AS patients with no cytogenetic deletion, however, have not been examined by molecular means for molecular abnormalities in the region between SNRPN and D15S113. It is this group of patients who may pinpoint the actual genetic abnormality responsible for AS.

Future studies in the quest to elucidate the gene(s) responsible for AS necessarily include a complete molecular analysis of this region. This

is quite a large region between D15S113 and SNRPN, approximately just less than 1 Mb in length (Mutirangura et al. 1993). In order to narrow down the candidate area, one might initially begin with exon trapping experiments to search for potential genes in the vicinity of CpG rich islands in this region. Putative exons could then be used to screen cDNA libraries. Isolated cDNA clones would then be mapped back to normal metaphase chromosomes by FISH in order to confirm that they were from the 15q11.2-q13 region. This procedure could then be followed by FISH of the clones to chromosomes of AS patients with known deletions. Loss of the clone in AS patients would demonstrate relevance but not confirm that the clone is the actual AS gene. Confirmation of the clone as the AS gene could be demonstrated by loss of expression of the clone by Northern blotting analysis in AS versus control patients. No expression of the clone in a hemizygous AS patient would be consistent with an imprinted, maternally expressed gene. However, since this is an imprinted gene, the examination of multiple tissue types might be required to detect expression. If a cDNA were identified, the detection of abnormalities in AS patients without deletions would then be investigated by restriction digests and Southern blotting with this clone. In addition, subtle mutations such as point mutations or the insertion/deletion of a small number of nucleotides could be identified by heteroduplex analysis of PCR products of AS patients.

**Trinucleotide Expansion Syndromes**

The failure to identify the genetic abnormality in approximately 15 to 35% of AS patients suggests that in addition to the study direction above, alternative and/or additional genetic mechanisms responsible for AS need to be explored. Given that AS is a neurogenetic disorder, it is

possible that the expansion of an unstable trinucleotide repeat is responsible for the clinical manifestations observed within this group.

There are several neurogenetic disorders known to be caused by the expansion of trinucleotide repeats within the disease gene. In disorders such as Huntington, Muscular dystrophy, Spinocerebellar ataxia 1 and 3, Spinobulbar muscular atrophy, Fragile X and E, Dentatorubral and Pallidoluysian atrophy, an increase in the number of tandemly-repeated trinucleotides may inactivate the gene or cause a gain of function (Müller et al. 1994). The genetic defect stems from an expansion of the repeat beyond the upper limit of the normal size range, with larger expansions associated with increasing severity and earlier age of onset. The severity of the congenital manifestations of AS may represent an extremely early age of onset in a disorder caused by the expansion of a trinucleotide repeat.

A trinucleotide repeat expansion could cause AS by various means. The presence of an expanded trinucleotide repeat may disrupt gene expression by causing the aberrant methylation of an adjacent CpG island. In the case of myotonic dystrophy, the expansion of a $(CTG)_n$ repeat in the 3'-untranslated region of the myotonin protein kinase gene (DMK) is thought to interfere with the expression of adjacent genes by causing the methylation of a downstream CpG island (Boucher et al. 1995). The IC region of proximal chromosome 15q is a region that also contains at least one CpG island that is methylated in a parent specific fashion. Abnormal trinucleotide expansion within this area could disrupt regional imprinting and/or transcription of the PW71 locus and SNRPN gene. Such an abnormality would have far reaching parent-specific and tissue-specific effects.

Alternatively, the chromosomal instability and the presence of a deletion hot spot seen in AS may be due to trinucleotide expansion. For example in Fragile X syndrome loss of activity of the FMR1 gene is in some cases caused by both expansion and deletion of the FMR1 CGG repeat (Quan et al. 1995). These deletions of the FMR1 gene, which involve an upstream deletion hot spot, may be related to the expansion of the repeat, occurring at an early stage of development from an expanded allele or during the expansion event itself. It is possible, then, that the expansion of a trinucleotide repeat could be associated with the deletions seen in AS.

