MOLECULAR ANALYSIS OF THE PRADER-WILLI SYNDROME CHROMOSOME REGION IN GENOMIC DNA OF PRADER-WILLI SYNDROME PATIENTS

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by

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To John, Todd, Boyd, and Sonya.

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ABSTRACT

Genomic DNA probes defining loci D15S9, D15S10, D15S11, and D15S12 which map to 15q11.2-12 were used to investigate the Prader-Willi syndrome Chromosome Region (PWCR) in patients with the Prader-Willi syndrome (PWS). First, dosage analysis of the four loci was determined by comparing the intensity of hybridization at a locus defined by a chromosome 15 probe and a locus defined by a probe to be used as a standard. Second, restriction fragment lengths at the four loci in normal controls were compared to restriction fragment lengths in the PWS patients to detect molecular rearrangements; third, seven PWS patients and two PWS families were studied using RFLP analysis for the detection of deletions and for determining the parental origin of the deleted chromosome. These analyses show that molecular rearrangements have occurred in eight of the nine PWS investigated patients but that the specific rearrangements differed from patient to patient. Six patients had a deletion of at least one locus, one patient had a deletion-duplication, one patient had a duplication, and one patient showed no abnormalities at any of the four loci tested. These molecular studies suggest that a physical disruption of the PWCR may cause the PWS not only in those patients reported to have a cytogenetic aberration but also for those identified as karyotypically normal. The question remains as to whether the one PWS patient with no

molecular abnormality yet detected has a recessive form of PWS or presents by another mechanism. The relationship of the molecular aberration at each of the four loci for each patient predicts the order of the four loci on chromosome 15 to be CEN→D15S9→D15S12→D15S11→D15S10→qter.

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ABBREVIATIONS

ß-ME-Betamercaptoethanol

EDTA-ethylenediamine tetraacetate

HCl-hydrochloric acid

M-molar

Mbp-Megabase pairs

mg-milligram

ml-milliliter

NaCl-sodium chloride

NaH_2PO_4-Sodium diphosphate

NaOH-sodium hydroxide

ng-nanogram

OD-optical density

PFU-plaque-forming units

SDS-sodium dodecyl sulfte

SSC-salt sodium citrate

TRIS-Tris(hydroxymethyl)aminomethane

ug-microgram

ul-microliter

I. INTRODUCTION

A. Literature review

1. Clinical characteristics and incidence of Prader-Willi syndrome(PWS)

In 1956 Prader, Labhart, and Willi reported a new syndrome which they called "Ein syndrom von adipositas, kleinwuchs, krytorchismus und oligophrenie nach myatonieartigem zustrand im neugeborenenalter". Zellweger and Schneider (1968) reviewed the ninety-four cases reported in the literature and described the chronological progression of PWS patients as being divided into two developmental phases. Phase One involves primarily the first year of life in which the young patient fails to thrive due to feeding difficulties, has a low birth weight, weak cry, hypoplastic genitals, and inadequate temperature regulation. Phase Two begins from one to four years of age at which time the infant gains strength and feeding difficulties disappear. Developmental milestones are delayed with sitting occurring around twelve months, walking at thirty months, and talking in short sentences by age three or four years. During this phase the PWS child is generally happy, pleasant, and good natured. Late in Phase Two the PWS patient develops hyperphagia and the resulting voracious appetite results in bizarre behaviour. The once happy child exhibits stubbornness and occasional temper tantrums. In late adolescence and early adulthood some

patients have severe personality and behavioral problems usually associated with the insatiable appetite.

From a review of the published cases Jancar(1971) reported the constant features of the syndrome to be congenital muscular hypotonia, obesity, hypogonadism, retarded skeletal growth and mental retardation. There was also a marked tendency for the patients suffering from Prader-Willi syndrome (PWS) to develop diabetes mellitus. Most of the patients have a characteristic facial appearance. Marked adiposity is present around the cheeks and under the chin. The palpebral fissures are almond-shaped with slightly overhanging lids. The mouth is fish-like and slightly open with a triangular shaped upper lip. Less consistent clinical features include blue eyes, blonde hair, strabismus, astigmatism, acromicria, and other skeletal and dental abnormalities, cortical atrophy, absence of auricular cartilage, chromosome anomalies, and unusual dermatoglyphic patterns on the fingers, hyperphagia, somnolence, abnormal EEG, convulsions, intrauterine inactivity, prolonged gestation, and low birth weight. A photographic study by Char(1984) shows the development of progressive obesity in a child with PWS. At age nine months, the child had hypotonia but no obesity. The prenatal history was normal except for decreased fetal movement. At birth a poor cry and floppiness was noted. Obesity began shortly after two years of age and progressed.

Cassidy (1984,1987b) concurs with previous clinical descriptions (Jancar, 1974; Zellweger, 1968) of PWS and further defines PWS IQ range to be normal in 12%, borderline in 29%, mild mental retardation in 41%, and moderate mental retardation in 12%. The minimal diagnostic criteria according to Cassidy(1987b) are hypotonia, dysmorphism, cognitive abnormalities, obesity, hypogonadism, and short stature.

Despite the fact that over seven hundred cases of PWS have been reported in the literature in more than two hundred articles in the last twenty-five years (Zellweger, 1984), the average age when PWS is diagnosed is approximately ten years of age in males but older in females. Thus the condition is not rare and has been estimated to occur in 1/10,000 live births (Holm, 1981).

2. Chromosome abnormalities in Prader-Willi syndrome patients.

In 1976 Hawkey and Smithies reported that approximately one out of six PWS patients studied cytogenetically had a chromosomal abnormality and suggested that an anomaly of 15p played a role in the pathogenesis of PWS. In 1979 Kucerova et al. stated that about 10% of the cases of PWS had a detectable abnormality of a D-group chromosome. In 1982 Kousseff and Douglas reported that 40 PWS patients studied cytogenetically had chromosomal abnormalities. Of the 40 patients studied 34 had abnormalities involving

chromosome 15. Initially the translocation 15q;15q was thought to be causally related to the PWS phenotype by virtue of the deletion of the short arms, but this hypothesis was not consistent with subsequent reports of apparently identical translocations of individuals with normal phenotypes, and translocations involving only one chromosome 15 and another autosomal chromosome in patients with PWS. Kouseff and Douglas(1982) concluded that the etiology of PWS remained obscure, and that the chromosomal abnormalities were associated either causally to the phenotype, or as a result of a derangement of chromosome 15, induced by a gene presumably responsible for PWS.

Ledbetter et al. (1981) reported an identical chromosome 15 deletion in four patients with breakpoints in bands 15q11 and 15q13 and concluded the deletion to be the cause of the PWS in these patients. In a later study 45 additional patients were studied by Ledbetter et al. (1982). They report that of the 45 patients studied with high resolution chromosome banding techniques, 23 had an interstitial deletion of proximal 15q with varying breakpoints. In all 23 cases part or all of band 15q11 was lost. These results supported their hypothesis that in many patients PWS is associated with a specific deletion of chromosome 15. They further refined the critical region to band 15q11.2.

In 1984 Cassidy et al. reported that all of nine

patients with a firm clinical diagnosis of PWS were found to have a deletion 15q11-13 in all or part of the cells analyzed. Two of the nine patients were mosaic for the deletion 15q12. They suggest that perhaps PWS patients previously reported not to have a 15q deletion may actually have had mosaicism for the deletion. It was hypothesized that previous researchers considered a patient had a 15q deletion only if a deletion was found in every cell examined. This approach eliminated the possibility of detecting mosaicism and may have led to those patients being falsely reported as cytogenetically normal. Therefore a higher percentage of PWS patients may have a chromosome abnormality.

Early in 1984 Mattei et al. reported 94 anomalies of chromosome 15 associated with PWS patients cited in the literature. They reported that even though the chromosomal anomalies appear very different, the proximal region of the long arm of chromosome 15 is constantly implicated. The anomalies of chromosome 15 associated with PWS included interstitial deletions,balanced and unbalanced translocations, Robertsonian translocations, small bisatellited additional chromosomes derived from chromosome 15 and pericentric inversions. Thus, Mattei et al. (1984) concluded that the proximal region of 15q is involved in all of the chromosome anomalies associated with PWS. They suggested that an alteration in the proximal 15q region is

probably the origin of the PWS phenotype even if no anomaly was detected and that DNA technology with specific probes could reveal a subtle or microscopically undetectable deletion in chromosomally normal PWS patients.

3. Familial cases of PWS and recurrence risk

Reports of familial incidence of PWS have been cited(Burke et al., 1987; Clarren & Smith, 1977; DeFraite et al.,1975; Hall & Smith, 1972; Hasegawa et al.,1984; and Lubinsky et al., 1987). A recurrence risk of 1.6% in sibs of probands was found by Clarren and Smith(1977). Cassidy(1987a) points out that this proposed empirical recurrence risk was formulated before the use of high resolution chromosome banding techniques and prior to the recognition that an interstitial deletion of chromosome 15 is present in approximately 50% of PWS patients (Ledbetter et al., 1981, 1982). No familial recurrence of PWS has been reported in families where the proband has a deletion of proximal 15q11-12. With only two well documented case of familial PWS (Lubinsky et al., 1987; Burke et al., 1987) in the over 1500 families with PWS on the registry of the Prader-Willi syndrome Association, Cassidy proposes a recurrence risk of less than 1/1000 and that when the proband has a 15q interstitial deletion, recurrence is not likely to occur.

4. Phenotype-karyotype correlation in Prader-Willi syndrome patients

Several studies (Butler et al., 1982; Butler et al., 1986a; Butler et al.,1986b; Kaplan et al.,1987; Niikawa & Ishikiriyama, 1985; Reynolds et al.,1987; Schwartz et al., 1985; and Smith & Simpson; 1984) have attempted to correlate phenotype characteristics with cytogenetic aberrations in order to subgroup PWS patients to aid clinical diagnosis of the syndrome, to determine the etiology of the syndrome, and to further define the map position of a potential gene in the proximal region of 15q.

a. Phenotype-karyotype correlation in PWS patients and non-PWS patients with deletions of proximal 15q.

To seek evidence of a phenotype-karyotype correlation in PWS Niikawa and Ishikiriyama (1985) reevaluated the clinical features of 27 PWS patients and classified each patient into one of three study groups. Karyotyping of patients and parents was done using high resolution banding techniques. They found that the patients that had met all diagnostic criteria for PWS had an interstitial deletion 15q11-12, of the 9 group two patients who were floppy infants age three or under who as yet had not developed hyperphagia or obesity, 50% had a 15q11-12 deletion and 50% had normal karyotype; the 3 PWS patients in the third study group who were lacking some features of PWS were karyotypically normal. They concluded that when PWS

patients met all PWS diagnostic criteria, the PWS phenotype correlates with chromosome 15 aberrations.

Schwartz et al. (1985) surveyed the literature and report finding 14 cases with 15q deletions with non-PWS phenotype. Most of the non-PWS cases had deletions including 15q13 with even larger deletions in some. They concluded that deletion size may correlate with the severity of the disorder in non-PWS patients and that deletion of only bands q11 or q11-12 may result strictly in the PWS phenotypes. An additional case of non-classical PWS with an interstitial 15q11-12 deletion was reported by Reynolds et al. (1987). Kaplan et al. (1987) reported three patients with deletions 15q11-12 which are usually associated with the PWS phenotype who do not have the PWS. One patient was diagnosed as Williams syndrome, one as Angelman syndrome, and the third had some of the features of PWS but not fitting all of the diagnostic criteria. Magenis et al. (1987) also report three patients with clinical characteristics more consistent of Angelman syndrome than PWS with a 15q11-13 deletion of one chromosome 15. Both Kaplan et al. (1987) and Magenis et al. (1987) propose that the phenotypic diversity seen among patients with an interstitial deletion of 15q11-12 might be accounted for by different molecular abnormalities within the critical region 15q11-13.

Butler et al. (1986b) compared the clinical

characteristics of 39 PWS patients with 124 individuals reported in the literature and concluded that deletion patients had lighter hair, eye, and skin color than non-deletion patients. Deletion patients were also more sun sensitive and had higher intelligence scores than PWS patients with normal karyotype. To determine the frequency of hypopigmentation in PWS patients, Wiesner et al. (1987) studied 30 individuals with PWS and found 48% to be hypopigmented. There was a positive correlation between hypopigmentation and the patients with a deletion of proximal 15q. Therefore hypopigmentation is an important feature in PWS and characterization of the mechanisms may aid in delineation of the gene locus for control of melanin formation and insight into the heterogeneity of PWS.

Clinical observation of small hand size in PWS patients (Cassidy, 1984) led Butler et al. (1982) to determine the metacarpophalangeal pattern profile (MCPP) on 16 PWS patients to evaluate its potential use as an additional diagnostic tool for PWS. Two distinctly different profiles were found when the PWS patients were grouped according to the cytogenetic findings. The patients with the deletion were homogeneous with respect to the MCPP but the non-deletion patients were heterogeneous. The MCPP discriminant analysis of normal individuals and PWS patients results suggest that PWS patients can be separated from the normals based on the profile. In a follow-up study Butler et al.

(1986a) determined the MCPP of 38 PWS patients, 16 Sotos syndrome patients, and 41 normal subjects. The results suggest that the classification of PWS patients and Sotos syndrome from normal subjects was possible.

Dermatoglyphic findings of 24 PWS patients (Smith & Simpson, 1982) and 32 parents of these patients showed that no abnormal pattern was characteristic of PWS patients and no features were exceptionally different between parents and PWS offspring. Reed and Butler (1984) compared dermatoglyphic patterns of 38 PWS patients, 17 with normal chromosomes and 21 with an interstitial deletion, and 270 normal controls. They concluded that their results were consistent with previous studies (Smith & Simpson; 1982, 1984) that dermatoglyphics cannot be used as a diagnostic tool for PWS.

5. Parental origin of aberrant chromosome 15 in PWS patients.

The parental origin of the chromosome 15 with the proximal 15q deletion in PWS patients was found by Butler et al. (1983) to be paternal in 13/13 cases and paternal in 6/7 cases reported by Niikawa and Ishikiriyama (1985). In the one case of maternal origin of the deleted chromosome 15 an interstitial deletion 15q11-12 was also found in the apparently phenotypically normal mother. Of the 20 patients studied (Butler et al., 1985; and Niikawa & Ishikiriyama, 1985) all other parents chromosomes were

normal. Thus, 19/20 chromosome deletions were de novo. Butler et al. (1986b) suggest that if the proximal region of chromosome 15 is sensitive to environmental stress, there would be increased mutational effect on male meiosis rather than female meiosis as male meiosis is continuous. The authors suggest that epidemiological studies of male parents of PWS patients might be informative.

A study of parental orgin of de novo chromosome rearrangements by Chamberlin and Magenis (1980) suggests that different types of chromosomal errors involve different mechanisms. Of the 42 cases of de novo chromosome rearrangements studied, Robertsonian tranlocations and non-disjunction events are most often maternal. Those chromosome rearrangements involving breakage and reunion that are not Robertsonian translocation are most often paternal in origin (13 paternal origin, 4 maternal origin). Chamberlin and Magenis (1980) suggest that differences in the meiotic process between females and males may account for differences in parental origin of chromosomal rearrangements resulting in non-disjunction events and chromosome breakage and reunion. The continued proliferation of sperm throughout adult life may increase the chance in sperm for rearrangements to occur. The breakage event in PWS patients may be the same mechanism as most aberrant chromosomes in PWS patients are paternal in origin.

Data relating to environmental conditions and births of PWS patients were reported by Butler et al. (1985). The birth month of 124 PWS patients (63 with normal chromosomes, 61 with a chromosome 15 deletion) was determined and compared to the general population. The birth month of PWS patients with normal chromosomes did not differ from the general population but a greater number of PWS patients with chromosome deletions were born in the autumn and not as many were born in winter as expected.

Strakowski and Butler (1987) studied 652 PWS patients for evidence of paternal hydrocarbon and lead exposure. The control group consisted of fathers of 268 Down syndrome patients and of 66 fragile X patients. Paternal lead exposure of PWS fathers was not greater than in controls but paternal occupational hydrocarbon exposure in the PWS group was greater. Twenty-four percent of fathers of PWS patients with known chromosome 15 abnormalities had occupational hydrocarbon exposure. They conclude that paternal occupational hydrocarbon exposure may be a causative factor in PWS by inducing environmental stress during paternal gametogenesis producing chromosome aberrations.

6. Metabolic anomalies in Prader-Willi syndrome patients.

Chasalow et al. (1987) report evidence of a possible defect in the sulfo-conjugate metabolic pathway. Studying steroid sulfate conjugates in serum samples of 17 PWS

children, they found that ten of the 17 PWS patients had a normal pattern of dehydroepiandrosterone (DHEA) but the remaining seven patients had unusual steroid sulfates. One of the seven PWS patients with abnormal steroid sulfate patterns had an unusual DHEA. Four other compounds were detected but were not identified. The authors suggest that the clinical manifestations in PWS patients may reflect different defects in the sulfo-conjugate metabolic pathway.

A causative relationship between linear growth retardation and short stature in PWS and a deficiency in growth hormone(GH) is suggested by Lee et al. (1987). Four patients being treated with GH showed significant increases in linear growth rate and somatomedin-C levels. Previous studies (Bray et al.,1983; and Tolis et al., 1974) have reported both normal and abnormal response to pharmacologic stimulus. Lee et al. (1987) suggest that pharmocological stimulus may not accurately reflect physiological GH secretion and further studies are recommended.

The biochemical pathway involved in the clinical manifestation of hypopigmentation in 12 PWS patients was investigated by Butler et al. (1987) by assaying plasma samples for levels of immunoreactive ß-melanocyte stimulating hormone. Levels were within the normal range for all patients tested. Thus hypopigmentation observed in PWS is not caused by a decrease in circulating plasma immunoreactive ß-melanocyte stimulating hormone.

B. Rationale and strategy for molecular approach to studying PWS patients.

1. Implication of chromosome aberrations in PWS

The results of cytogenetic findings in six surveys (Butler & Palmer, 1983; Fear et al., 1983; Mattei et al., 1983; Ledbetter et al., 1981, 1982; and Niikawa & Ishikiriyama, 1985) involving 144 PWS patients indicates that 51% of PWS patients have a deletion of 15q11.2, 15q11-12 or 15q11-13; 3% have a Robertsonian translocation (some balanced and some unbalanced resulting in a deletion of the proximal region of 15q); 1% with an extra isodicentric chromosome 15pter-q11::q11-15pter; 1% with 18p+; 2% of chromosome preparations were inadequate to determine if any chromosome anomalies were present; and 41% had normal chromosomes. Thus even with high resolution chromosome banding techniques chromosome aberrations have not been detected in at least 1/3 of PWS patients.