Since a large portion of the 15q11.2-13 region is not fully characterized at the molecular level, and there is no report of a concerted effort to identify polymorphic, potentially unstable trinucleotide repeats in this region, such repeats may well be found in various loci , associated with any number of genes within this region and show involvement in the etiology of AS.

## A SYSTEMATIC APPROACH TO THE DIAGNOSIS OF PWS AND AS

**Chapter 4**

The data, presented in chapter 4 of this thesis supported the expectation that the majority of PWS and AS patients would show a chromosome deletion (Nicholls 1993). As an initial test, high resolution chromosomes banding offers a global, as well as a specific examination of the primary cause of these syndromes. FISH analysis, done in conjunction with standard cytogenetics was shown to be a logical approach to establishing a microdeletion. The results of these studies demonstrated that quantitative Southern blotting analysis of the 5'-SNRPN methylation parent-specific pattern is a powerful diagnostic tool, capable of accurately

diagnosing PWS in all cases studied, regardless of etiology. Cytogenetic techniques, however, are required to detect structural rearrangements and address issues of recurrence risks associated with cases of PWS and AS. In addition, microsatellite analysis by PCR was necessary to differentiate an imprinting error from UPD.

The protocol set forth in chapter 4 as a systematic approach to the diagnosis of PWS and AS begins initially with cytogenetic HRCB and FISH of chromosome 15 proximal probes analysis. The rationale for this initial step is based on studies in the literature that demonstrate that the overwhelming number of patients referred for PWS do not have PWS (Gillessen-Kaesbach et al. 1995). Furthermore since approximately 40 % of cases of mental retardation are due to a chromosome abnormality (Connor and Ferguson-Smith 1984), karyotype analysis, as an initial PWS and AS test, will not only detect approximately 60-80% of PWS and AS patients, it will also provide an automatic global view of any chromosome abnormality in the event that the patient does not demonstrate a chromosome 15 deletion. Patients for whom the referring physician feels confident of the diagnosis and for whom cytogenetic and FISH results are normal, will necessarily need 5' SNRPN methylation analysis by quantitative Southern blotting in order to rule out UPD of chromosome 15. Imprinting abnormalities will need to be detected by PCR of chromosome 15-specific microsatellites.

The protocol set forth does not presume to infer that only one approach is correct in all cases. When testing is initiated by DNA methylation analysis of the 5' SNRPN gene, the referring clinician should be made aware, however, that follow-up karyotype analysis is necessary to establish recurrence risks of PWS, AS, and potentially other chromosome

syndromes, within the family. As has been discussed, recurrence risks differ depending on the etiology of the syndrome.

**Structural rearrangements of 15q11.2-q13**

As I have indicated in this thesis, there are multiple case reports involving chromosome 15 structural rearrangements. The inherent instability of 15q11.2-q13 that so frequently involves this region in deletions, duplication, inversions, translocations and the formation of extra bisatellited chromosomes has yet to be resolved. The presence of inverted repeat elements related to the Alu family have been demonstrated at the IR10 locus, near the distal PWS/AS critical region common breakpoint (Donlon et al. 1986). It has been proposed that a deletion of 15q11.2 may result from unequal sister-chromatid exchange or simple looping out of DNA mediated by direct or inverted repeats. With regard to the observed telomeric translocations (Reeve et al. 1993), no telomeric-related sequences have been demonstrated in this region that might explain this unusual finding. In agreement with this finding, extra bisatellited chromosomes, which are formed presumably by breakage and fusion, behave as though no telomeric sequences are present.

While the promised results of gene therapy for congenital structural abnormalities are clearly in the distant future, it will be imperative in any future attempts to insert DNA sequences within this region to develop an understanding of the mechanisms which promote, or alternatively, passively allow, the mobility of DNA sequences within this region.