2. Mode of inheritance of PWS

Several hypotheses as to the etiology of PWS can be formulated. McKusick (1983) lists PWS as an autosomal recessive. Expression of the PWS phenotype could be the result of homozygosity of two mutant alleles or by the disruption of a normal allele in a heterozygote. The autosomal recessive mode of inheritance is supported by the one-third of PWS patients with apparently normal karyotypes as well as those with an interstitial deletion of the

proximal region of 15q. Such patients would express the PWS phenotype as a result of the presence of an hemizygous mutant allele. If PWS is an autosomal recessive disorder increased consanguinity might be expected among parents of PWS patients but this has not been observed. Also more cases of familial PWS might be expected. Only two well documented cases of familial PWS have been reported(Burke et al., 1987; Lubinsky et al., 1987) out of over 1500 families with PWS (Cassidy, 1987a). Patients reported as being trisomic or tetrasomic for the proximal region of 15q do not support an autosomal recessive mode of inheritance.

A second possibility is that of a dominant mode of inheritance of PWS evoked by disruption of 15q11-12 by translocation, duplication, deletion, or inversion (Fraccaro et al., 1983; Mattie et al., 1983). Some aberrations may be submicroscopic. A dominant inheritance pattern may be created but not passed onto offspring because of decreased reproductive fitness in PWS patients; therefore, familial patterns are not often reported. There are no reports of PWS patients reproducing. Support for this mode of inheritance is based on evidence that most patients with an interstitial deletion of 15q11-13 are phenotypically PWS. Exceptions have been cited by Kaplan et al. (1987), Magenis et al. (1987), Reynolds et al. (1987), Schwartz et al. (1985). If PWS is an autosomal recessive it would be expected that some of the time the normal allele would be

deleted presenting as a normal phenotype; to date no normal individuals have shown the 15q11-12 deletion with the exception of the mother of a PWS patient having an interstitial deletion of 15q11-12 (Niikawa and Ishikiryama, 1985). The karyotypically normal individuals who have a submicroscopic deletion or rearrangement could fit into either mode of inheritance.

Rare cases of PWS patients (Kousseff et al.,1987; Pettigrew et al.,1987) with a duplication of the proximal region 15q present an unusual circumstance where a trisomic or tetrasomic chromosome region mimics a phenotype more commonly associated with monosomy for the critical region 15q11-12. Thus an alternate etiology to gene dosage is required. Position effect, gene disruption, as well as lability of chromosome 15 as a secondary effect have been suggested (Kousseff and Douglas, 1982; Murdock and Wurster-Hill, 1986).

Schmickel (1986) suggests the possibility that PWS is a contiguous gene syndrome resulting from the disruption of genes within the region involved in the characteristic chromosomal rearrangement or breakage. Schmickel (1986) points out that contiguous gene syndromes are usually sporadic rather than familial and a wide spectrum of severity is observed among affected individuals. A heterogeneous group of phenotypes present because they are caused by several genes, the severity reflects the number of

genes involved. Usually two groups of contiguous gene syndrome patients are identified, those with and those without visible chromosome anomalies. The general features of contiguous gene syndromes are consistent with the heterogeneity of PWS patients both phenotypically and karyotypically. The heterogeneity of cytogenetic findings among PWS patients is cited by Kousseff et al. (1987) as evidence that PWS is a contiguous gene syndrome rather than an aneuploid syndrome.

Lastly, it is possible that heterogeneity resultsd in more than one group of patients presenting with the PWS phenotype. One group results from disruption of a gene or genes in the proximal region of chromosome 15q; another group might present as the result of a gene mutation on chromosome 15 or elsewhere.

3. Molecular consideration for an approach to investigate etiology of PWS.

a. Estimation of the size of the critical region 15q11-13.

Cytogenetic studies of PWS patients have conclusively implicated the proximal region of chromosome 15q. The region 15q11-12 has been designated as the Prader-Willi syndrome Chromosome Region (PWCR). Cytogenetic analysis is limited by the resolution of a banded region of a chromosome. In the case of the PWCR locus no further resolution is possible with present cytogenetic technology.

A different approach is required if any further clarification of this critical region in PWS patients is to be accomplished. Assuming that the human autosomal haploid chromosomes comprise 3 X 10^9 base pairs, the size of band 15q11 is estimated to be 5,000Kb and 15q12 is estimated to be 3,000Kb. Thus the critical region PWCR 15q11-12 is estimated to be 8.0Mbp of DNA.

b. Reverse genetics

Molecular analysis of inherited human disorders has proceeded mainly by the study of specific proteins associated with the phenotype and thus the corresponding genes. The thalassemia syndromes (Orkin & Kazazian, 1984) and familial hypercholesterolemia (Brown & Goldstein, 1986) are two disorders in which molecular disruptions aided in the isolation and characterization of the corresponding genes. Success in this process depends on understanding the affected proteins associated with the inherited disorder prior to molecular analysis. In many inherited disorders with well defined phenotypes the underlying biochemical malfunction is unknown and often animal models that mimic the disease are lacking. In these cases the isolation of the gene without any knowledge as to the specific underlying protein defect permits access to understanding the disorder. This approach sometimes called "reverse genetics" (Orkin, 1986) proceeds by establishing the map position of the potential gene by identifying a specific region within the

disease locus in which mutations are correlated with the disease. Assignment within several million bases is usually possible (White et al., 1985; 1986).

Orkin (1986) reviews the success of "reverse genetics" in Duchenne muscular dystrophy(DMD) and chronic granulomatous disease(CGD), and retinoblastoma(Rb). The key to identifying the chromosomal location of each locus was the cytogenetic deletions identified in DMD, CGD, and Rb patients. These deletions helped to localize the gene locus and also aided in the cloning of DNA segments useful in the gene search by allowing enrichment for such segments in cloning experiments (Kunkel et al.,1985b). The specific deletion regions also serve to identify a target region on a specific chromosome to which anonymous DNA segments should map to be of use in the gene search.

The cytogenetic aberrations identified in PWS patients have clearly implicated the region 15q11-12 as a potential gene locus making PWS a genetic disorder that can be approached using "reverse genetics". The identification and characterization of cloned DNA segments which map to the critical region 15q11-12 could be used to analyze PWS patients in order to find the smallest region of overlap that is associated with the syndrome. This might allow the identification of the gene.

c. Strategy to enrich for probes mapping to the critical region 15q11-13.

The first reports of the isolation and localization of DNA segments from a specific human chromosome were those of DNA cloned into Charon 4A vector using DNA from a human-hamster hybrid cell line carrying a portion of the human chromosome 11 (Gusella et al., 1980). Clones containing human sequences were chosen by virtue of hybridization to HeLa DNA but not to hamster DNA. Wolf et al. (1980) prepared a genomic DNA library using a 48,XXXX cell line to enrich for X sequences. Using flow-sorted chromosomes to prepare chromosome-specific libraries was successfully done first with the X chromosome (Davies et al.,1981; and Kunkel et al.,1982) and more recently for other chromosomes (Van Dilla et al., 1987). The intent is to enrich a DNA library so that it contains an increased frequency of clones arising from the chromosome of interest as compared to a total genomic library. Chromosome 15 comprises approximately 3.4% of the haploid autosomal complement (Maynard-Smith et al., 1961). To search for cloned DNA sequences mapping to 15q11-12 from a total genomic library is limited by the small representation of chromosome 15 sequences. Estimates of the size of the PWCR (15q11-12) suggest that it comprises about 8,000Kb of DNA or 0.3% of the total genomic DNA. A genomic DNA library from flow-sorted chromosome 15's would therefore contain about 8% of DNA sequences from 15q11-12,

about a 30 fold enrichment.

Kunkel et al. (1985b) used a phenol-enhanced reassociation technique (PERT) to enrich for sequences from Xp21 in a search for the DMD gene. This approach is not applicable for enrichment of 15q11-13. The PERT technique depends on differential reassociation of DNA molecules that are present in one individual but not in another. The isolation of the PERT probes (Kunkel et al., 1985b) utilized a male patient with a minute deletion spanning the DMD locus reassociated with XXXXY DNA from another cell line. To date no individuals or human cell lines are available that do not have at least one normal chromosome 15. However individuals have been reported to be trisomic or tetrasomic for 15q11-13. Such a case is a transformed lymphoblast line GM6246 available from the Human Genetic Mutant Cell Repository, Camden, NJ. Lalande et al. (1985) report the successful flow-sorting of the inverted duplicated chromosome 15 fragment 15qter→q13::q13→15qter (GM6246) from which DNA could be extracted to use for preparation of a library enriched for 15q11-13 DNA sequences.

The strategy for enriching for chromosome 15 sequences from the region 15q11-13 was to prepare a library from flow-sorted chromosome 15's and to screen for sequences mapping to the region of interest. The study described here began with the preparation of a genomic DNA library prepared from flow-sorted chromosome 15's which had been flow-sorted from

a human lymphoblastoid cell line REN2 48XXXY, screening of the library just described along with a chromosome 15 library received from the Los Alamos National Laboratory, characterization of clones from these two libraries, and characterization of four probes received from Drs. Sam Latt and Timothy Donlon, Children's Hospital in Boston, from the library prepared from the inverted duplicated chromosome 15 fragment described by Lalande et al. (1985).

C. Objectives of this study.

The objectives of this study were to: 1) characterize four genomic DNA probes that map to the PWCR 15q11-12; 2) analyze the PWCR of PWS patients using genomic DNA probes which recognize the loci D15S9, D15S10, D15S11, and D15S12 to detect deletions, duplications, or base changes at each locus, 3) analyze by densitometry the loci (D15S9, D15S10, D15S11, and D15S12) in genomic DNA isolated from PWS patients to identify deletions or duplications; 4) use restriction fragment length polymorphisms (RFLP) in two PWS families to detect deletions, duplications, or base changes as well as parental origin of the deleted chromosome 15; 5) use restriction length polymorphisms to type PWS patients (without available family data) for their apparent genotype at each locus (D15S9, D15S10, D15S11, and D15S12); and 6) obtain a restriction map of each of the four loci (D15S9, D15S10, D15S11, and D15S12) in genomic DNA using conventional gel electrophoresis; and 7) order on the

chromosome the four loci D15S9, D15S10, D15S11, and D15S12 using PWS patient locus-deletion data.

II. MATERIALS AND METHODS

A. Isolation and extraction of DNA

1. Plasmid preparations

The isolation of DNA cloned into plasmids was completed according to the method of Garger et al. (1983) with the following modifications: 1) 10ml of LB broth with 25ug/ml of ampicillin was inoculated with one colony and allowed to grow overnight at 37°C; 2) the overnight culture was added to 500mls of prewarmed LB broth containing 25ug/ml of ampicillin and allowed to grow at 37°C for approximately three hours until the OD_{600} is 0.4; 3) chloramphenicol was added to a final concentration of 200ug/ml and culture was allowed to grow overnight at 37°C; 4) the overnight culture was centrifuged at 5K for 10-15 minutes at 4°C and the pellet was resuspended in 12mls of glucose buffer(50mM glucose, 25mM Tris-Cl, pH 8.0, and 10mM EDTA); 5) 2mls of glucose buffer containing 40mg of lysozyme (freshly prepared) was added to the resuspended pellet and incubated for 10 minutes at room temperature; 5) cells were lysed by the addition of 27.6mls of 1% SDS in 0.2N sodium hydroxide(NaOH) and placed in an ice-slurry mixture for 15 minutes; 6) protein, chromosomal DNA, and high molecular weight RNA were precipitated by addition of 14mls of potassium acetate (3M potassium, 5M acetate;pH 4.8) and left on ice for 15 minutes; 7) the insoluble contaminants were removed by centrifugation at 10K for 10 minutes; 8) the

supernatant was carefully removed and extracted with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1); 9) after mixing thoroughly the layers were separated by centrifugation at 6K for 5 minutes; 10) the aqueous phase was removed and the DNA was precipitated by the addition of a 0.6 volume of isopropyl alcohol; 11) the precipitation was allowed to proceed overnight at -20°C when the precipitate was removed by centrifugation at 10K for 20 minutes at 4°C; 12) the supernatant was removed, the pellet was dried briefly under a vacuum, and resuspended in TE(10mM Tris-HCl, pH8.0;1mM EDTA); 13) when the DNA was in solution RNase was added to a final concentration of 100ug/ml and incubated for 15 minutes at 37°; 14) the DNA was placed at 4°C for storage.

2. Extraction of genomic DNA from patients, parents, and controls

The source of the genomic DNA was either lymphocytes from 5-7mls of whole blood or fibroblasts from several 100mm tissue culture plates. DNA was extracted from whole blood according to the following method (Personal communication, David Hoar): 1) Blood was collected in EDTA vacutainer tubes. 2) Seven mls of whole blood were placed into 50ml capped tubes to which 5 volumes of prewarmed (37°C) ammonium chloride:tris solution(9:1, 0.155M ammonium chloride, 0.170 M Tris-Cl, pH 7.65) was added. 3) After incubation for 5 minutes at 37°C, the tubes were centrifuged

at 2000rpm for 10 minutes. 4) The supernatant was removed leaving 4-5mls of supernatant above the pellet. 5) The cell pellet was resuspended gently in 10mls of saline solution (0.85% NaCL) and centrifuged at 2000rpm for 10 minutes. 6) The supernatant was removed and the cells washed once more in saline solution and resuspended in 2mls of High TE Buffer (Tris 100mM, EDTA 40mM, pH 8.0). 7) The cells were lysed immediately by the addition of the Lysis Buffer (Tris 100mM, 0.2%SDS, pH 8.0). 8) An equal volume of saturated phenol was added to the lysed cell mixture and mixed for about 5 minutes and then centrifuged for 5 minutes at 2000rpm. 9) The upper aqueous layer was removed and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). 10) The upper aqueous layer was removed and sodium chloride was added to final concentration of 0.1M. 11) An equal volume of isopropanol was added to precipitate the DNA. 12) The pellet was carefully removed from the solution, placed in a 500ul Eppendorf, dried briefly, and resuspended in Low TE Buffer (Tris 10mM, EDTA 1mM, pH 8.0).

The extraction of DNA from fibroblasts followed the same procedure except cells were removed from tissue culture plates by the addition of 1% trypsin for 5 minutes at 37°C. Cells were washed once in 10mls ammonium chloride:Tris solution (9:1, 0.155M ammonium chloride, 0.170M Tris, pH 7.65) and once in 10mls saline (0.85% sodium chloride). Lysis of the cells and completion of the DNA extraction

followed the blood DNA extraction method.

B. Restriction of DNA with endonucleases, Southern blotting, nick translation of probes, and blot hybridization.

1. Restriction of DNA with endonucleases

Approximately 5-10ug of DNA was restricted with appropriate enzyme using 3 units of enzyme per ug of DNA in a total volume of 30ul. Conditions of the reaction were performed according to the manufacturers recommended conditions using reaction buffers supplied by the manufacturer. The digests were set up early in the day and boosted with 2 units of enzyme per ug of DNA for overnight completion of restriction.

2. Gel electrophoresis

Restricted DNA was electrophoresed in a 1% agarose gel in tris acetate buffer of total volume of 125mls to which ethidium bromide was added to a final concentration of 100ug/ml. The gels were electrophoresed for 16-18 hours at 20 volts and 25 milliamperes at room temperature.

3. Southern blotting

DNA was blotted onto either Zeta-probe, Hybond, or Gene Screen plus according to the manufacturers' recommended procedure. Gels were generally blotted for 4 hours at which time the blotting membrane was removed and treated according to manufacturers' procedures. All blots regardless of the membrane type(Zeta-probe, Hybond, or Gene Screen plus) were

prehybridized for a minimum of 1.5 hours at 42°C in prehybridization solution (47% formamide, 10% dextran suflate, 3X SSPE, 1% SDS, 0.5% blotto, and 200ug/ml of denatured salmon sperm DNA) recommended for high sensitivity hybridization by the manufacturer of Zeta-probe.

4. Nick translation of probe DNA

Approximately 100ng of DNA was nick translated by the method of Maniatis et al. (1982) except that the total reaction volume was 20ul. The nick translation reaction buffer (NTK) was added to the DNA solution followed by 50uCi of dCTP and 50uCi of dATP. Diluted DNase II was added to a final concentration of 0.1ug/ml at room temperature for 2 minutes; 9 units of DNA polymerase I were added and the reaction volumewas incubated for 1.5 hours at 16°C. At the end of the incubation time the reaction was stopped by adding 5 uls of Stop buffer (50mM EDTA, 50% glycerol) and placing at 65°C for 10 minutes. Separation of the labelled DNA from the unincorporated dNTP's was accomplished by column chromatography through a Sephadex G-75 column. Sephadex G-75 was prepared according to Maniatis et al.(1982, p. 464). Chromatography was monitored by a Geiger counter and an aliquot of approximately 1000ul was collected. Specific activities were routinely 1-3 X 10^8.

5. Hybridization and washing of Southern blot, and autoradiography

Southern blots were hybridized in 9.4mls of Zeta-probe

hybridization solution (same as prehybridization solution) to which the denatured nick-translated DNA was added. Blots were hybridized for a maximum of 16 hours in a shaking water bath at 42°C. At the end of the hybridization blots were washed two times in 2 X SSC, 1% SDS, at room temperature for 10 minutes, all remaining washes were at 65°C for 15 minutes, 1 X 0.5 X SSC, 1% SDS, and 1 X 0.1 X SSC, 1% SDS. Autoradiography was at -70°C with double intensifying screens of Dupont Cronex lightning Plus GB on preflashed Kodak-Omat AR X-ray film. Exposure times varied from 16 hours to 5 days as required and are given specifically for each experiment. The X-ray film was developed according to the procedure outlined by Kodak.

6. Insert isolation

Insert DNA from plasmid to be used as a probe was electroeluted from the gel according to Maniatis et al. (1982, pp. 164-165) except that the dialysis bag was split open and the gel slice was placed into the trough. The edges of the dialysis membrane were propped up with slices of the gel so that the small amount of buffer did not run out. After electroelution from the gel slice the buffer was collected into a 1.5ml Eppendorf. The solution was extracted twice with isoamyl alcohol to remove the ethidium bromide. The DNA was precipitated by the addition of 5M NaCl to a final concentration of 0.1M and two volumes of isopropyl alcohol. The DNA was allowed to precipitate

overnight at -20°C, centrifuged for 15 minutes at 15K at 4°C, dried under vacuum briefly, and resuspended in 20ul of Low TE Buffer (Tris 10mM, EDTA 1mM, pH 8.0).

C. Patient materials

The PWS patients being reported here range in age from 2 years to 20 years. A brief description of clinical characteristics pertinent to syndrome diagnosis is included for each patient studied. Table I. lists the karyotype for each patient as well as the patient number as assigned for this study. The patients were karyotyped in the Clinical Genetics Laboratory, Children's Hospital, Winnipeg, Manitoba, Dr. Mano Ray, Director.

1. Patient 1 (MO).

MO born 28/2/84 presented with hypotonia, strabismus, micropenis, and hypoplastic scrotum. He has developed mild obesity with weight >95th %tile and height is 10th %tile. Mental assessment places patient as borderline retarded. No chromosome anomaly was detected.

2. Patient 2 (DS).

Even though DS (born 15/12/73)is karyotypically normal her clinical phenotype is consistent with PWS. At fourteen she is grossly obese and short in stature, her weight is at the 95th %tile and her height is at the 5th %tile. She has bitemporal narrowing, almond-shaped eyes, tented upper lip and a high narrow palate. Her hands and feet are relatively small. The patient is mildly mentally retarded.

Table I. Karyotypes[3] of Prader-Willi syndrome patients included in present study.

Patient	Sex	Karyotype
1(MO)	M	46,XY[1]
2(DS)	F	46,XX[1]
3(NB)	F	45,XX,-15,-5,+t(5;15)(p15.2;q12)[1]
4(LD)	M	46,XY,del(15)(q11q13)[1]
5(RH)	F	46,XX,del(15)(q11q13)[1]
6(EB)	F	46,XX,del(15)(q11q13)[1]
7(CC)	F	46,XX,del(15)(q11-13)[1]
9(AS)	F	undetermined[2]
10(AK)	F	46,XX[1]

[1]Patient diagnosed as PWS at the Children's Hospital, Winnipeg, Manitoba.
[2]Patient diagnosed as PWS at the Children's Hospital of Eastern Ontario,Ottawa, Ontario.
[3]Karyotypes were done in the Clinical Genetics Laboratory, Children's Hospital, Winnipeg, Manitoba, Dr. Mano Ray, Director.

3. Patient 3 (NB)

NB born 09/01/73 had feeding difficulties in the early neonatal period accompanied by hypotonia. Slight developmental delay was noted. Chromosome analysis revealed an unbalanced translocation karyotype 45,XX,-5,-15,+t(5;15)(p15.2;q12). Appetite control problems developed around age 5 or 6 as well as behavioral problems associated with insatiable appetite. As a young adolescent stature is short and the patient is mildly retarded. The unusual personality feature of skin picking is a constant aggravation.

4. Patient 4 (LD).

LD (born 13/01/73) presented with hypotonia, poor suck and was a poor eater; development was delayed. Hyperphagia developed in early childhood and he is very obese; his weight is greater than the 95th %tile and his height is less than 5th %tile. LD has small hands and feet, small penis, and underdeveloped scrotum. Skin picking causes skin eruptions. He is moderately retarded. Chromosome analysis reveals the characteristic PWS deletion 15q11-13.

5. Patient 5 (RH).

RH was diagnosed as PWS at age 14 (born 30/4/68) with gross obesity, voracious appetite, short stature, small hands and feet, strabismus, and scoliosis. Chromosome analysis reveals a 15q11-13 deletion.

6. Patient 6 (EB).

EB (born 11/5/80) was hypotonic and a poor feeder early in infancy but has been hyperphagic since about age 2. Development delay was recorded and speech delay is still apparent. The patient is borderline to mildly retarded. Hands as well as feet are small (hands 10th %tile; feet 3rd and 10th %tile). Weight is > 95th %tile and height 50th %tile. Chromosome analysis reveals a 15q11-13 deletion.

7. Patient 7 (CC).

CC (born 11/6/85) presented with hypotonia, developmental delay, and feeding difficulties. Chromosome analysis reveal an abnormal karyotype 46,XX,del(15)(q11q13).

8. Patient 9 (AS).

AS was diagnosed as PWS at the Children's Hospital in Ottawa.

9. Patient 10 (AK).

AK was born 6/5/68 and had significant feeding difficulties in early infancy followed by slow but steady weight gain. Developmental delay was noted and patient did not walk until 2 years of age. She is short in stature, has an obsession with food, and is mildly retarded. At age 19, her height is below the 5th %tile and her weight is greater than the 50th %tile. Some secondary sexual developmental is evident but she has not yet menustrated. Chromosome analysis reveals a normal karyotype using high resolution banding techniques.

III. DESCRIPTION OF TWO CHROMOSOME 15 LIBRARIES (λMUGS & λLAGS)

A. Introduction

1. Library building and clone screening

a. Selection of cloning vector EMBL3.

Lambda bacteriophage vectors such as EMBL3 (Frischauf et al.,1983) can be used to clone fragments created by a number of different restriction enzymes. To take full advantage of the vector and especially to allow the establishment of libraries from small amounts of material, Frischauf et al.(1983) modified the usual library cloning protocol substituting biochemical and genetic selection in place of the usual size separation steps. Selection for chimeric phage is provided by two different features of the phage. Red and gam genes carried by the middle fragment provide genetic selection; this selection is based on the Spi+(susceptible to P2 inhibition) phenotype. Growth of phage displaying this phenotype is restricted to bacterial hosts NM539 or Q359 (Frischauf et al., 1983). The genes responsible for P2 inhibition (genes responsible for the Spi+ phenotype) are present in the stuffer region of the EMBL3 vector. When the red and gam genes are removed and replaced with insert DNA during a recombinant construction, the phage express the Spi- phenotype and can be plated on a P2 lysogen. Only recombinant phage (Spi-) with the central

region containing a foreign insert have the ability to grow on the P2 lysogen and there is selection against nonrecombinant phage. The polylinker sequences of EMBL3 allow inactivation of the middle fragment by cleavage with both BamHI and EcoRI. Precipitation by isopropyl alcohol leaves the short cohesive fragments in the supernatant, thereby reducing the possibility of the middle fragment being ligated into a recombinant phage.

b. Preparation of library from nanogram quantities of DNA.

Davies et al. (1984) and Lalande et al. (1984) report that nanogram amounts of DNA are recovered from the extraction of 1 X 10^7 flow-sorted chromosomes. A total genomic library was prepared to determine the parameters necessary to build a library from nanogram amounts of DNA extracted from flow-sorted chromosomes. The cloning involved the following steps: 1) Placental DNA was partially digested with 3 units of MboI in the buffer recommended by the manufacturer. Aliquots of 400ul, 300ul, and 200ul were incubated for 10, 20, and 30 minutes. The reactions were visualized on a gel to select the aliquots of restricted DNA with a length distribution averaging approximately 20Kb. The use of 3 units of MboI for an incubation period of 10 minutes ws found to produce a suitable distribution pf restriction fragments of DNA. The appropriate aliquot of restricted DNA was precipitated and resuspended in Low TE

Buffer (Tris 10mM, 1 mM EDTA, pH 8.0). 2) Vector DNA (EMBL3) was digested to completion with BamHI and EcoRI, recovered by precipitation, and resuspended in 10ul of TE. 3) Insert DNA was treated with one unit of calf intestinal alkaline phosphatase, precipitated, and resuspended in TE. 4) DNAs were mixed in a 3:1 ratio of vector to insert and 1 unit of T4 ligase was added and reaction was left overnight at 15°C. 5) The ligation mixture was packaged according to the method of Maniatis et al. (pp.262-263, 1982) and plated out on both the permissive host (NM538) and the restrictive host (NM539); the library was titred and amplified according to Maniatis et al. (p.293, 1982).

The preparation of the chromosome 15 library from flow-sorted chromosomes followed the procedure described above for the preparation of the total genomic DNA library with the following modifications. Chromosome 15s were flow-sorted from a 49,XXXXY lymphoid cell line with purity of greater than 75% chromosome 15s, contaminated mainly with chromosomes 16 and 18 and slight contamination with chromosome 14 (Judith Fantes, M.R.C.Population Cytogenetics Research Unit, Edinburough, personal communication). DNA was extracted from approximately 1 X 10^6 flow-sorted chromosomes according to the following steps: 1) Two aliquots containing approximately 0.5 X 10^6 chromosomes in a volume of 400 ul of PBS (phosphate buffered saline) were used. 2) To each aliquot 8ul of Proteinase K was added to a final

concentration of 100ug/ml and incubated at 57°C for 2-3 hours. 3) After digestion the reaction volume was extracted twice with phenol, once with phenol:chloroform and once with chloroform. 4) Mussel glycogen was added to a final concentration of 0.03ug/ul. 5) NaCl was added to a final concentration of 0.1M and the DNA was precipitated by the addition of a 2.5 volume of 95% isopropanol. 6) The DNA was allowed to precipitate for one hour at -70°C, centrifuged, dried briefly, and resuspended in 200ul of TE. 7) The total volume of the resuspended DNA was restricted by the addition of 0.2 units of MboI at 37°C for 10 minutes. 8) The restriction was stopped by placing at -70°C for 10 minutes. 9) The completion of the library building was consistent with the method described above for the total genomic library. Visualization of the cloning steps by gel electrophoresis was eliminated because of the nanogram amounts of DNA predicted to be recovered from the DNA extraction procedure.

Three aliquots of the resulting library packaging extract were plated out on the permissive host NM538 and the remaining packaging extract was plated out on the restrictive host NM539. The clones growing on the permissive host represents both recombinant and nonrecombinant phage. Clones growing on the restrictive host NM539 are recombinants. The total number of independent clones was estimated to be 4×10^4. The total

number of independent recombinant phage was estimated to be 3.6×10^3. The amplified library stock was determined to contain $2.2\text{-}8.0 \times 10^7$ pfu/ml. These clones were designated as λMUGS.

The flow-sorted chromosome 15 library received from the Los Alamos National Laboratory was prepared from a human-hamster hybrid cell line containing the human chromosome 15. The library was prepared in Charon 21A yielding 1.64×10^4 independent recombinants with the most likely comtaminants to be hamster sequences. These clones were designated as LAGS.

c. Screening of amplified library in bacteriophage vector.

The amplified stock of recombinant λMUGS phage were plated at 5,000-10,000 pfu/150mm plates on lawns of NM539 and the λLAGS phage were plated onto lawns of LE392. Filter imprints were blotted onto Screen Plaque according to manufacturers recommended procedure and screened with ^{32}-P labelled total genomic DNA according to Benton and Davis (1977).

d. Isolation of bacteriophage DNA.

Bacteriophage DNA was isolated using the rapid, small scale plate lysate isolation method described by Maniatis et al. (pp. 371-372, 1982). Yields of 3mg/ml of good quality DNA was obtained from five confluent 150mm plates.

B. Results

The strategy used in characterization of clones from these two chromosome 15 libraries is a modification (Figure 1.) of the strategy described by Maniatis et al.(p.320-321, 1982). The total number of recombinant clones in the λMUGS library was estimated to be 3,600 which compares favorably with the 9,000 clones generated from a flow-sorted X chromosome library (Kunkel et al., 1982).

1. Plaque screening

Plaque screen blots (Figure 2.) were hybridized with total genomic DNA and plaque signals were scored after 24 hrs and after 5-7 days (Table II). Only those clones with no signal or very faint signal were considered to be of interest and were predicted to contain low copy or unique sequences.

2. Southern blot analysis of insert sequence type.

To more accurately classify the cloned sequence, the clones scored as no signal or faint signal by the plaque screening as well as several random clones were restricted with EcoRI, run in 1% agarose gels, and blotted onto either Hybond or GeneScreen Plus. The blots were hybridized to total genomic DNA. The recombinant phage insert size was measured from the gel for each clone and the sequence type was recorded (Table III.) from the autoradiograph (Figure 3.). The labeled DNA was removed from the blots with λLAGS clones and reprobed with total genomic hamster for the

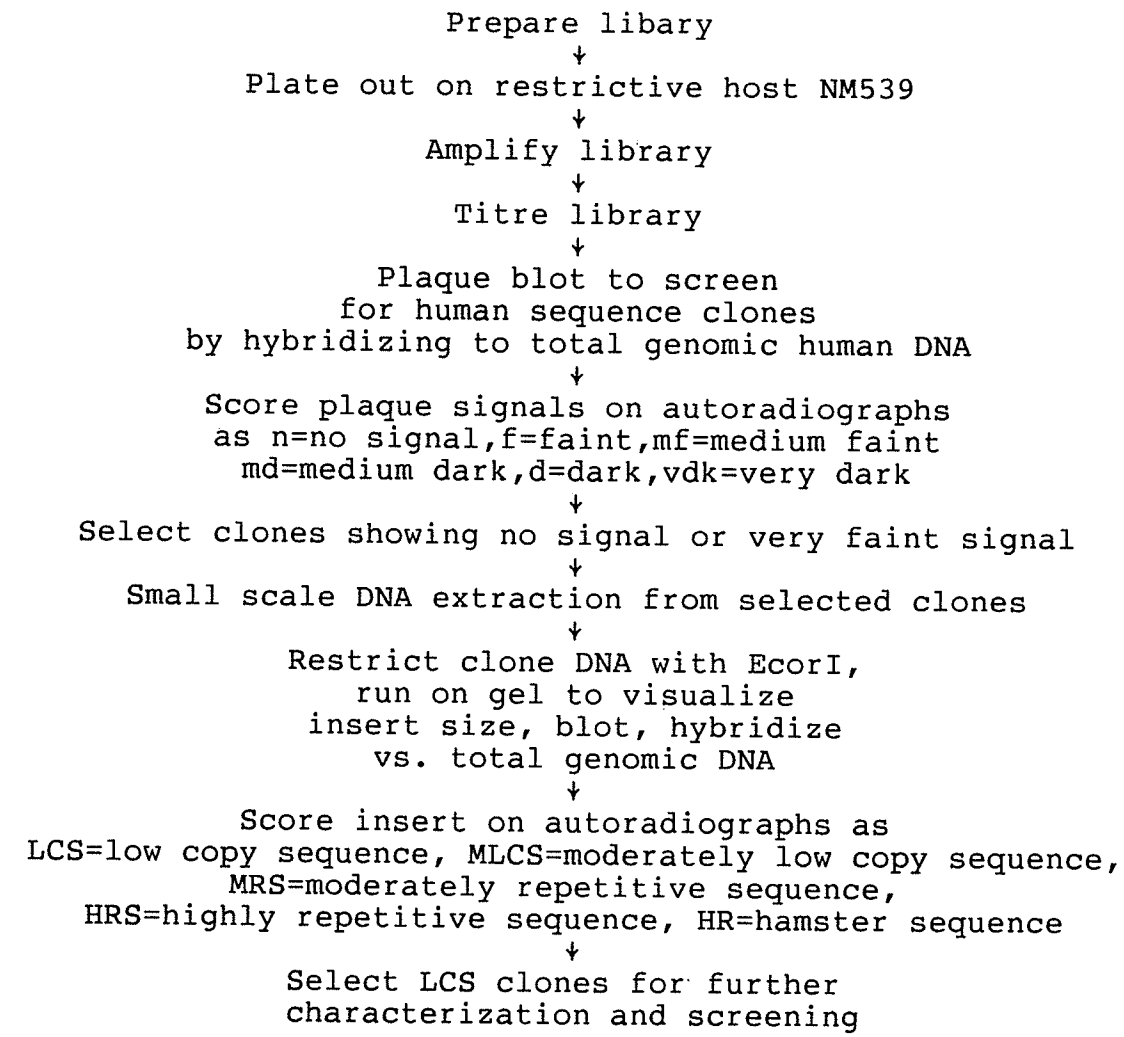


Figure 1. Flow diagram identifies the steps involved in screening and characterizing two chromosome 15 libraries (λLAGS & λMUGS).

Figure 2. Autoradiograph of a typical plaque blot hybridizided to total genomic human DNA illustrating the scoring method employed for plaque screening; abbreviations: n=no signal, f=faint, mf=medium faint, m=medium, md=medium dark, d=dark, vdk=very dark. Exposure time was 48h at -70°C with one intensifying screen.

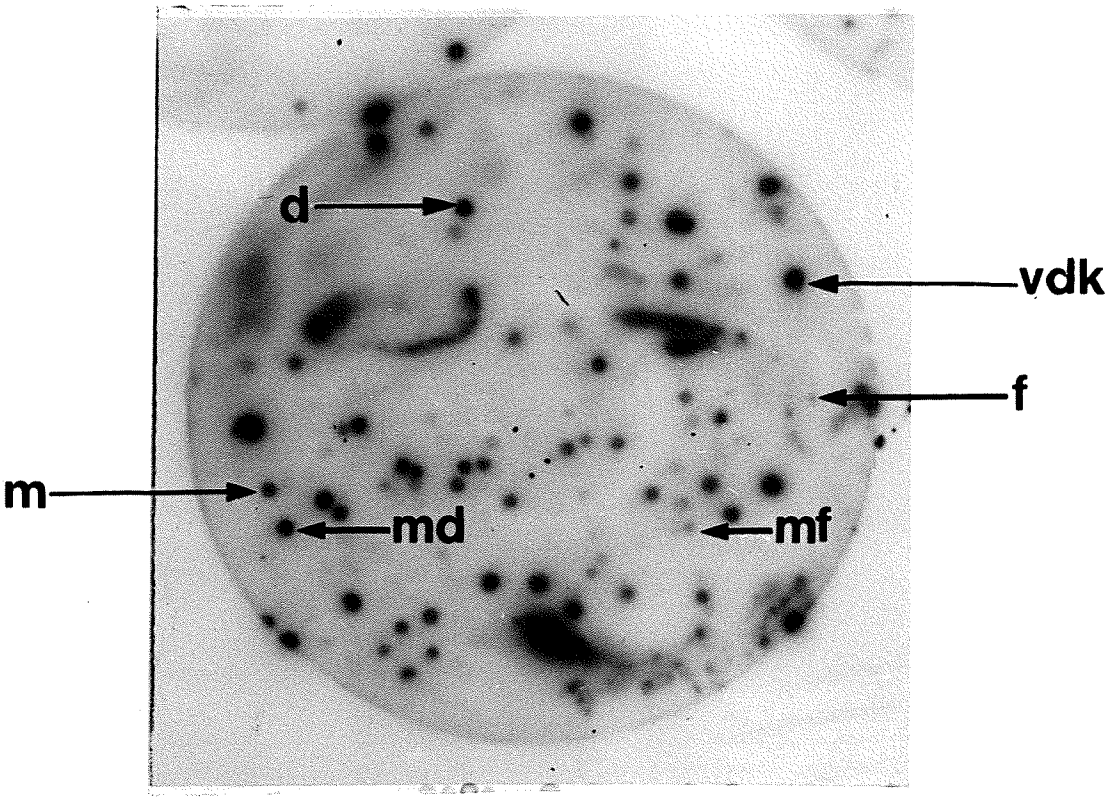

d
vdk
f
m
md
mf

Table II. Summary of results of plaque screening for human insert DNA as detected by hybridization to total genomic human DNA and determination of insert sequence type.