## THE CHROMOSOME 15Q11.2-13 IMPRINTED DOMAIN

The 15q11.2-q13 region of the genome has clearly been established as an imprinted region, as cited above. The evidence includes the parent-of-

origin specific: expression of PWS and AS syndromes, replication of alleles, the SNRPN gene, mRNA transcription of the IPW gene, as well as parent-specific methylation at the D15S9 (ZNF127), D15S63 (PW71), and SNRPN loci.

Buiting and colleagues (1995) have suggested that there is an imprinting domain that acts in *cis* throughout the 15q11.2-q13 region. This region is just proximal to SNRPN and therefore is not in the currently defined AS critical region. These data suggest an additional genomic location within the 15q11.2-q13 region, that when defective, results in AS. Perhaps the actual imprinted genes responsible for AS are between SNRPN and D15S113 and a defect in these genes can result in AS. Alternatively, AS may also result when there is a deletion or mutation in the imprinting domain such that the regulation of the distal AS genes is adversely affected. Deletions in the imprinting domain have been shown to alter the methylation pattern of adjacent parent-specific methylation loci (Buiting et al. 1995). This suggests that conformational changes due to a deletion within the imprinting domain may alter the regulation of adjacent genes, including genes further distal within the AS critical region. The vast majority of AS patients have not been molecularly examined for an abnormality within the imprinting domain. Cloned fragments are available from this region, and it seems reasonable to screen all AS patients, for which no defect has yet been determined, initially with the clones from the smallest region of deletion overlap within the imprinting domain.

**Maintenance of the parent-specific imprint**

It is initially difficult to imagine how an imprint may be set in parent-specific fashion and then reset through successive generations.

However, if one synthesizes from various genetic fields and is allowed a bit of speculation, a working theory can be developed. Chromosomes do not appear to move about the nucleus in freely and randomly. Rather they appear to be tethered to the nuclear scaffolding at nuclear scaffold-associated regions (SARs), also known as nuclear matrix attachment regions (MARs) (Grunstein 1990). SARs have been implicated in mediating the formation of the base of chromosomal loops, the binding of topoisomerase and chromatin unfolding, all events being necessary precursors to gene transcription. It may be that within the nuclei of different tissue-specific cells, that through the mediation of SARs, different regions of chromosomes attach to the nuclear matrix. The attachment sites may be different in oocytes versus sperm cells. If the attachment sites define the conformation of a particular chromosome domain within the cell, then different attachment sites within different cells would lead to parent-specific chromosome domains.

The studies presented by Kafri et al. (1992) imply that many of the sites that are methylated in adult somatic tissue are not methylated in the germ cells, with the sperm cell showing more methylation than the oocyte. This groups suggests that this may be due to loss of a methylating enzyme activity. In any event, when the sperm and oocyte join, they show different levels of sites of methylation than somatic tissues. After zygote formation and some time in the blastula stage, methylation of DNA is initiated. 5' CpG islands of housekeeping and tissue-specific inactive genes remain unmethylated. Studies have not specifically looked at the timing of gene imprinting. However, the timing of methylation of nonimprinted genes would suggest that it occurs during this time of embryonic development as well. I suggest that it may be that the putative

parent-specific chromosome conformations defined in the germ cells that are continued in the zygote and that, for imprinted regions, the conformation in the oocytes compliments the conformation in the sperm cell. Furthermore, it is the conformation of the chromosome domain that determines whether methylating enzymes have access to the region. This model, in which the cell type determines the chromatin organization in the nucleus, could be carried over to explain tissue-specific methylation as well.