Plaque signal λLAGS15 clones after 50 hours	Plaque signal λMUGS15 clones after 5 days
None A4,5,8,9,11,12;B6,7	2,3,4,5,7,12,15,16,17,37,45,46
Faint A1,2,3,6,7,13,B3,9 2C5,2C9	1,6,9,11,13,19,21,22,25,27,31,32, 34,36,41,42,49,50,52-66
Medium A10,2B1,2C2,2C4 Faint	10,14,23,24,26,28,29,33,39,40,47-48
Medium 2C3	
Dark 2C6	8,18,20,30,35,38
Very dark 2C8	43,44,51

Table III. Results of screening insert for size* and sequence type as determined by hybridization to total genomic DNA.

Low copy sequence(LCS)=12
 Plaque signal:
 1/12 very dark
 8/12 none
 3/12 faint

Medium low copy sequence(MLCS)=2
 Plaque signal:
 1/2 faint
 1/2 none

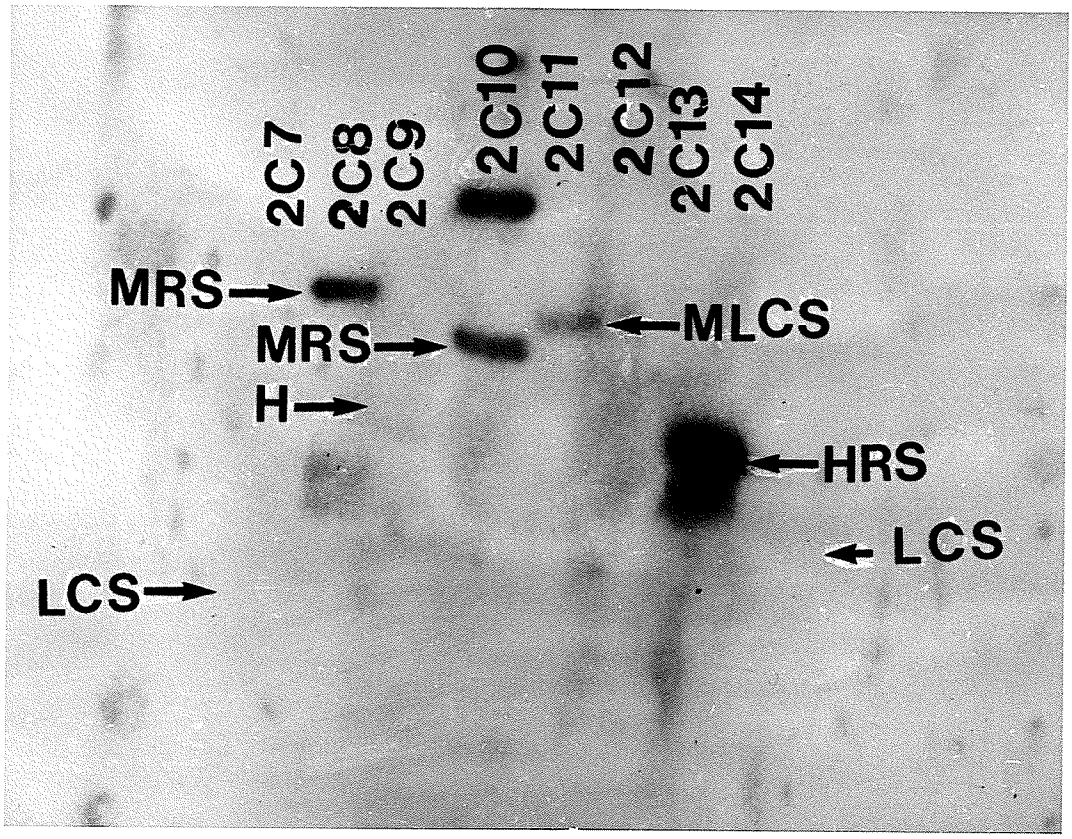
Moderately repetitive sequence(MRS)=5
 Plaque signal:
 1/5 medium faint
 1/5 medium dark
 1/5 dark
 2/5 none

Highly repetitive sequence(MRS)=3
 Plaque signal:
 1/3 medium
 1/3 medium faint
 1/3 none

Hamster sequence(HS)=2
 Plaque signal:
 1/2 dark
 1/2 faint

*Average insert size=3.8±1.9 n=27

Figure 3. Autoradiograph of a Southern blot of eight clones hybridized to total genomic human DNA illustrating the scoring of insert sequence type. Exposure time was 48h at -70°C using one intensifying screen. Abbreviations:LCS=low copy sequence, MLCS=medium low copy sequence, MRC=moderately repetitive sequence, HRS=highly repetitive sequence, H=hamster(Scored from blot reprobed with total genomic hamster DNA).


2C7
2C8
2C9
2C10
2C11
2C12
2C13
2C14
MRS
MRS
H
MLCS
HRS
LCS
LCS

detection of contaminating hamster sequences. The plaque signal of each clone and the resulting analysis of the insert sequence type by Southern blot analysis was compared (Table IV).

The scoring of the insert sequence type of a clone by the plaque signal was found to be less sensitive than by Southern blot analysis of the insert. Therefore, a second round of screening is required if only low copy or unique sequence clones are to be selected for further characterization. It was also determined that clones yielding no plaque signal when hybridized to total human genomic DNA were sometimes found to be hamster sequences.

3. Screening λLAGS15 clones and λMUGS clones for overlapping and homologous sequences to loci **D15S10** (pTD 3-21), **D15S9** (pML34), **D15S11** (IR4-3), and **D15S12** (IR10-1).

The four genomic DNA probes pTD3-21, pML34, IR4-3, and IR10-1 were used to screen the partially characterized clones and amplified library stock from the λMUGS library and the λLAGS library to detect overlapping sequences. To expedite the screening process, a dot blot of 57 clones was prepared using a dot blot apparatus. The procedure used for preparation of the blot was as described by the manufacturer of Hybond for dot blotting. The dot blot was hybridized sequentially using insert DNA from each of the genomic probes pTD3-21, pML34, IR10-1, and IR4-3. Blots were washed at high stringency with the final wash being 1h at 65°C in

Table IV. Comparison of plaque signal/Southern blot signal as determined by hybridization to total genomic DNA

Plaque Signal	Insert Sequence Type LCS	MLCS	MRS	HRS	H
Faint or none n=17	11	2	2	1	1
Medium faint n=2			1	2	
Medium n=1				1	
Medium dark n=1			1		
Dark n=2			1		1
Very dark n=1	1				

Abbreviations:LCS=low copy sequence, MLCS=medium low copy sequence, MRC=moderately repetitive sequence, HRS=highly repetitive sequence, H=hamster(Scored from blot reprobed with total genomic hamster DNA).

0.1XSSC, 0.1% SDS.

No strong responses were detected but a number of clones responded with a faint signal. Those clones showing a faint response were further screened by Southern blot analysis and no responses were detected. At the same time that the partially characterized clones were screened, blots of amplifified library stocks were screened with pTD3-21, IR4-3, pML34, and IR10-1. Approximately 100,000 clones were screened but no strong signals were detected.

C. Conclusions

Clones from two chromosome 15 libraries were characterized and screened for low copy sequence clones and for overlapping sequences with the genomic probes pTD3-21, pML34, IR10-1, and IR4-3. No clones with overlapping sequences were detected. The false positive weak signals on the dot blot was probably slight homology of pBR322 to the bacteriophage vectors either EMBL3 or Charon 21A. Kunkel et al. (1985a) report a similar response and suggest that slight homology of Charon 30 phage to pBR322 accounts for the phenomena. Other investigators (Mayal, personal communication,1987) have reported a similar response when screening a total genomic DNA library in Charon 21A with a unique human DNA probe.

Recently Donlon et al. (1986) report that clones from the proximal region of 15q might be eliminated by a screening process that only considers negative plaques or

clones which are negative on Southern blot analysis. Most clones isolated from the library that mapped to 15q11-13 were not low copy sequence clones but were moderately repetitive with some showing inverted repeats. Therefore, a reverse screening strategy in which clones with repetitive sequences are selected for further characterization might yield more clones which map to the proximal region of 15q.

IV. THE CHARACTERISTICS OF FOUR LOCI D15S9, D15S10, D15S11, AND D15S12 AS RECOGNIZED BY GENOMIC DNA PROBES pTD3-21, pML34, IR4-3, AND IR10-1, RESPECTIVELY.

A. Introduction

The genomic DNA probes pML34, pTD3-21, IR4-3, and IR10-1 were received from Drs. S. A. Latt and T. D. Donlon at the Children's Hospital in Boston. The isolation of inverted duplicated chromosome 15's by flow sorting for use in preparation of chromosome 15 library enriched for 15q11-13 sequences from which these clones were prepared is described by Lalande et al. (1985). The cloning of the library from which these clones were isolated is described by Donlon et al. (1986). Briefly pTD3-21 is a 2.2Kb insert in the HindIII site of pBR322 which recognizes a 2.2Kb HindIIII fragment in genomic DNA; pML34 contains a 6.4Kb HindIII insert cloned into the HindIII site of pBR322 which recognizes a 6.4Kb fragment in genomic DNA; subclone IR10-1 is a 0.8Kb HindIII-BamHI fragment of IR10 subcloned into the polylinker of pUC18 and recognizes a 3.8Kb HindIII fragment; and IR4-3 a 0.5Kb fragment of probe IR4 subcloned into the polylinker of pUC18 recognizes a 4.6/4.8Kb doublet in HindIII digest genomic DNA as well as homologous fragments of unknown origin below 2Kb and between 15-20Kb.

B. Confirmation mapping of loci D15S9 (pML34), D15S10 (pTD3-21), D15S12 (IR10-1), and D15S11 (IR4-3) to chromosome 15.

The mapping of each locus was accomplished by hybridizing the human insert DNA from each plasmid clone (pTD3-21, pML34, IR10-1, and IR4-3) to a Southern blot of a panel of hybrid cell lines containing various complements of human and hamster chromosomes. The autoradiography of the Southern blot of the hybrid panel was scored by indicating if a hybridization signal was present or absent in each lane of DNA (Figure 4.). Identical results were obtained for all loci (Table V.). Four discordancies were noted in the scoring of cell lines 79.05b, 134.02a, 84.16a, and 190.03; cell lines 79.05b, 134.02a, and 85.16a were scored as no signal on the Southern blot but contain 1, 2, or 3 copies of chromosome 15 in 20 cells. Cell line 190.03 with unidentifiable human chromosome fragments was scored as having a hybridization band so the possibility exists that a portion of the proximal region of chromosome 15 is present. These four cell lines, however, were negative for chromosome 15 isozymes. Thus, concordancy was 100% to lines containing chromosome 15s and positive for chromosome 15 isozymes.

Therefore, probes pML34, pTD3-21, IR4-3, and IR10-1, refining loci D15S9,10,11,and 12 were confirmed to map to chromosome 15. Donlon et al. (1986) reported that all four loci were deleted in a cell line with a 15q11.2 deletion.

Figure 4. Autoradiograph of hybrid panel Southern blot hybridized vs pTD3-21 (D15S10). Each lane contains 5ug of hybrid DNA restricted with HdIII. The identity of the DNA in each lane is as follows: 1)41.06, 2)45.01, 3)45.43, 4)76.14, 5)76.31, 6)76.33, 7)79.05b, 8)80.05b, 9)80.14c, 10)80.17a, 11)82.82a, 12)85.16a, 13)89.27, 14)100.02b, 15)102.05b, 16)103.04 17)111.02a, 18)112.10a, 19)120.33, 20)120.35, 21)133.05, 22)134.02a, 23)1103 hamster, 24)P23 human placental DNA. Each lane is scored as + or - indicating presence of hybridization to the human-hamster cell line DNA. The tabulation of the mapping results based on these data is displayed in Table V.

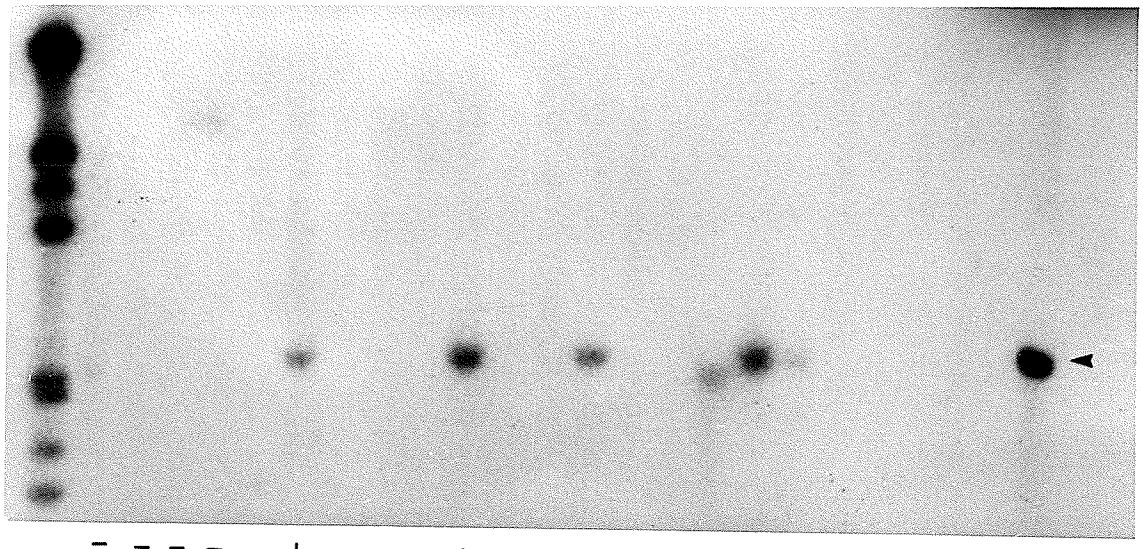

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Table V. Segregation of human sequences homologous to D15S10.

Cell Line	Response to D15S10	Identifiable, intact human chromosome[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
41.06	+	-	-	+	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+	-	+	-	-	+	-
45.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
45.43	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	-
76.14	-	-	-	+	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+	-	+	+	-	+	+
76.33	+	+	-	+	+	+	-	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	-
79.05b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	+	-
80.05d	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	+	+
80.14c	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	-
80.17a	+	-	-	-	+	+	-	-	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	-
82.82a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
85.16a	-	-	+	+	+	+	-	-	+	-	+	+	-	+	-	+	-	+	-	-	-	+	-	+	-
89.27a	+	-	-	-	-	+	-	-	-	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-
100.02b	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-
102.05b	-	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	+	-	-
103.04	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	-
103.07b	-	-	-	+	-	+	+	-	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	-	+
111.02a	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	+	-	-
120.33	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	+	+	-	+	-	+	+	+	-
120.35	-	-	-	+	-	+	+	-	+	-	-	-	+	+	+	-	-	+	+	+	-	-	+	+	-
133.05	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
134.02a	-	+	-	-	+	+	-	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-
190.03	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
76.31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Percent discordancy		35	35	48	26	30	57	49	39	30	39	43	26	43	43	17	43	57	39	35	39	52	39	61	43

[a]Method by which the chromosome content of each cell line was determined has been reported by Riddell et al. (1985).

Since these data are consistent with the chromosome mapping of Donlon et al, these four loci were believed to map to 15q11.2-12.

C. Strategy for detecting and characterizing restriction fragment length polymorphism(RFLP's)

1. Introduction

The strategies developed by Aldridge et al. (1984) were used to detect restriction fragment length polymorphisms (RFLP's). If a probe is going to be useful for the analysis of a disease locus, it is useful to have a battery of enzymes that can be used that reveal RFLP's at a given locus. In the case of PWS where only small nuclear pedigrees are available, a high frequency of variants in a two-allelle polymorphism is required so that segregation of the alleles can be followed through a small pedigree for linkage, deletion detection, and detection of the parental origin of the aberrant chromosome. Most RFLP screening strategies have recommended large screening panels of DNA cut with a few restricion enzymes (Skolnick and White, 1982) thus ensuring that low-frequency polymorphisms will be detected. Aldridge et al. (1984) proposed using a very small screening panel(2 females, 1 male) to detect RFLP's at previously characterized X-loci, a strategy that favors the detection of high-frequency two-allele polymorphisms. The hemizygous male in the case of X-linked probes ensures that high frequency polymorphisms which might go undetected in a

small number of females are flagged. The use of a hybrid cell line hemizygous for chromosome 15 would have been another way to be able to flag high frequency minor allelles if small sample size was employed. This was not used in this study.

The strategy used in the present study for the detection of polymorphisms employed a larger sample size thereby decreasing the chance of missing a high frequency 2-allelle polymorphism. A panel of DNA from seven unrelated individuals was restricted with ten enzymes allowing four enzymes to be screened by a single hybridization. Thus, the probability of detecting RFLP's with a minor allelle with a frequency of 0.3 is greater than 95% and there is a probability of 90% of detecting a minor allele with a frequency of 0.2 (Aldridge et al., 1984). The disadvantage of this approach is the possibility that low-frequency minor alleles will be detected. Such minor alleles will have limited use for studying PWS patients and their families. On the other hand, because of the limited number of probes mapped to 15q11-13 this approach increases the chance of detecting a higher number of RFLP's at a given locus and may increase the probability of detecting informative RFLP's.

The restriction enzymes selected for studying were (i) enzymes which had been reported by Aldridge et al. (1984) to have detected polymorphisms for chromosome X and chromosome 13 loci (ii) enzymes with recognition sites containing CpG

or TpG doublets or (iii) enzymes recognizing a 3 purine/3 pyrimidine string. The eight enzymes declared by Aldridge et al. (1984) to be most likely to detect polymorphisms were MspI, TaqI, PstI, PvuII, BglI, EcoRI, XmnI, and HindIII.

2. Results of screening for RFLP's and restriction fragment lengths detected at loci **D15S9** (pM134), **D15S10** (pTD3-21), **D15S12** (IR10-1), and **D15S11** (IR4-3).