## EVIDENCE OF IMPRINTING IN HYDATIDIFORM MOLES AND OVARIAN TERATOMAS

Studies reported in the literature which have used hydatidiform moles and ovarian teratomas to observe parent-specific expression of imprinted genes have been presented in this thesis (Mutter et al. 1993; Mowery-Rushton et al. 1996; Glenn et al. 1996). The results reported by these studies were different from what the authors expected. The parent-specific imprinting of genes was not maintained in these tissues. I do not find this surprising. And while I understand the choice to use these tissues because they have only maternal or paternal genetic contribution, I believe that an important aspect of cell regulation has been missed when interpreting the results of these studies. The authors expected that the ovarian teratoma, an all maternal tissue, would maintain a maternal imprint and that the hydatidiform mole, an all paternal tissue, would maintain a paternal imprint. I suggest that it was precisely because the genetic contribution was all of one gender that the genes could not maintain their imprint. This is in support of the model I have presented

above. I propose that without the defined male and female chromatin conformation and complementarity, proper imprinting was not possible.

## THEORIES FOR GENOMIC IMPRINTING

The genomic imprinting of the 15q11.2-q13 region remains the most enigmatic aspects of studying this chromosome. This is not the only imprinted region of the human genome and various additional chromosome regions, beyond those that are currently recognized, are certain to be identified. Imprinting, however, is not a generalized method of regulating gene expression throughout the genome. Several theories have been proposed to explain what the purpose of imprinting might be.

It has been suggested that imprinting evolved in mammals to restrain the proliferative growth of the placenta (Hall 1990). This limitation is necessary to ensure that the mother survives the pregnancy and is capable of future pregnancies.

A second theory suggests that DNA methylation is the genomic imprint (Barlow 1993). In this scenario, imprinting evolved as an extension of the host defense role that DNA methylation plays in bacteria against invading organisms. Imprinting would be the basis of an immune response.

Finally, it has been suggested that imprinting ensures diversity (Hall 1990). By having certain genes that are vital for the survival of the embryo expressed in a parent-specific manner, sexual reproduction becomes a requirement of the species. Since humans are heterozygous for several recessive lethal alleles, parthenogenic reproduction would reduce genetic diversity and increase the risk of homozygosity for deleterious genes, leading to an increase in the expression of recessive disorders.

While these theories are merely that, I believe that the rationale of imprinting, presented here as the first and third theories, are substantiated by several findings. With regard to the first theory, results of cytogenetic and histologic studies of ovarian teratomas and hydatidiform moles support this reasoning. As has been previously briefly discussed in this thesis, the hydatidiform mole in which all chromosomes are paternal in origin can be described as a collection of fluid-filled cysts derived from the chorionic villi of the placenta. It is all extraembryonic membrane. Ovarian teratomas are embryonic tumors which develop from a female germ cell. They are maternal in origin and contain no extraembryonic tissue. The implication is that paternally derived genes are important for the development of the extraembryonic tissues and the maternally derived genes are important for the development of the embryo proper. These studies as well as the pronuclei transplantation studies (McGrath and Solter 1986) do suggest that contribution of both the maternal and paternal genomes are necessary for normal embryonic development.

The third theory of genomic imprinting suggesting that organisms are protected from the deleterious effects of lethal homozygosity has been demonstrated in nature. Malsegregation due to a nondisjunction event can in fact lead to UPD and the subsequent expression of a recessive disorder, as in the case of the PWS patient with Bloom syndrome (Woodage et al 1994).

This second theory, by Barlow (1993), is more difficult for me to appreciate. It does not address the criteria for parent-specific imprinting of alleles, a crucial aspect of the imprinting process. Nor do I see that imprinting as host-defense role is in keeping with mechanisms commonly utilized by human immunological systems.

Imprinting may be a way of regulating dose effect. Parent-specific regulation would be a method for insuring that only one gene is expressed.

## FUTURE AREAS OF RESEARCH IN PWS AND AS

My experience with many of the PWS patients and families who have been a part of the OHSU study cohort, either through clinic visits, scientific conferences or the OHSU PWS support group, suggests that the area of research most important to these families is that which will modify certain behaviors that are characteristic of these syndromes. These behaviors, which include hyperphagia, obesity and aggressive outbursts, are life-threatening to the patient and extremely disruptive to the family. I believe that it is the responsibility of the scientific community to respond to the requests put forth by these patients and their families.