The four probes pML34, pTD3-21, IR10-1, and IR4-3 were used to screen for RFLP's at each locus using a panel of genomic DNA(placental) from seven unrelated individuals restricted with the eight enzymes MspI, TaqI, PstI, PvuII, BglI, XmnI, EcoRI, and HindIII. The restriction fragment sizes were determined using the method of Southern (1979). When DNA fragments are electrophoresed at high voltage the curvature in the plot of log L(fragment size) against m(mobility) is exaggerated as plots of L against 1/m. Southern (1979) determined that the data could be made to fit a straight line if L was plotted against $1/(m-m_o)$ where m_o is a factor calulated to give the best fit to a straight line. Linear relationships for double-stranded DNA's have been demonstrated by Southern's method in the range 10-50Kb in 0.5% agarose, 1-20Kb in 1% agarose gels, and 0.2-6Kb in 2% agarose gels. Formulas from Southern (1979) were used and three standard points (phage restriction fragment lengths) that span the range of and as close as possible to the unknowns that were to be measured were selected for m_1,

m_2, and m_3. Direct calculation of L from measurements of m and vice versa were calculated using the following formulas:

$$L=k_1/(m-m_o) + k_2$$

$$k_1=L_1-L_2/\ 1/(m_1-m_o) - 1/(m_2-m_o)$$

$$k_2=L_1-k_1/(m_1-m_o)$$

$$m_o=m_3-m_1A/1-A$$

$$A=L_1-L_2/L_2-L_3\ X\ m_3-m_2/m_2-m_1$$

Using values k_1, k_2, and m_o values, L(unknown) can be calculated for any measured mobility within a 2% error (Table VI.).

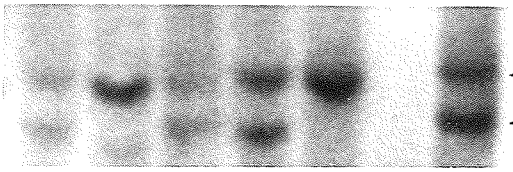
Apparent TaqI and XmnI RFLP's were detected at locus D15S10; EcoRI and TaqI RFLP's at locus D15S12; and a PvuII RFLP at locus D15S11. ScaI RFLP's at loci D15S9 and D15S12 and a RsaI RFLP at locus D15S11 are reported by Nicholls et al. (1987). To verify polymorphisms as segregating alleles in a pedigree, four sets of control couples were used to detect informative matings and family blots were prepared from informative matings. The frequency of major and minor alleles were determined from autoradiographs of the blots of the original screening panels (Figures 5a.& 6a.). The autoradiographs of family blots demonstrated the segregation of the alleles at D15S11 (IR4-3)(Figure 5b.) and at D15S10 (pTD3-21) (Figure 6b.) in a Mendelian fashion. The minor alleles for the XmnI fragments at locus D15S10 and the TaqI and EcoRI fragments at locus D15S12 were uninformative in

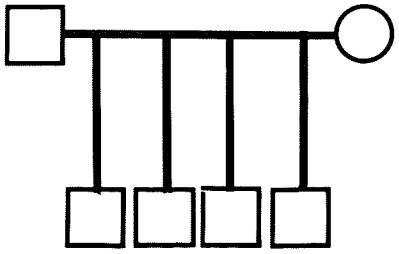
Table VI. Restriction fragment lengths detected at loci D15S10, D15S12, D15S11 & D15S9 as defined by genomic DNA probes pTD3-21,IR10-1, IR4-3 and pML34.

Probe	Locus	Enzyme	Fragment Sizes
3-21	D15S10	HindIII	2.2kb
		BamHI	2.8kb
		PstI	9.0, 1.6kb
		PvuII	12.0kb
		EcoRI	18.0, 8.0kb
		MspI	15.0kb
IR10-1	D15S12	HindIII	3.8kb
		BamHI	18kb
		PstI	1.28, 0.96kb
		PvuII	1.95kb
		MspI	1.0kb
		XmnI	4.0kb
		BglI	1.95kb
IR4-3	D15S11	HindIII	4.8/4.6kb
		BamHI	>21.7kb
		PstI	9.0,5.1,3.6,3.2kb
		EcoRI	15,12,3,2.5kb
		MspI	6.0,1.3kb
34	D15S9	HindIII	6.4kb
		BamHI	12.0kb
		PstI	8.0,3.0kb
		PvuII	4,2.8,0.59,0.5kb
		MspI	3.4,3.2,0.96kb
		TaqI	5.4kb
		XmnI	4.2,1.7,0.9kb
		BglI	3.6,3.4kb

Figure 5. (A) Autoradiograph of Southern blot of PvuII restricted genomic DNA of six unrelated individuals hybridized vs IR4-3 (D15S11). Allele 1=9.4Kb, Allele 2=7.1Kb; frequency of Allele 1=7/12(0.6) and Allele 2=5/12(0.4); Exposure time was 72h at -70°C using two intensifying screens. (B). Autoradiograph of Southern blot of DNA from five family members showing segregation of PvuII alleles at D15S11 (IR4-3). Father's genotype is A1, mother is A2, and sons are all heterozygous A1A2. A constant band is present just below the A2 hybridization band showing as a doublet in the sons and the mother since they have an A2 allele but as a single band in the father since he does not have an A2 allele.

A.





B.

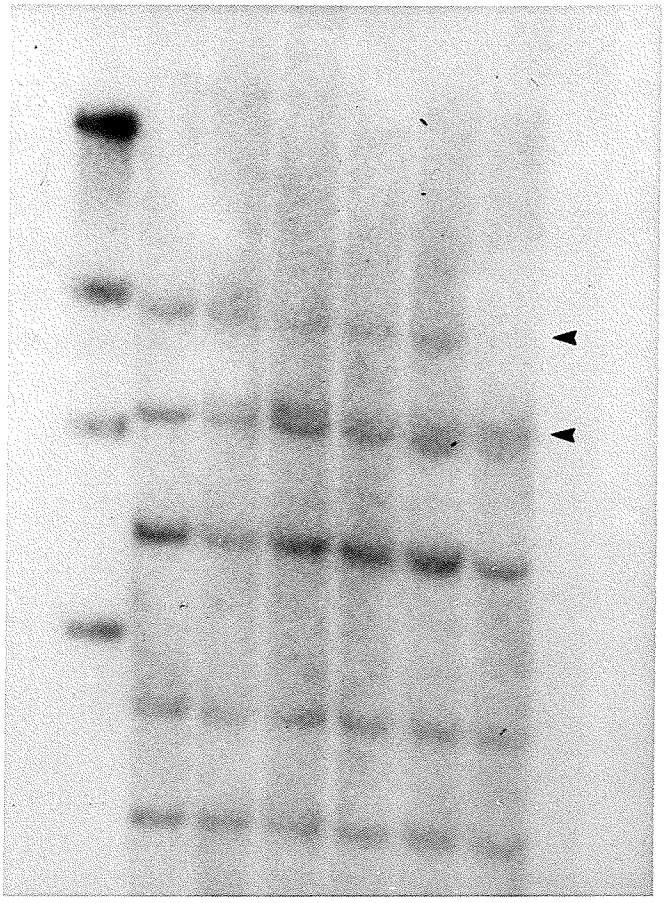
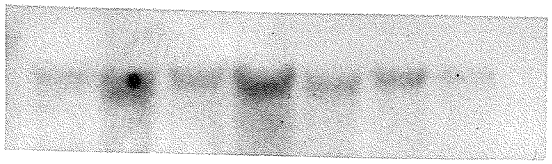
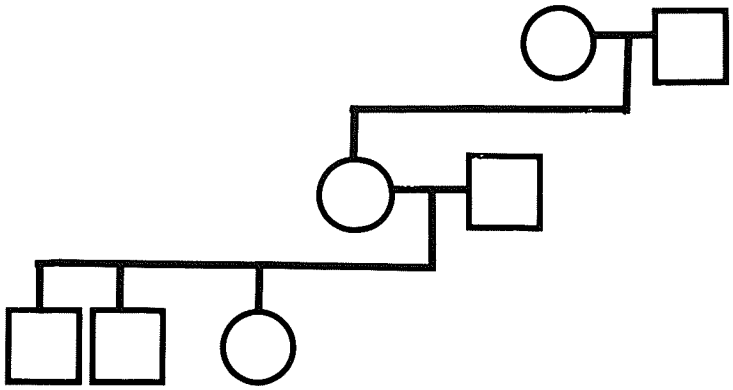


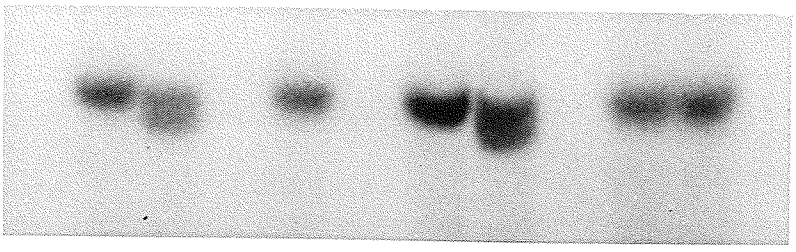
Figure 6. (A) Autoradiograph of Southern blot of TaqI restricted DNA of seven unrelated individuals hybridized vs pTD3-21 (D15S10). Allele 1=9.0Kb, Allele 2=8.8Kb; Allele 1 frequency=0.86 and Allelle 2=0.14; Exposure time was 24h at -70°C using two intensifying screens. (B) Autoradiograph showing segregation of TaqI alleles at D15S10 (pTD3-2). Maternal grandparents genotypes are A1, mother is A1, father is A1A2, one daughter and one son are A1 and one son is A1A2.

A.





B.



test families and were not further analyzed as variants occur with such low frequency so as to make them of little use in PWS families.

Polymorphism information content(PIC) was calculated for each marker locus according to the method described by Botstein et al. (1980)(Table VII).

D. Restriction maps of region containing each locus D15S9, D15S10, D15S11, and D15S12.

1. Introduction

A preliminary restriction map was constructed using the cloning information from Donlon et al. (1986) and the restriction fragment length data described above. To better define the locus, double digests of genomic DNA were used to determine the position of restriction sites relative to the cloned segment within the locus.

A restriction map of each locus (D15S9, D15S10, D15S11, and D15S12) was predicted to be useful in the analysis of molecular rearrangements at these loci in PWS patients. The detection of some large restriction fragments detected at some of the loci made it possible to examine a fairly large area surrounding each locus for molecular changes at the PWCR in PWS patients as well as the possibility of comparing the four loci for similarities or overlapping segments.

Preliminary studies indicated that double digests using various combinations of the five enzymes HindIII, EcoRI,

Table VII. RFLP's at loci D15S10, D15S12, D15S11, & D15S9.

Probe	Symbol	Enzyme	Constant Band(Kb)	Allele Symbol	Size(Kb)	Frequency	PIC	Number of Chrs.
3-21	D15S10	TaqI[1]	0.59	A1	9.0	0.85	.25	34
				A2	8.8	0.15		
		XmnI	3.0	A1	1.33	0.07	.13	14
				A2	1.23	0.93		
IR10-1	D15S12	EcoRI		A1	12.2	0.1	.18	20
				A2	12.0	0.9		
		TaqI		A1	3.2	0.93	.13	14
				A2	3.0	0.07		
		ScaI[2]		A1	17.5	0.18	.14	28
				A2	16.0	0.68		
				A3	12.5	0.14		
IR4-3	D15S11	PvuII	6.8	A1	9.4	0.54	.48	28
			5.1	A2	7.1	0.46		
			3.6					
			3.2					
			1.9					
		RsaI[2]		A1	1.2	0.5	.47	28
				A1	1.0	0.5		
		BglI	18.0	A1	10.0	0.93	.13	14
			3.6	A2	8.0	0.07		
		XmnI		A1	9.0	0.93	.13	14
				A2	6.0	0.07		
34	D15S9	ScaI[2]	10.0	A1	6.5	0.71	.33	28
				A2	6.3	0.29		

[1]RFLP was reported by Latt HGM9, 1987; and Gregory and Hamerton,Abst.CSG, 1987; [2]Latt, HGM9, 1987.

TaqI, XmnI, and PstI would yield a reasonable map of each locus.

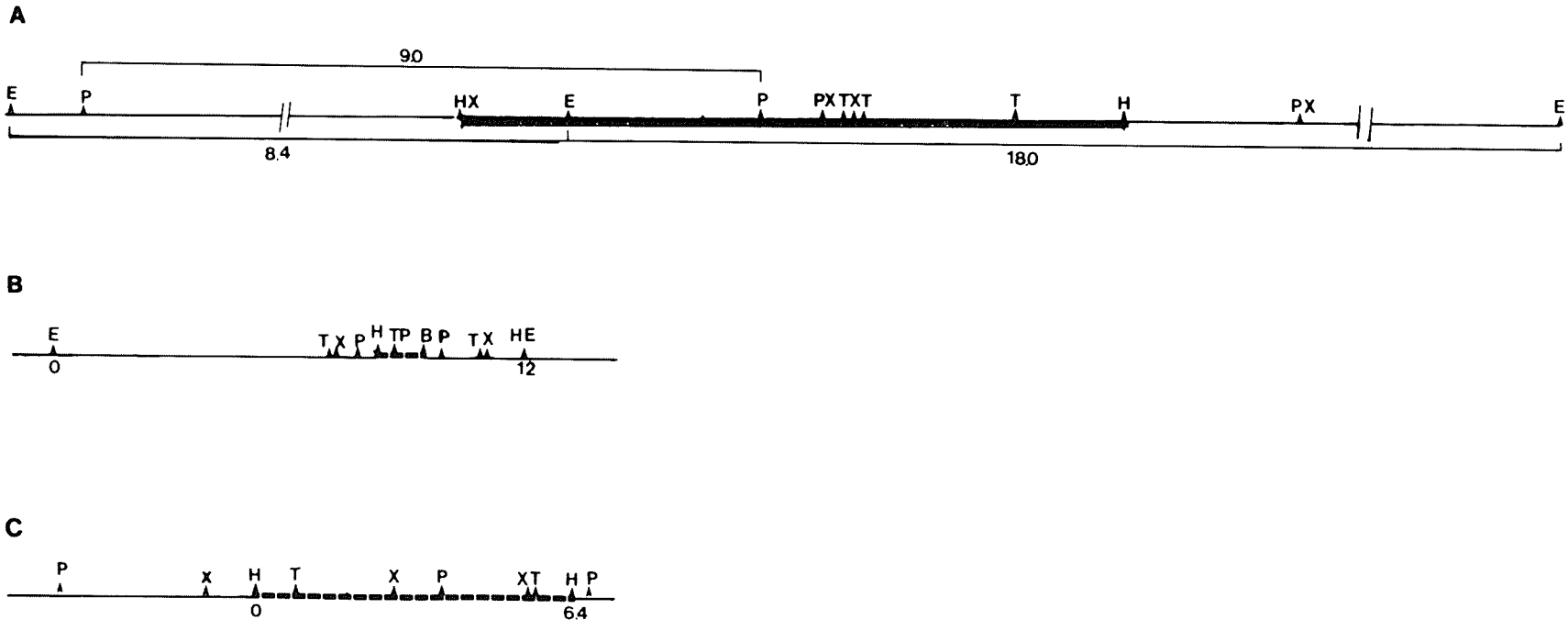
2. Procedures used for restriction analysis of loci.

The digests of 5 ug of DNA were set up as follows: 1)HindIII; 2)XmnI; 3) TaqI; 4) EcoRI; 5)PstI; 6) PstI/XmnI; 7) PstI/EcoRI; 8)TaqI/EcoRI; 9)TaqI/PstI; 10)TaqI/XmnI; 11)HindIII/EcoRI; 12)HindIII;PstI; 13)HindIII/XmnI; and 14) HindIII/TaqI. Digests using two enzymes were done simultaneously if the buffers required were compatible; in cases in which the enzyme buffers were not compatible, low salt requiring enzymes were used first and upon completion the salt concentration requirements of the second enzyme were adjusted and restriction completed.

3. Results

A partial restriction map of each locus D15S10, D15S12, and D15S9 was prepared (Figure 7). Some fragments were not resolved using routine gel electrophoresis but were inferred from other fragment sizes. A restriction map of the locus D15S11 as defined by IR4-3 is most difficult to assess and is not completely resolvable. According to the information supplied by Donlon et al.(1986), IR4-3 is a 0.4Kb BamHI/HindIII segment subcloned from IR4 into pUC18 by the addition of a polylinker and the insert is released by a HindIII/EcoRI digest. The original IR4 clone contained repetitive DNA with AluI repeats. The restriction fragment

Figure 7. Partial restriction map of locus (A) D15S10 (pTD3-31), bold line indicates the 2.2Kb cloned fragment; (B) D15S12 (IR10-1), 0.8Kb subcloned fragment BamHI/EcoRI indicated by bold dashed line; (C) D15S9 (pML34). 6.4Kb HindIII fragment indicated by bold dashed line.


A
9.0
E
P
HX
E
P
PX TXT
T
H
PX
E
8.4
18.0
B
E
T X P H TP B P T X HE
0
12
C
P
X
H
T
X
P
XT
H
P
0
6.4

lengths detected by IR4-3 in genomic DNA suggest that IR4-3 recognizes more than one locus. The size of the cloned insert is too small to span a locus as large as is indicated by the size and number of fragments recognized (Table VI.). Perhaps the locus recognized by the cloned segment is held within a sequence of DNA that is repeated. The presence of AluI repeats and inverted repeats in the segment from which IR4-3 was subcloned supports the possibility that a repeated segment might be present.

E. Discussion

A disease locus may be most easily analyzed when numerous polymorphic loci map to the appropriate chromosome region and can be shown to be linked to the locus in question. In the case of the PWCR locus the four loci that map to the chromosome region 15q11-12 have nine codominant alleles cosegregating in a Mendelian fashion. None of the four loci mapped to 15q11.2-q12 characterized here are highly informative (PIC > 0.5), but four are reasonably informative (0.5 >PIC> 0.25), and five are slightly informative (PIC < 0.25). However more than one RFLP is defined for three of the four loci increasing their usefulness for analysis of the PWCR in PWS patients.

The immediate objectives of this study were to characterize the locus defined by each of the four probes pTD3-21, pML34, IR10-1 and IR4-3 so that molecular rearrangements in the genomic DNA of PWS patients could be

detected by changes in restriction fragment lengths, to analyze PWS patients by using RFLP data at each locus, and to use densitometric analysis to detect deletions or duplications. With a limited number of probes mapping to a chromosome region that spans as much as 9.0Mbp of DNA it was decided to first analyze the critical region in PWS patients to determine if the loci D15S9, D15S10, D15S11, and D15S12 are deleted in patients with cytogenetic aberrations of 15q11-13 and to determine if submicroscopic deletions are present in karyotypically normal patients. The determination of restriction fragment lengths at each locus in normal controls could be used to detect molecular rearrangements in PWS patients that result from a change in a restriction site.