The biochemical defect that induces the morphological and behavioral abnormalities in these patients is completely unknown. It is recognized, however, that many of the physical and behavioral abnormalities associated with PWS are abnormalities of hypothalamic functioning. If the SNRPN gene, which is at present the primary candidate gene for PWS (Özçelik et al. 1992), exerts effect on this phenotype it may very well be in this region of the brain. Paternal-specific expression of the SNRPN gene has been observed in the brain(McAllister et al 1988; Schmauss et al. 1990), but its role in this specific tissue has not yet been established. It is, therefore, reasonable to suggest that the direction of studies move towards the establishment of what, if any, baseline normal expression of this gene occurs in the hypothalamus. Since the SNRPN gene codes for a protein associated with a spliceosome unit (McAllister et

al. 1988), abnormal functioning due to loss of the expression of this gene may result in abnormal splicing of mRNA and/or premature digestion of mRNA molecules. In vitro RT-PCR of mRNA from cells of the hypothalamus of PWS patients, when compared to normal hypothalamic tissue, may explain if and how the genetic abnormality in the SNRPN gene is manifesting in this tissue type.

The 5' end of the SNRPN gene has now been shown to have more than one transcription start site (Glenn et al. 1996). Future studies, which focus on normal transcriptional differences from this gene may elucidate the tissue specificity of the SNRPN gene expression. This information may serve to pinpoint targeted tissues that are affected by the genetic abnormality and in which abnormal biochemical responses occur. In keeping with this same direction of study, PWS patients, for which it has been demonstrated that only the promoter region of the SNRPN gene is lost, need to be molecularly re-evaluated given this new data. These patients may show loss of only one transcription site and indicate a finer definition of the different tissue specific expression of this gene.

I suggest further investigation into the model which suggests that there is an imprinting center, inclusive of the parent-specific sites of methylation in the 15q11.2-q13 region, at which regulatory mechanisms function in *cis* fashion to coordinate the parent-specific gene regulation of this region. Research of this model may provide valuable insights into genetic regulation of imprinting, as well as the genetic abnormalities of these syndromes. I suggest that it is plausible that there is actually physical overlap of the PWS/AS critical region and that it may very well be in the putative imprinting center. Transcription regulation is in this region. Not only is it parent-of-origin specific, but it regulates the expression of a

gene which encodes for a protein that is part of the cellular machinery that could have tremendous influence not only on ultimately what proteins are made, but also in what tissues. I is conceivable that different mutations within the same imprinting center, that are parent-of-origin specific, might alter a completely different cascade of biochemical pathways and produce a completely different phenotype.

In addition to exploring the imprinting center with regard to the etiology of AS, I have previously suggested a finer molecular search for mutations in the 15q11.2-q13 region distal to SNRPN and proximal to GABRB3. It may be that AS is produced by abnormalities of as yet unmapped genes within this region. Consistent with known methods for disrupting gene expression, it may be that AS is produced by abnormalities within structural genes as well as by disruption of the regulatory regions.

Since AS is a neurogenetic disorder, I have offered that the basis of the genetic abnormality may be an abnormal trinucleotide repeat expansion. Therefore, further investigation into the possibility of this proposed etiologic mechanism requires an initial systematic screen for the presence and, then range limit, of normal trinucleotide repeat length throughout the 15q11.2-q13 region, followed by analysis in AS patients.

## CONCLUDING THOUGHTS

This study of the proximal region of chromosome 15 has brought about far more interesting questions than the simple answers that I accumulated. Through the synthesis of the experimental results contained in this thesis, I was able to provide a systematic and comprehensive approach to both prenatal and postnatal diagnosis of PWS and AS. I completed a study which compared the cytogenetic results of

PWS and AS patients with the Southern blotting results of 5'SNRPN methylation assay. This type of study had not yet been performed in PWS and AS, thus, the results that I obtained provided data concerning the accuracy of this molecular test in these syndromes. In addition, I provided further evidence for the involvement of structural rearrangements in nondisjunction in PWS and AS. Through the studies of AS patients I have developed a new set of reliable primers for characterization of a region of 15q11.2-q13 implicated in AS.