VI. RFLP STUDIES OF TWO PWS PATIENTS AND THEIR PARENTS.

A. Introduction

The four genomic DNA probes pML34, pTD3-21, IR4-3, and IR10-1 detect RFLP's at loci D15S9, D15S10, D15S11, and D15S12 at PIC values of 0.5 to <0.25 (See above). Thus the chance of detecting a useful RFLP in a given family with a patient with PWS for the purpose of detecting molecular aberrations or to determine parental origin of aberrant chromosome 15 is limited. Since at least one normal chromosome 15 has been reported to be found in all PWS patients, the detection of a deletion event at a given locus is dependent on finding homozygosity of one allele at that locus in the parent transmitting the aberrant chromosome 15. Using a modification of the mating tables of Botstein et al. (1980) the probability of offspring being informative for deletion detection can be calculated (Table VIII.)

B. Results of testing of loci D15S10 (pTD3-21), D15S9 (pML34), D15S12 (IR10-1), and D15S11 (IR4-3) for detection of deletions and parental origin of aberrant chromosome 15.

Two nuclear families were available for study. DNA's isolated from the patient and both parents were restricted, blotted onto GeneScreen Plus and hybridized as described above. The genotypes of probands #1 and #6 and each of their parents were determined by Southern blot analysis (Figure 8.) for the TaqI alleles at locus D15S10, the ScaI

Table VIII. The probability of deletion detection in PWS offspring as determined from mating types** of parents at D15S10, D15S12, D15S11, and D15S9.

Locus	Genotype of parent donating aberr.chr**	Genotype of mate	Genotype of offspring
D15S10 (TaqI) A1(9.0Kb) A2(8.2Kb)	A1*A1 or A2*A2	A1A2 or A2A2 or A1A1	A1*A2 or A2*A1
Probability of offspring being informative:			(0.85)(0.15)+ (0.15)().85)=0.24
D15S10 (XmnI) A1(1.33) A2(1.23)	A1*A1 or A2*A2*	A1A2 or A2A2 or A1A1	A1*A2 for A2*A1
Probability of offspring being informative:			(0.07)(0.93)+ (0.93)(0.07)=0.14
D15S12 (EcoRI) A1(12.2) A2(12.0)	A1*A1 or A2*A2	A1A2 or A2A2 or A1A1	A1*A2 or A2*A1
Probability of offspring being informative:			(0.1)(0.9)+ (0.9)(0.1)=0.18
D15S12 (TaqI) A1(3.2) A2(3.0)	A1*A1 or A2*A2	A1A2 or A2A2 or A1A1	A1*A2 or A2*A1
Probability of offspring being informative:			(0.93)(0.07)+ (0.07)(0.93)=0.14
D15S12 (ScaI) A1(17.5) A2(16.0) A3(12.5)	A1*A1 or A2*A2 A3*A3 or A1*A2 A1*A3 or A2*A3	A2A2 or A1A3 A1A1 or A2A2 A3A3 or A1A2	A1*A2 or A2*A1 A2*A3 or A3*A1 A3*A2 or A1*A3
Probability of offspring being informative:			(0.18)(0.68)+ (0.68)(0.14)+ (0.14)(0.18)=0.25

Locus	Genotype of parent donating aberr.chr**	Genotype of mate	Genotype of offspring
D15S11			
PvuII	A1*A1 or A2*A2	A1A2 or A2A2	A1*A2 or A1A2*
A1(9.4)		or A1A1	
A2(7.1)			
Probability of offspring being informative:			(0.54)(0.46)+ (0.46)(0.54)=0.5
D15S11			
RsaI	A1*A1 or A2*A2	A1A2 or A2A2	A1*A2 or A2*A1
A1(1.2)		or A1A1	
A2(1.0)			
Probability of offspring being informative:			(0.5)(0.5)+ (0.5)(0.5)=0.5
D15S11			
XmnI	A1*A1 or A2*A2	A1A2 or A2A2	A1*A2 or A2*A1
A1(9.0)		or A1A1	
A2(6.0)			
Probability of offspring being informative:			(0.93)(0.07)+ (0.07)(0.93)=0.13
D15S11			
BglI	A1*A1 or A2*A2	A1A2 or A2A2	A1*A2 or A2*A1
A1(10.0)		or A1A1	
A2(8.0)			
Probability of offspring being informative:			(0.07)(0.93)+ (0.93)(0.07)=0.13
D15S9			
ScaI	A1*A1 or A2*A2	A1A2 or A2A2	A1*A2 or A2*A1
A1(6.5)		A1A1	
A2(6.3)			
Probability of offspring being informative:			(0.71)(0.29)+ (0.29)(0.71)=0.41

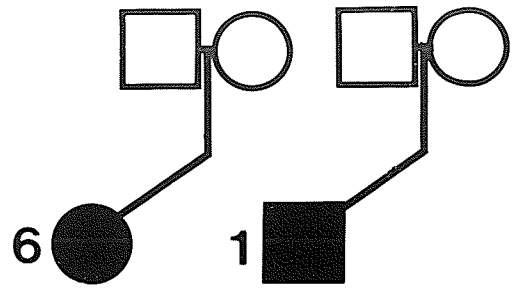
*Aberrant chromosome 15
**Modification of mating tables of Botstein et al., 1980.

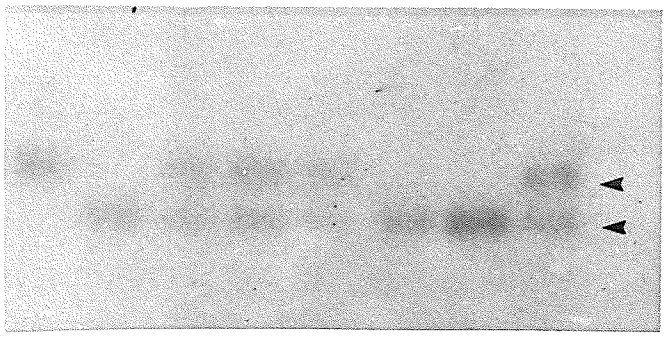
alleles at locus D15S9 and D15S12, and the RsaI alleles at locus D15S11 (Table IX).

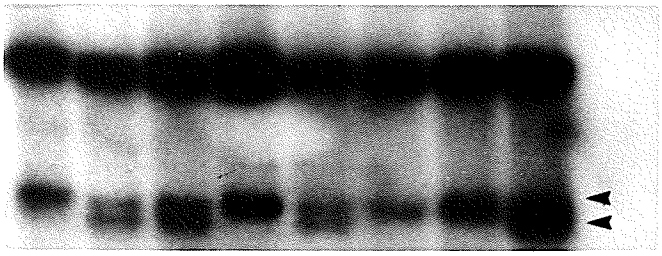
From the family data locus D15S10 was shown not to be deleted in proband #1 as he was heterozygous, locus D15S11 (IR4-3) was not deleted in proband #1 (MO) as he was heterozygous but was deleted in proband #6 (EB)and the origin of the aberrant chromosome 15 was determined to be paternal. The proband #6 (EB) has one RsaI allele size 1.2Kb (A1) at locus D15S11, her father (RB) is homozygous A2(1.0kb band), and her mother (LB) is heterozygous A1A2 Figure 8.). Since the father is homozygous A2 and the proband does not have an A2 allele, a deletion of the locus D15S11 must have occurred on the paternally transmitted chromosome 15. This evidence that the deleted chromosome 15 is of paternal origin in PWS patient #6 agrees with reports in the literature that 19/20 aberrant chromosome 15's as evidenced by cytogenetic analysis in PWS patients are paternally derived (Butler & Palmer,1983; and Niiakawa & Ishikiriyama, 1985).

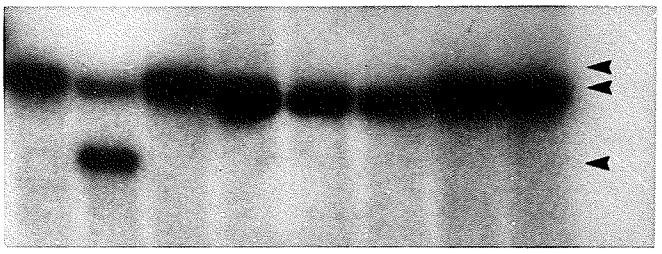
The loci D15S9 and D15S12 were uninformative in both probands. The lack of heterozygosity neither confirms nor rejects the possibility of a molecular deletion at these loci. Since hemizygosity could not be detected in either patient, densitometry was required to determine how many copies of D15S9 and D15S12 were present in these patients. The densitometric analysis follows in section VIII.

Figure 8. Autoradiographs of the Southern blots of DNA's from families of PWS probands 1 and 6 two control DNAs in lanes 7 and 8.and. (A) RsaI alleles at locus D15S11 (IR4-3); proband 6 is A1, father is A2, mother is A1A2; proband 1 is A1A2, father is A1A2, and mother is A2, controls are A2 and A1A2. (B) ScaI alleles at locus D15S9 (pML34); proband 6 genotype is A1, father is A1A2, mother is A1A2; proband 1 genotype is A1, father is A1A2, mother is A1, controls are A1 and A1A2. (C) ScaI alleles at locus D15S12 (IR10-1); proband 6 genotype is A1, father is A1A3, mother is A1; proband 1 genotype is A2, father is A2, mother is A2, controls are A1A3 and A2.


6
1


A.
1.2
A 1
1.0
A 2


B.
6.5
A 1
6.3
A 2


C.
17.5
A 1
16.0
A 2
12.5
A 3

alleles at locus D15S9 and D15S12, and the RsaI alleles at locus D15S11 (Table IX).

From the family data locus D15S10 was shown not to be deleted in proband #1 as he was heterozygous, locus D15S11 (IR4-3) was not deleted in proband #1 (MO) as he was heterozygous but was deleted in proband #6 (EB)and the origin of the aberrant chromosome 15 was determined to be paternal. The proband #6 (EB) has one RsaI allele size 1.2Kb (A1) at locus D15S11, her father (RB) is homozygous A2(1.0kb band), and her mother (LB) is heterozygous A1A2 (Figure 8). Since the father is homozygous A2 and the proband does not have an A2 allele, a deletion of the locus D15S11 must have occurred on the paternally transmitted chromosome 15. This evidence that the deleted chromosome 15 is of paternal origin in PWS patient #6 agrees with reports in the literature that 19/20 aberrant chromosome 15's as evidenced by cytogenetic analysis in PWS patients are paternally derived (Butler & Palmer,1983; and Niiakawa & Ishikiriyama, 1985).

The loci D15S9 and D15S12 were uninformative in both probands. The lack of heterozygosity neither confirms nor rejects the possibility of a molecular deletion at these loci. Since hemizygosity could not be detected in either patient, densitometry was required to determine how many copies of D15S9 and D15S12 were present in these patients. The densitometric analysis follows in section VIII.

Table IX. Genotypes of two PWS patients and their respective parents as determined by RFLP studies of loci D15S10, D15S9, D15S12, and D15S11 as defined by pTD3-21, pML34, IR10-1 & IR4-3.

Patient Number	Relationship to Proband	Genotype D15S10 TaqI[1]	Genotype D15S9 ScaI[2]	Genotype D15S12 ScaI[3]	Genotype D15S11 RsaI[4]
6(EB)		A1	A1[a]	A1[a]	A1[b,d]
	Father(RB)	A1	A1A2	A1A3	A2
	Mother(LB)	A1A2	A1A2	A1	A1A2
1(MO)		A1A2[c]	A1[a]	A2[a]	A1A2[c]
	Father(LO)	Und	A1A2	A2	A1A2
	Mother(KO)	Und	A1	A2	A2

[1]A1=9.0kb, A2=8.2kb
[2]A1=6.5kb, A2=6.3kb
[3]A1=17.5kb, A2=16.0kb, A3=12.5kb
[4]A1=1.2kb, A2=1.0kb
[a]uniformative for deletion detection
[b]deletion detected
[c]heterozygostity indicates no deletion
[d]paternal origin of aberrant chromosome 15
Und=undetermined; proband was heterozygous so parents' genotypes were not determined.

C. Discussion

The use of RFLP's to analyze the PWCR is limited by the probability that the parental mating types produce offspring with an informative genotype. Even though the probability that informative loci will occur is at best 0.50 in the offspring at the four loci described, RFLP analysis offers a more direct deletion-detection system than densitometric analysis and also can yield information as to the parental origin of the aberrant chromosome.

The use of RFLP studies for deletion-detection were successful for detecting a deletion at one locus in the proband #6 and one locus was shown not to be deleted in the proband #1. The origin of the aberrant chromosome 15 was determined to be paternal in proband #6.

VII. RFLP STUDIES OF PWS PATIENTS WITHOUT FAMILY DNAs.

A. Introduction

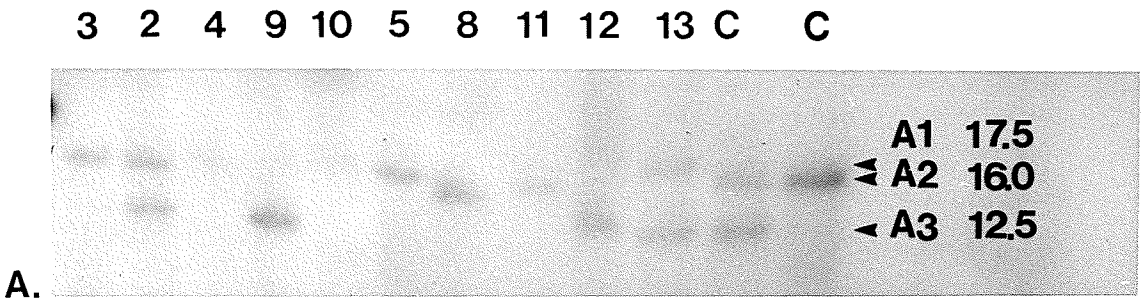
It is possible to type PWS patients for their apparent genotype at each locus (D15S9, D15S10, D15S11, and D15S12) within the PWCR by RFLP analysis. The strategy depends on the assumption that a proband heterozygous for alleles at a locus indicates that there is no deletion at that locus and that no detectable rearrangement has taken place at a restriction site. Six PWS patients for whom no RFLP data from any family members was available were analyzed. Each PWS patient was scored as heterozygous if two bands were present and either homozygous or hemizygous if only one band size was present.

B. Results

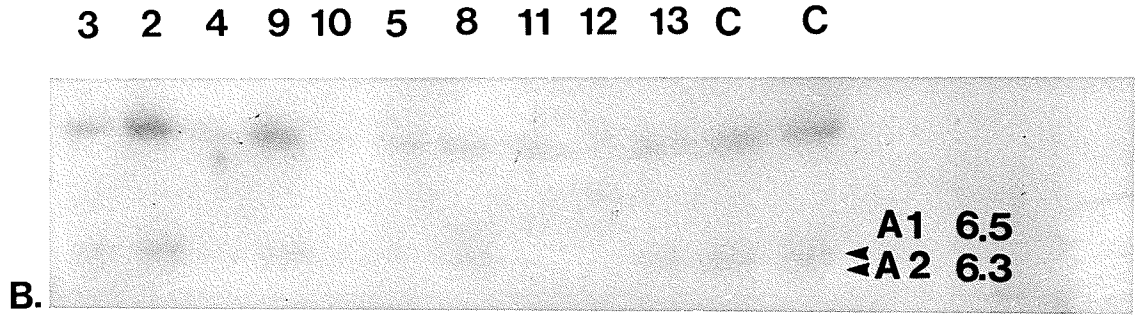
The six PWS patients (#'s 2,3,4,5,9,10) were scored as to the alleles present at each of the four loci D15S9, D15S10, D15S11, and D15S12 (Table X.) from autoradiographs of Southern blots hybridized vs pM134, pTD3-21, IR4-3 (not shown) and IR10-1 (Figure 9).

The expected heterozygosity for the ScaI alleles at the D15S12 is 0.42. Patient #2 was the only heterozygote detected at D15S12; of the other five patients, four had allele A1 and one had allele A2. None of the six patients were heterozygous at D15S9 (pML34); all patients were typed A1. The expected frequency of heterozygosity at D15S9 is 0.41. Patient #2 was the only patient who was heterozygous

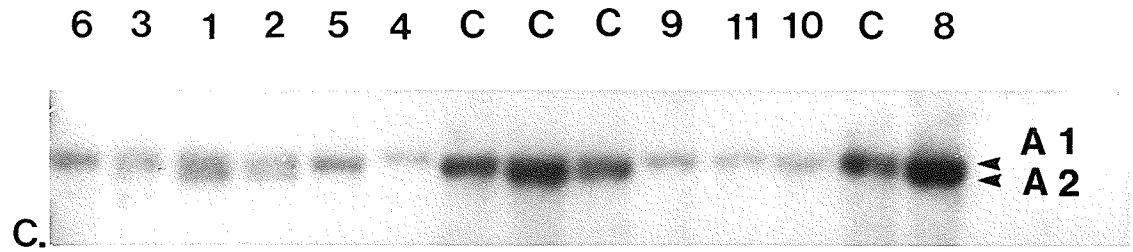
Figure 9. (A) Autoradiograph of Southern blot of patient and normal control DNA's restricted with Sca I and hybridized to IR10-1 (D15S12); lane numbers correspond to patient number*, normal controls are designated by C; PWS patients were scored for genotype of ScaI alleles at D15S12 locus; A1=17.5, A2=16.0, A3=12.5; (B) Autoradiograph of Southern blots of patient and normal control DNA's restricted with Sca I and hybridized to pML34 (D15S9)is shown; lane numbers correspond to patient number*, normal controls are designated by C; PWS patients were scored for genotype of ScaI alleles at D15S9 locus; A1=6.5, A2=6.3. (C) Autoradiograph of Southern blot of PWS patients and normal controls DNA's restricted with Taq I and hybridized to pTD3-21(D15S10); lane numbers correspond to patient number*, normal controls are designated by C; PWS patients were scored for genotype of TaqI alleles at D15S10 locus; A1=9.0, A2=8.8; lane numbers correspond to patient number*, normal controls are designated by C; *Patients 8,11,12, and 13 are not described in this study.


3 2 4 9 10 5 8 11 12 13 C C
A1 17.5
A2 16.0
A3 12.5

A.


3 2 4 9 10 5 8 11 12 13 C C
A1 6.5
A2 6.3

B.


6 3 1 2 5 4 C C C 9 11 10 C 8
A 1
A 2

C.

Table X. Summary of results of RFLP studies in 6 PWS patients for alleles at loci D15S12(IR10-1), D15S9(pML34), D15S10(pTD3-21),and D15S11(IR4-3).