My scientific research of chromosome 15 has given me a far greater respect and sense of awe for the complexity of the genetic mechanisms that regulate the genes located within the 15q11.2-q13 region. This region of the genome is affected by the rules of classical Mendelian genetics of assortment and segregation but also has a rigid set of imprinting laws that, unless rigorously followed, preclude normal gene expression and embryonic development.

**APPENDIX A**
Holm et al. (1993)

DIAGNOSTIC CRITERIA FOR PRADER-WILLI SYNDROME
REVISED SCORING SYSTEM, JUNE 1991

Diagnostic criteria for Prader-Willi syndrome have been developed in two sets. The first set pertains to patients who are within the ages 0-36 months and the second set is relevant for patients who are within the ages of 3 years to adulthood. The examiner selects the age-appropriate list of criteria, completes the questioner and then records the diagnostic score, using the system given at the end of the respective criteria list.

PATIENT IDENTIFICATION :_____SEX :__AGE:__yr__mo

DATA SOURCE: Patient examined on _____ (date)
[ ] History from parent
[ ] Other historians (specify)

IMPRESSION:
[ ] Typical Prader-Willi syndrome
[ ] Not Prader-Willi syndrome
[ ] Atypical Prader-Willi syndrome
[ ] Unsure

SCORE
[ ] **0-36 months** TOTAL SCORE___
FROM MAJOR GROUP:___
[ ] **3 years to adulthood** TOTAL SCORE___
FROM MAJOR GROUP:___

COMMENTS (please explain your reasons for putting a patient in the atypical or unsure category):

PRADER-WILLI SYNDROME DIAGNOSTIC CRITERIA

Age 0 to 36 months

**MAJOR CRITERIA:**

| | | | | |
|---|---|---|---|---|
| 1. | Neonatal and infantile central hypotonia with poor suck and lethargy, gradually improving with age | yes | no | uk |
| 2. | Feeding problems with need for special feeding techniques and poor weight gain/failure to thrive. | yes | no | uk |
| 3. | Characteristic facies with dolichocephaly, almond-shaped eyes, narrow face, small appearing mouth, down-turned corners of mouth. | yes | no | uk |
| 4. | Genital hypoplasia: | | | |
| | Female: hypoplasia of labia minora and/or clitoris. | yes | no | uk |
| | Male: cryptorchidism, small penis, scrotal hypoplasia. | yes | no | uk |
| 5. | Global developmental delay. | yes | no | uk |
| 6. | Excessive or rapid weight gain on weight-for-length chart after 12 months (excessive is defined as crossing two centiles). | yes | no | uk |
| 7. | Deletion 15q11-13 on high resolution (>550 bands) or other cytogenetic/molecular abnormality of the Prader-Willi chromosome region (PWCR), including maternal disomy. | yes | no | uk |

**MINOR CRITERIA:**

| | | | | |
|---|---|---|---|---|
| 1. | Weak cry <6 months | yes | no | uk |
| 2. | Esotropia | yes | no | uk |
| 3. | Negative neuromuscular studies | yes | no | uk |

**SCORING:**

Item 1 to 6 are weighted at 1 point each. The presence of an abnormality in the PWCR (7) is weighted at 2 points. Minor criteria are scored at 1/2 point each. Six points are required for diagnosis.