Patient	D15S12 IR10-1 ScaI[1]	D15S9 34 ScaI[2]	D15S10 3-21 TaqI[3]	D15S11 IR4-3 RsaI[4]
3(NB)	A1	A1	A1	A1
2(DS)	A1A3	A1	A1A2	A1A2
4(LD)	A1	A1	A1	A1
9(AS)	A3	A1	A1	Und
10(AK)	A1	A1	A1	A1
5(RH)	A1	A1	A1	A1A2

[1]Allele sizes:A1=17.5kb, A2=16.0kb, A3=12.5kb
[2]Allele sizes:A1=6.5kb, A2=6.3kb
3Allele sizes:A1=9.0kb, A2=8.2kb
[4]Allele sizes:A1=1.2kb, A2=1.0kb
Und=undetermined

at locus D15S10 for the TaqI alleles. All of the other patients were scored as having an A1 allele. The expected heterozygosity for the TaqI alleles at the D15S10 locus is 0.25.

Locus D15S11 has the highest PIC value of the four loci tested in these PWS patients. For the RsaI alleles the expected heterozygosity at this locus is 0.50. The patients #2 and #5 are heterozygous at locus D15S11 while patients #3, #4 and #10 were scored as having only allele A1. The allele type was not determined for the patient #9.

C. Discussion

In the present study loci D15S9, D15S10, D15S11, D15S12

VIII. DENSITOMETRIC ANALYSIS OF FOUR LOCI D15S9, D15S10, D15S12, AND D15S11 AS DEFINED BY PROBES pML34, pTD3-21, IR10-1, AND IR4-3, RESPECTIVELY.

A. Introduction

Three approaches to the molecular analysis of the PWCR of PWS patients are possible. RFLP studies both with and without family DNAs have been described above. For completeness, all three loci in all patients were tested by densitometric analysis even if some loci had been informative by the RFLP studies.

Junien et al. (1983) have successfully used dosage analysis for the mapping of sequences to chromosome 7 and a ß-globin gene to the X chromosome. The evidence of increasing hybridization intensity as judged by intensity of exposure of bands on autoradiographs was indication for gene dosage. The procedure has also been used successfully for subregional mapping to the X chromosome (Riddell et al., 1986).

A number of parameters must be considered before densitometric scanning of autoradiographs may be used to determine gene dosage. It is possible that the signal intensity for the same fragment may vary from lane to lane even if DNA quantities in each lane are the same. Junien et al. (1983) suggested that it was more suitable to compare hybridization intensities at an experimental locus and a control locus within the same lane. These authors used the

4.8kb fragment of DXS(X-specific DNA) as a standard locus and compared it with the 1.4kb fragment of COL1A2. Densitometry analysis using peak area indicated that the ratio of peak area of DXS/peak area of COL1A2 (1.4kb fragment) was constant for normal men (0.43±0.7) and for normal women (0.789±0.033). The mean value for males was approximately half the value for females. Comparing HBB with COL1A2 in a patient with trisomy 11p, an overall increase of 35% was detected in the patient in comparison to normals. Likewise, the mean ratio of the peak areas was found to be 0.54 for monosomic/disomic, 1.35 for trisomic/disomic for the ratio of COL1A2/HBB for the trisomy 11p. For the trisomy 7 patient the trisomic/disomic ratio for HBB/COL1A2=1.68. These results demonstrated that it was possible to measure accurately a half-normal or one and one-half normal value related to a monosomy or trisomy by densitometric analysis of autoradiographs.

Laskey and Miller (1975) demonstrated that the relationship between absorbance of the fluorographic image and the concentration of radioactivity in a gel is not proportional. Similarly the absorbance of the film image is not proportional to exposure time. A different curve is obtained for each concentration of radioactivity when plotted against absorbance of the film image indicating that the fluorographic image absorbance is dependent not only on the number of disintegrations which occur but also on the

rate at which they occur. Exploiting the photographic procedure these authors predicted that pre-exposing the film to a very short flash of light(<1s) should increase the sensitivity of the film to small amounts of radioactivity and further that the absorbance of the image should be proportional to the amount of radioactivity even at low concentrations. They found that for fluorographic exposure at -70°C pre-exposure to give absorbances of 0.15(A_{540}) to a maximum of 1.5 absorbances after exposure to radioactivity results in a completely linear relationship between fluorographic image absorbance and the quantity of radioactivity.

B. Procedure.

The method developed for dosage analysis by densitometric scans of autoradiographs is a modification of methods of Junien et al.(1983) along with recommendations of Laskey (1980). Patient and control DNA's were restricted with either HindIII or PstI as indiciated, run in 1% agarose gels, and blotted onto Hybond or GeneScreen Plus as described above. Blots were hybridized as previously described to a standard probe pX83 (DXS47) and an experimental chromosome 15 probe after nick translation. Radiolabelling pretrials determined the amount of each probe (standard and experimental) to yield specific activities of approximately equal levels. Specific activities were routinely 1-3 X 10^8. Blots were washed at high stringency 2

X 2XSSC, 0.1% SDS at room temperature for 15 minutes, 1 X 2XSSC, 0.1% SCS at 65°C for 15 minutes, 1 X 0.5XSSC, 0.1% SDS at 65°C for 15 minutes, and finally 1 X 0.1XSSC, 0.1% SDS at 65°C for 15 minutes. Blots were exposed for 24, 48, and 72h or 24, 48, and 96h using preflashed X-ray film. The X-ray film was hypersensitized according to the recommendations of Laskey (1980) just before use by exposure to a single instantaneous flash of light from an electronic photographic flash unit through an orange filter (Kodak Wratten no.21) at a distance of 30inches. To obtain a fog level as even as possible, Whatman no.1 filter paper was placed over the filter to serve as a translucent diffuser. The intensity of the flash was adjusted to increase the absorbance of the film to 0.15-0.3(A_{540}) above the absorbance of the unexposed film (Laskey & Mills, 1975) by varying the distance between film and light source. Distances of less than 50cm should not be used as shorter distances result in uneven illumination. Preflashing of the film was done immediately before use. Laskey (1980) reports that the storage of pre-exposed film decreases the shelf life and the fog level rises more rapidly than pre-exposed film used immediately. Efficiency of ^{32}P detection was increased by enclosing the film between two intensifying screens (DuPont Cronox Lightning Plus) arranged : screen A, sample, film, screen B. The resolution is slightly reduced but the sensitivity is maximized (Laskey, 1980).

The autoradiographs were scanned using a Gilford spectrophotometer. Each lane was scanned three times for each exposure time. Means and standard deviation were determined for peak area. To insure that exposure times were within the linear range of the X-ray film, linear regression analysis was applied. Values used in the dosage analysis were those calculated from the linear regression analysis. The mean peak area as determined by the densitometry readings was plotted against the length of exposure time in hours where:

intercept(a) of regression line =

$$n \cdot \Sigma xy - \Sigma x \cdot \Sigma y / n \cdot \Sigma x^2 - (\Sigma x)^2$$

gradient(b) of regression line= $\Sigma y - b \cdot \Sigma x / n$

correlation coefficient(r)=

$$n \cdot \Sigma xy - \Sigma x \cdot \Sigma y / \sqrt{\{n \cdot \Sigma x^2 - (\Sigma x)^2\} \cdot \{n \cdot \Sigma y^2 - (\Sigma y)^2\}}$$

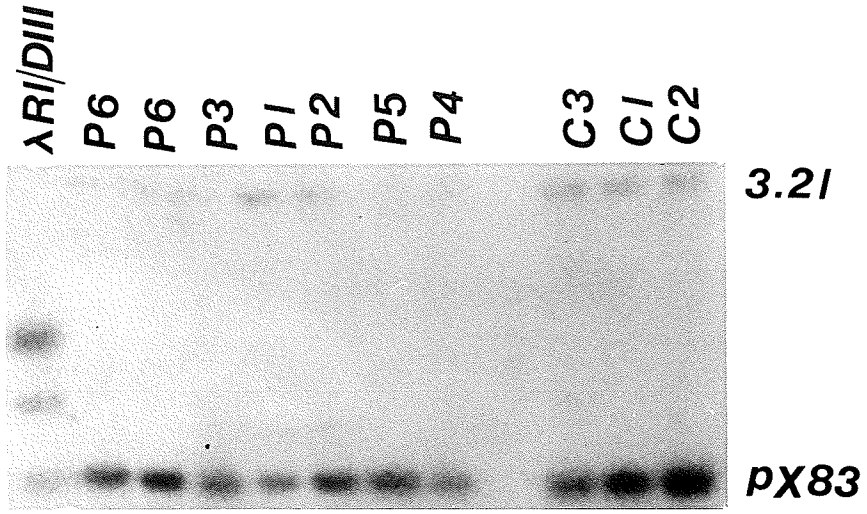
estimate(x)= (Y-a)/b

estimate(y)= a + bx

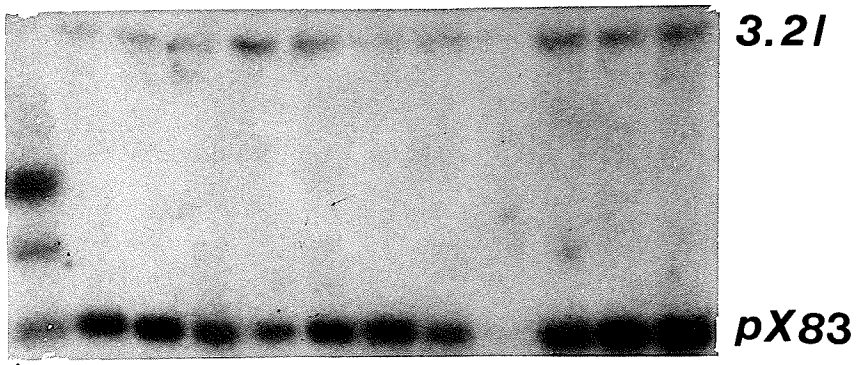
C. Results

The autoradiographs of the blots hybridized to a standard probe pX83d recognizing a sequence on the X chromosome (DXS47) and the experimental probes pTD3-21 (D15S10)(Figure 10.), IR10-1 (D15S12)(Figure 11.), pML34 (D15S9)(Figure 12.) and the standard probe pSW50 recognizing a sequence on chromosome 8 (D8MGV1) and the experimental probe pTD3-21 (D15S10)(Figure 13.) were scanned using a Gilford spectrophotometer at wavelength 600nm with an

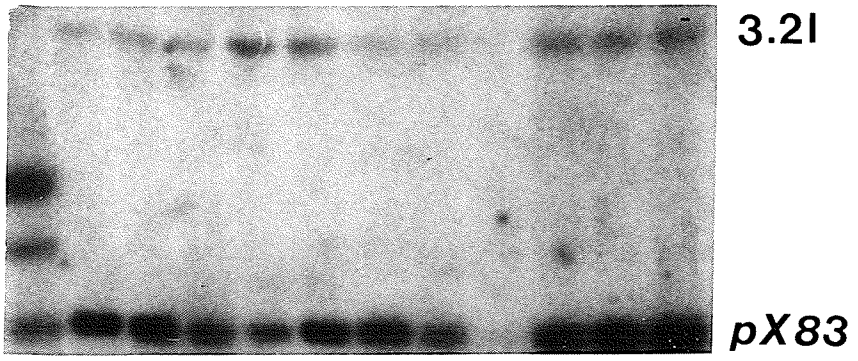
Figure 10. Autoradiograph of a blot hybridized with the control probe pX83d (DXS47) and the experimental chromosome 15 probe pTD3-21 (D15S10) showing exposure times of (A) 24h; (B) 48h; and (C) 72h; Methods:Patient* and control** DNAs were extracted from 5-10mls of whole blood, restricted with PstI using conditions recommended by the manufacturer. Approximately 5ug of restricted DNA was run on a 1% agarose gel, and blotted onto Hybond using conditions recommended by the manufacture. *Lanes containing patient DNA's are designated by P followed by patient number; **Lanes containing normal control DNA's are designated by C.



A.24h

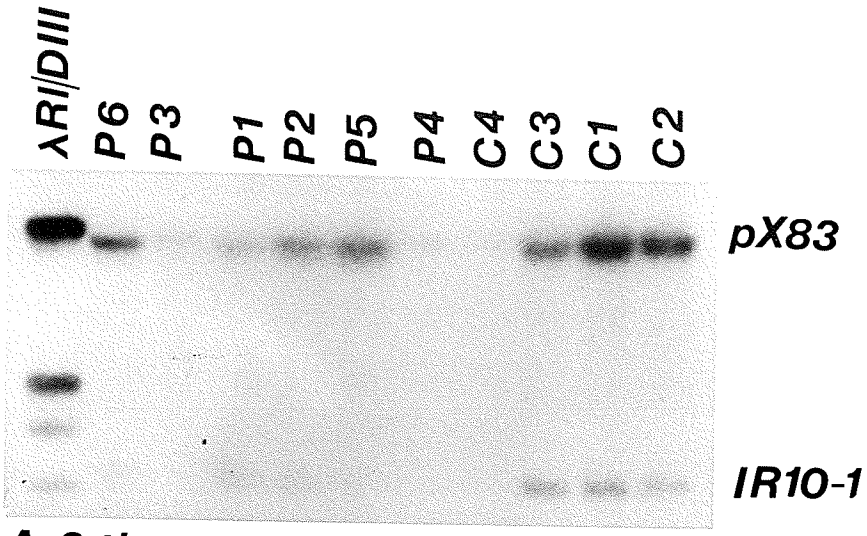


B.48h

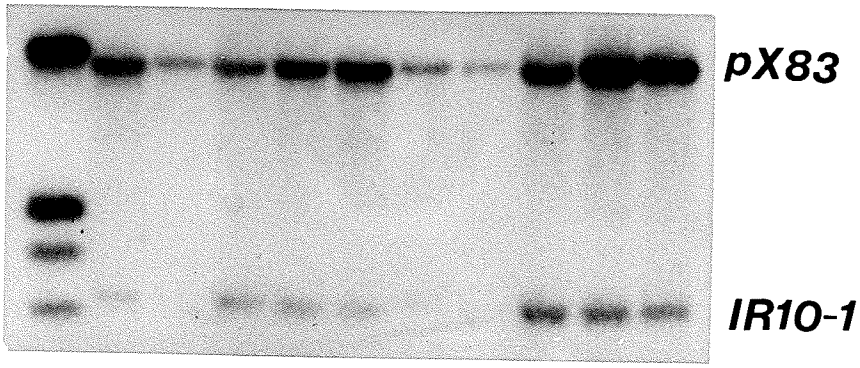


C.72h

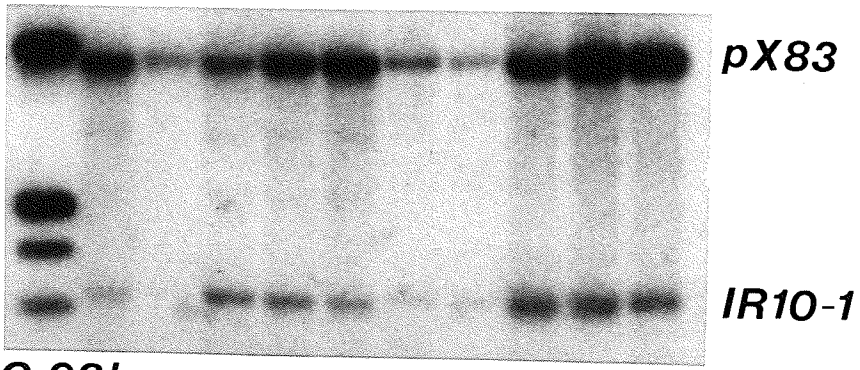
Figure 11. Autoradiograph of a blot hybridized with the control probe pX83d (DXS47) and the experimental chromosome 15 probe IR10-1 (D15S12) showing exposure times of (A) 24h; (B) 48h; and (C) 96h; Methods:Patient* and control** DNAs were extracted from 5-10mls of whole blood, restricted with HindIII using conditions recommended by the manufacturer. Approximately 5ug of restricted DNA was run on a 1% agarose gel, and blotted onto Hybond using conditions recommended by the manufacture. *Lanes containing patient DNA's are designated by P followed by patient number; **Lanes containing normal control DNA's are designated by C.



A. 24h



B. 48h



C. 96h

Figure 12. Autoradiograph of a blot hybridized with the control probe pX83d (DXS47)and the experimental chromosome 15 probe pML34 (D15S9) showing exposure times of (A) 24h; (B) 48h; and (C) 72h; Methods:Patient* and control** DNAs were extracted from 5-10mls of whole blood, restricted with HindIII using conditions recommended by the manufacturer. Approximately 5ug of restricted DNA was run on a 1% agarose gel, and blotted onto Hybond using conditions recommended by the manufacture. *Lanes containing patient DNA's are designated by P followed by patient number; **Lanes containing normal control DNA's are designated by C.