**SUPPORTIVE FINDINGS** (increase the certainty of diagnosis):

| | | | | |
|---|---|---|---|---|
| 1. | Decreased fetal movement | yes | no | uk |
| 2. | Lethargy <6 months | yes | no | uk |
| 3. | Temperature instability | yes | no | uk |
| 4. | Scoliosis | yes | no | uk |

## PRADER-WILLI DIAGNOSTIC CRITERIA

### Age three years to adulthood

**MAJOR CRITERIA:**

| | | | | |
|---|---|---|---|---|
| 1. | History of neonatal central hypotonia and poor suck with later improvement. | yes | no | uk |
| 2. | History of feeding problems and poor weight gain | yes | no | uk |
| 3. | Obesity (primarily central) beginning between ages 1 and 6 without intervention. | yes | no | uk |
| 4. | Characteristic facial features-narrow bifrontal diameter, thin upper lip, down-turned mouth, almond-shaped eyes (>3). | yes | no | uk |
| 5. | Hypogonadism-with any of the following: | yes | no | uk |
| | (a) Genital hypoplasia: | | | |
| | Male: scrotal hypoplasia, cryptorchidism, small penis and testes for age (<5th percentile). | | | |
| | Female: hypoplasia of labia minora and/or clitoris. | | | |
| | (b) Incomplete pubertal development without intervention after 16 years of age. | | | |
| | Male: decreased facial and body hair, lack of voice change. | | | |
| | Female: amenorrhea/oligomenorrhea after age 16. | | | |
| 6. | Mild to moderate mental retardation or learning | yes | no | uk |
| 7. | Hyperphagia/food foraging/obsession | yes | no | uk |
| 8. | Deletion 15q11-13 on high resolution (>550 bands) or other cytogenetic/molecular abnormality of the Prader-Willi chromosome region (PWCR), including maternal disomy. | yes | no | uk |

**MINOR CRITERIA:**

| | | | | |
|---|---|---|---|---|
| 1. | History of decreased fetal movement or infantile lethargy or weak cry, improving with age. | yes | no | uk |
| 2. | Characteristic behavior problems-temper tantrums, violent outbursts, obsessive/compulsive behavior; tendency to be argumentative, oppositional, rigid, manipulative, possessive, stubborn; perseverates, steals, lies (>5 of these symptoms present). | yes | no | uk |
| 3. | Sleep disturbance or sleep apnea. | yes | no | uk |
| 4. | Short stature for genetic background by age 15 (without intervention). | yes | no | uk |
| 5. | Hypopigmentation-fair skin and hair compared to | yes | no | uk |
| 6. | Small hand (<25%) and/or feet (<10%) for height | yes | no | uk |
| 7. | Narrow hands with straight ulnar border. | yes | no | uk |
| 8. | Eye abnormalities (esotropia, myopia). | yes | no | uk |
| 9. | Thick viscous saliva with crusting at corners of the | yes | no | uk |
| 10. | Articulation defects. | yes | no | uk |
| 11. | Skin picking. | yes | no | uk |

**SCORING:**

Eight major criteria are weighted at 1 point each. Eleven minor criteria are weighted at 1/2 point each. Total score of eight is necessary for the diagnosis. Major criteria items must comprise five or more points of the total score.

**SUPPORTIVE FINDINGS** (increases the certainty of diagnosis):

| | | | | |
|---|---|---|---|---|
| 1. | High pain threshold. | yes | no | uk |
| 2, | Decreased vomiting. | yes | no | uk |
| 3. | Altered temperature sensitivity. | yes | no | uk |
| 4. | Scoliosis and /or kyphosis. | yes | no | uk |
| 5. | Early adrenarche. | yes | no | uk |
| 6. | Osteoporosis. | yes | no | uk |
| 7. | Unusual skills with jigsaw puzzles. | yes | no | uk |
| 8. | Normal neuromuscular studies. | yes | no | uk |

**APPENDIX B**

Williams et al. (1995)

ANGELMAN SYNDROME: Consensus for Diagnostic Criteria

TABLE I Developmental History and Laboratory Findings*

1. Normal prenatal and birth history with normal head circumference. Absence of major birth defects.
2. Developmental delay evident by 6-12 months of age.
3. Delayed but forward progression of development (no loss of skills ).
4. Normal metabolic, hematologic and chemical laboratory profiles.
5. Structurally normal brain using MRI or CT (may have mild cortical atrophy or dysmyelination).

*These findings are useful as inclusion criteria but deviations should not exclude diagnosis.

TABLE II Angelman Syndrome: Clinical Characteristics

A. Consistent (100%)

-Developmental delay, functionally severe

-Speech impairment, none or minimal use of words; receptive and non-verbal communication skills higher than verbal ones

-Movement or balance disorder, usually ataxia of gait and/or tremulous movement of limbs

-Behavioral uniqueness: any combination of frequent laughter/smiling; apparent happy demeanor; easily excitable personality, often with hand flapping movements; hypermotoric behavior; short attention span

B. Frequent (more that 80%)

-Delayed, disproportionate growth in head circumference, usually resulting in microcephaly (absolute or relative) by age 2

-Seizures, onset usually<3 years of age

-Abnormal EEG, Characteristic pattern with large amplitude slow-spike waves (usually 2-3/s), facilitated by eye closure

C. Associated (20-80%)

-Flat occiput

-Occipital groove

-Protruding tongue

-Tongue thrusting; suck/swallowing disorders

-Feeding problems during infancy

-Prognathia

-Wide mouth, wide-spaced teeth

-Frequent drooling

-Excessive chewing/mouthing behaviors

-Strabismus

-Hypopigmented skin, light hair and eye color (compared to family), seen only in deletion cases

-Hyperactive lower limb deep tendon reflexes

-Uplifted, flexed arm position especially during ambulation

-Increased sensitivity to heat

-Sleep disturbance

-Attraction to/fascination with water

**Appendix C**

The following is a list of the number of studies and the individuals who completed the studies that are included in this thesis.

## Angelman Syndrome

| Number | Study type | Technical assistance |
|---|---|---|
| **52** | **G-Band** | |
| 39 | | Clinical laboratory staff |
| 7 | | SuEllen Toth-Fejel |
| 4 | | Research laboratory staff |
| 1 | | Idaho State Laboratory, Boise, ID |
| 1 | | Kaiser Permanente Laboratory, Portland, OR |
| **25** | **FISH** | |
| 14 | | Research laboratory staff |
| 6 | | Clinical laboratory staff |
| 5 | | SuEllen Toth-Fejel |
| **24** | **Special Stains** | |
| 22 | | SuEllen Toth-Fejel |
| 2 | | Research laboratory staff |
| **16** | **5' SNRPN methylation** | |
| 16 | | SuEllen Toth-Fejel |

## Prader-Willi Syndrome

| | | |
|---|---|---|
| **56** | **G-Band** | |
| 54 | | Clinical laboratory staff |
| 1 | | SuEllen Toth-Fejel |
| 1 | | Kaiser Permanente Laboratory, Portland, OR |

| | | |
|---|---|---|
| **28** | **FISH** | |
| 9 | | Clinical laboratory staff |
| 11 | | Research laboratory staff |
| 8 | | SuEllen Toth-Fejel |
| **23** | **Special Stains** | |
| 19 | | SuEllen Toth-Fejel |
| 3 | | Research laboratory staff |
| 1 | | Clinical laboratory staff |
| **19** | **5′ SNRPN methylation** | |
| 19 | | SuEllen Toth-Fejel |
| **1** | **VNTR Southern blotting** | |
| 1 | | Jan Wolford |

**Clinical Laboratory Staff:**
Connie Durum
Debbie Buckmaster
Teresa Dougherty
Mike Brown
Mary Black
Anna Liza de la Cruz
Lee Allen
Richard Sherman
Vicki Hafits
Jodi Robinson
Bonnie Comegys
Françoise Fowler
Leslie Smith
Dana Bangs
Nancy Unsworth
Elizabeth Clark
Helen Lawce
Leila Ghodsi

**Research Laboratory Staff:**
Catherine Mace
Kristine Gunter
Carol Reifsteck
Lee Allen
Diane Tomar
XueYa Luo
Ken McMillin

**DNA Diagnostic Laboratory Staff:**
Jan Wolford

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