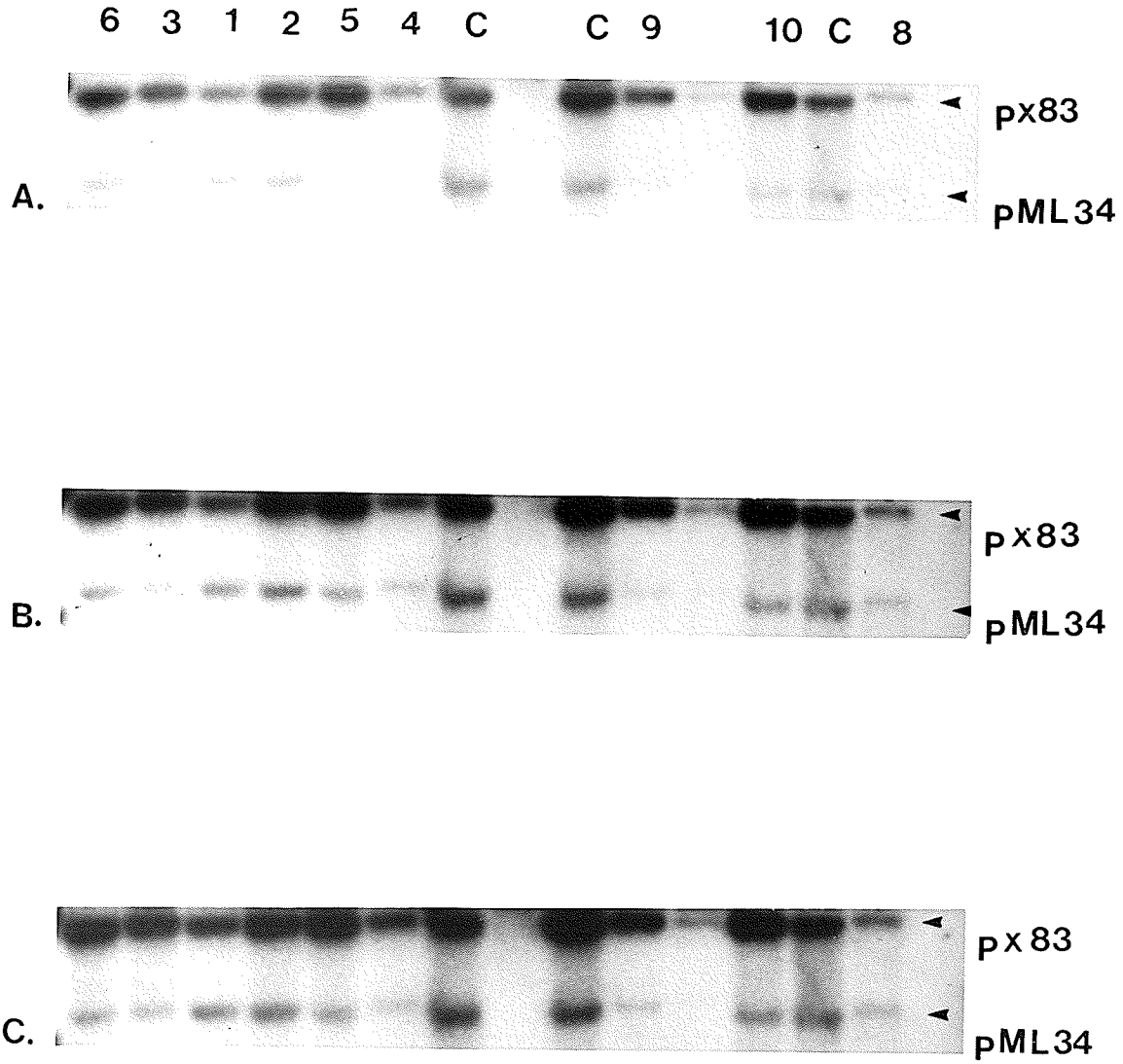
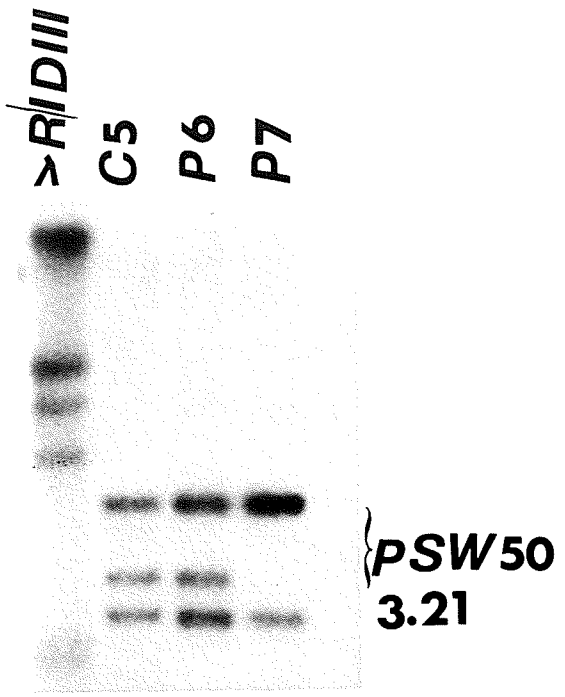
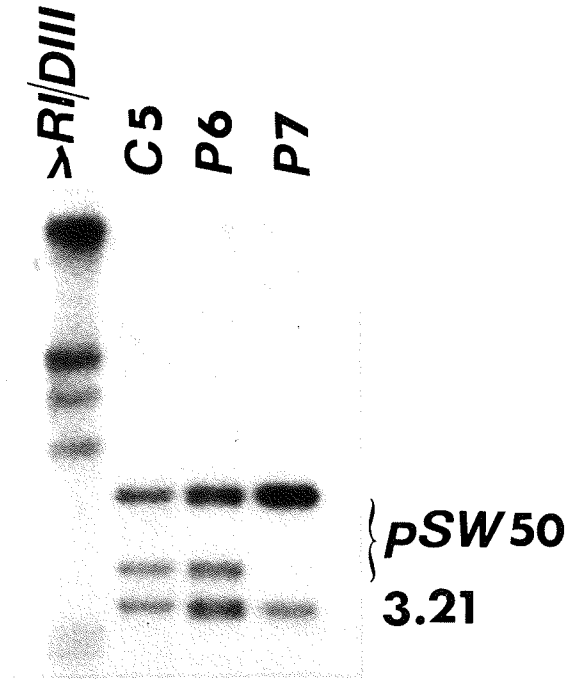

6 3 1 2 5 4 C C 9 10 C 8
A.
pX83
pML34
B.
pX83
pML34
C.
pX83
pML34

Figure 13. Autoradiograph of a blot hybridized with the control probe pSW50 (D8MGV1)and the experimental chromosome 15 probe pTD3-21 (D15S10) showing exposure times of (A) 16h; (B) 24h; Methods:Patient* and control** DNAs were extracted from 5-10mls of whole blood, restricted with HindIII using conditions recommended by the manufacturer. Approximately 5ug of restricted DNA was run on a 1% agarose gel, and blotted onto Hybond using conditions recommended by the manufacture. *Lanes containing patient DNA's are designated by P followed by patient number; **Lanes containing normal control DNA's are designated by C.



A.16h



B.24h

aperature of 1mm x 2mm and a band width of 0.5nm. For each patient and control, the mean peak area was determined for the chromosome 15 locus and the X chromosome locus. The ratio of the peak area of the chromosome 15 locus/peak area of standard locus was determined using estimated values from the linear regression line (Figure 14.). For the controls an expected ratio of the chromosome 15 locus peak area/chromosome X locus peak area is 1.0 for females (2 chromosome 15's/2 chromosome X's) and 2.0 for males (2 chromosome 15's/1 chromosome X). Using the ratio of one control as a standard all of the four controls were tested against the other three normal controls (Appendix B). The mean ratio for the controls was 1.0(±0.2). The mean ratio for each patient was determined using all four of the controls. If a patient's mean dosage was greater than two standard deviations from the norm, the locus was recorded as duplicated; if two standard deviations or more below the norm, the locus was interpreted as being deleted (Table XI.).

D. Discussion

Dosage analysis of three of the loci mapped to the PWCR have allowed further resolution of the deletion in four patients with apparently similar karyotypes del(15)(q11q13). Each case differs at the molecular level (Table XI). In one case all three of the loci are deleted. In 3 cases, two out of the three loci are deleted; these are loci D15S9 and

Figure 14. Schematic description of the method used to generate the ratio used in dosage calculation of chromosome 15 loci in PWS patients. The autoradiograph of each exposure time (24, 48, & 72h) is cut into strips to be scanned by the densitometer. The strips with two hybridization bands are illustrated by (d); directly above each hybridization band is the computer-generated peak area for the chromosome 15 locus followed by the peak area of the standard locus. Line (a) illustrates the regression line generated when plotting the peak area of the chromosome 15 locus vs exposure time and line (b) illustrates the regression line of the peak area of the standard locus vs exposure time. The ratio (c) is used in the dosage calculation of the chromosome 15 locus in each PWS patient by comparing the patient's ratio to the ratio in normal controls.

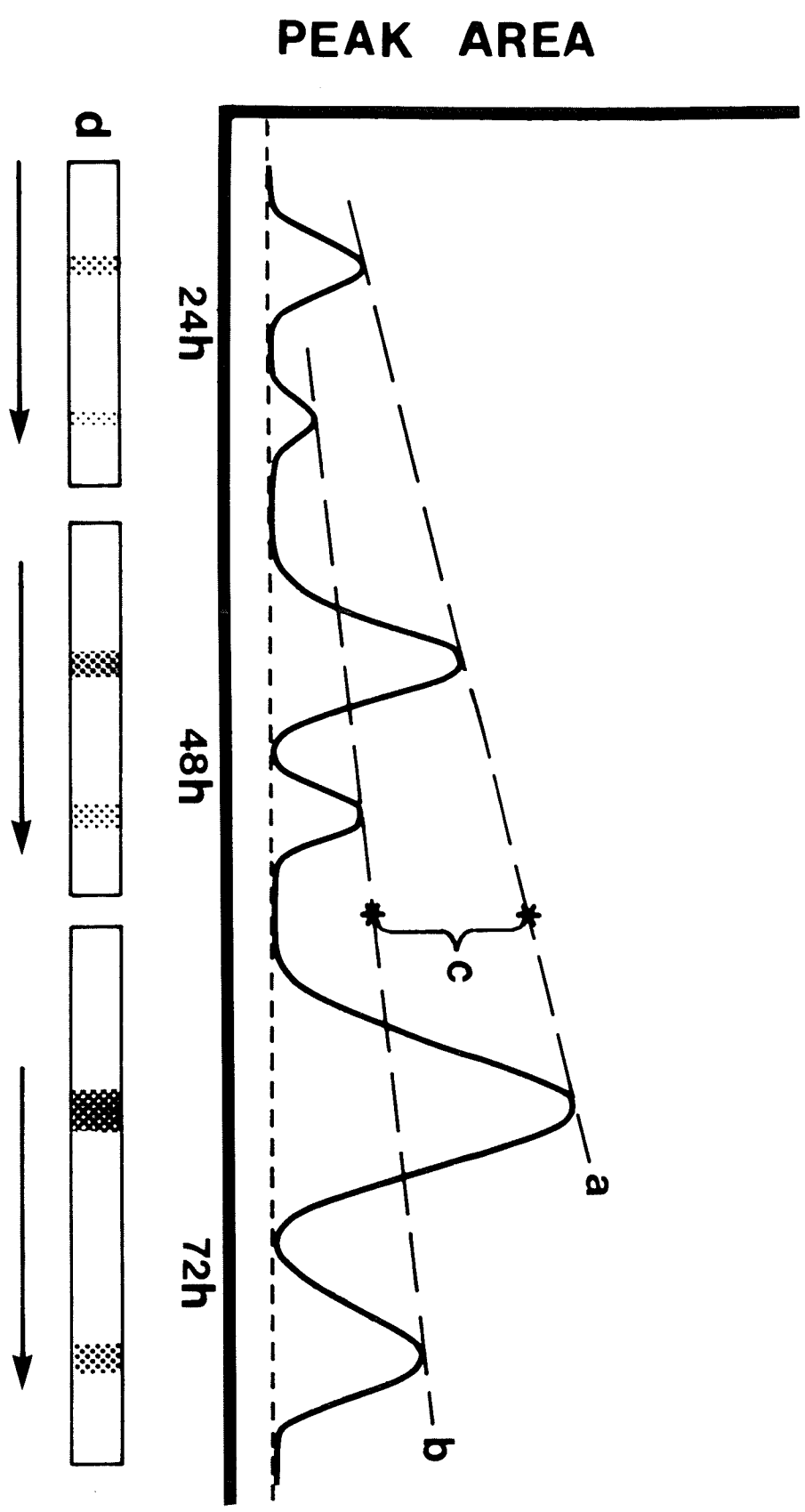

PEAK AREA
d
24h
48h
72h
c
a
b

Table XI. Summary of dosage of loci D15S9, D15S12, and D15S10 as defined by hybridization to pML34, IR10-1 and pTD3-21 respectively.

Case No.	Sex	D15S9 Mean Experimental Ratio D15S9/DXS47	D15S9 Dosage	D15S12 Mean Experimental Ratio D15S12/DXS47	D15S12 Dosage	D15S10 Mean Experimental Ratio D15S10/DXS47	D15S10 Dosage
1	M	1.7 ± 0.4	N	1.5 ± 0.6	N	4.1 ± 0.6	Amp
2	F	0.8 ± 0.2	N	1.3 ± 0.5	N	1.3 ± 0.2	N
3	F	0.3 ± 0.1	Del	0.7 ± 0.3	N	1.2 ± 0.2	N
4	M	0.3 ± 0.1	Del	1.0 ± 0.1	Del	1.0 ± 0.1	Del
5	F	0.3 ± 0.1	Del	0.5 ± 0.3	Del	0.8 ± 0.1	N
6	F	0.5 ± 0.1	Del	0.5 ± 0.2	Del	1.0 ± 0.2 (PstI) 1.6 ± 0.3 (HdIII)	N Dup
7	F	ND	ND	0.4	Del	0.6 ± 0.2	Del
9	F	0.2 ± 0.1	Del	0.2 ± 0.1	Del	ND	ND
10	F	0.4 ± 0.1	Del	0.2 ± 0.1	Del	ND	ND

The mean experimental dosage for each of the three loci for controls was 1.0 ± 0.2. If a patient's mean dosage was greater than two standard deviations from the norm, the locus was recorded as duplicated; if two standard deviations or more below the norm, the locus was interpreted as being deleted. For males, the expected ratio is 2.0 and for females 1.0. Abbreviations: Del=deletion, Amp=amplification, Dup=duplication, N=normal, ND=not done.

D15S12 in two cases and D15S10 and D15S12 in the other.

The karyotypically normal patients also are a heterogeneous group with molecular deletions in one, a molecular duplication in another, and the third with no alteration yet detected.

Case #6 is of particular interest; in this patient D15S11, D15S12 and D15S9 are deleted while D15S10 shows an apparent duplication when the HindIII fragment is analyzed but normal hybridization when the PstI fragment is analyzed. The cloned 2.2Kb HindIII fragment of pTD3-21 has an internal PstI site. Two PstI fragments (9.0Kb and 1.5Kb) are defined at the locus by pTD3-21. The 9.0Kb fragment was used for the densitometric scans. One possible explanation for these results is that a duplicated seguence homologous to a DNA sequence between the internal PstI site and the flanking HindIII site is present as an inverted repeat. The restriction fragment lengths defined at locus D15S10 by pTD3-21 for proband #6 were compared to normal controls and no apparent differences were observed. The complexity of the molecular rearrangement in patient #6 is not entirely understood but it is possible that an inverted repeat unit of a segment homologous to the cloned sequence has been inserted creating a duplicated region of the segment from the internal PstI site and the HindIII site. This event would not alter any of the flanking restriction sites but would be detected by the densitometric scans as a

duplication. Donlon et al. (1986) demonstrated by heteroduplex formation that inverted repeat elements are present in the proximal region of 15q. The presence of these inverted repeats could cause deletions, deletion-duplications, or more complex rearrangements. Whether this is the mechanism resulting in a duplication at locus D15S12 remains uncertain but molecular evidence of deletions and deletion-duplications in PWS patients are consistent with the gross rearrangements detected cytogenetically in PWS patients.

VIII. DISCUSSION AND CONCLUDING REMARKS

Genomic DNA probes which map to 15q11.2-12 were used to investigate the PWCR in patients with the Prader-Willi syndrome. Methods used included first, dosage analysis in which the number of DNA copies was measured by comparing the intensity of hybridization at a locus defined by a chromosome 15 probe and a locus defined by a standard probe. Second, restriction fragment lengths at four loci in normal controls were compared to restriction fragment lengths in the PWS patients to detect molecular rearrangements; third, two PWS families were studied using RFLP analysis for the detection of deletions, and for determining the parental origin of the deleted chromosome; and fourth, seven PWS patients without available family data were studied by RFLP analysis for the detection of heterozygosity indicating that a deletion event had not occurred.

A. Molecular aberrations in the Prader-Willi syndrome Chrosome Region.

The results of the analysis of each locus for each patient were consistent for each of the four molecular methods that were used (Table XII.). As an example, patient #2 was scored as having normal dosage by densitometry for the locus D15S10 and was heterozygous by the RFLP studies; patient #5 was predicted to have a deletion at the locus D15S12 by densitometry and was hemizygous as determined by the RFLP studies.

Table XII. Summary of dosage of loci D15S10, D15S12, D15S9 and D15S11 as determined by densitometry and RFLP's studies in nine PWS patients.

Patient Number	Sex M/F	D15S10 (3-21)	D15S12 IR10-1	D15S9 34	D15S11 IR4-3
1(MO)	M	Dup[2]	N[1]	N[1]	N[2]
2(DS)	F	N[2]	N[2]	N[1]	N[2]
3(NB)	F	N[1]	N[1]	Del[1]	Uni[1]
4(LD)	M	Del[1]	Del[1]	Del[1]	Uni[1]
5(RH)	F	N[1]	Del[1]	Del[1]	N[2]
6(EB)	F	Dup[1]	Del[1]	Del[1]	Del[1,3,4]
7(CC)	F	Del	Del	NT	NT
9(AS)	F	NT,Uni[1]	Del[1]	Del[1]	Und
10(AK)	F	NT,Uni[1]	Del[1]	Del[1]	Uni[1]

Abbreviations: N=normal; Del=Deletion; NT=Not tested; Dup=duplication; Uni=uninformative; Und=undetermined.
[1]Presence of only one allele size of RFLP
[2]Heterozygote for RFLP
[3]Deletion detection by RFLP family data
[4]Paternal origin of aberrant chromosome 15

Locus D15S11 was difficult to analyze by densitometric scanning of autoradiographs as the hybridization signal became too weak or disappeared completely when blots were washed at high stringency. Therefore dosage at locus D15S11 was analyzed only by RFLP studies.

Characterizing these PWS patients according to the results of the molecular analysis rather than by cytogenetics, eight out of the nine PWS patients had a molecular disruption of at least one locus studied. Six patients had a deletion of at least one locus, one patient had a deletion-duplication, one patient had a duplication, and one patient showed no abnormalities at any of the four loci tested. These molecular studies suggest that a physical disruption of the PWCR may account not only for those patients reported to have a cytogenetic aberration but as well for those identified as karyotypically normal.

The question still remains whether patient #2 represents an unusual example of a PWS patient in whom no molecular disruption has occurred and truly is a homozygous recessive case of PWS; whether the disruption lies outside of the region spanned by the four loci studied but within the PWCR or whether this patient represents an example of PWS caused by a gene at another locus entirely.

If it can be assumed that the presence of a hybridization band at a locus shown to be deleted by densitometry must represent the allele on the normal

chromosome 15, then the haplotypes represented by the alleles carried by the normal chromosome 15 can be determined in six of the PWS patients (Table XIII). Five of the six patients (#3,4,5,6,10) carried the same haplotype on the normal chromosome 15 for the loci D15S12, D15S9, and D15S10 (A1/A1/A1). The PWS patients were more variable at locus D15S11 and additional data on their haplotypes at this locus were not as informative. However, three out of the six patients carried A1 alleles on their normal chromosome 15's at D15S9, D15S10, D15S11, and D15S12. Locus D15S11 was shown not to be deleted in two of the five patients whose haplotypes are described making the haplotypes at D15S11 uninformative. The three remaining PWS patients who were informative at all loci carried identical haplotypes on the normal chromosome 15 at D15S9, D15S10, D15S11, and D15S12. Data from more PWS patients as well as analysis of other loci mapping to PWCR are needed if we are to draw any conclusions from these observations.

B. The ordering of the four loci D15S9, D15S10, D15S11, and D15S12 within the PWCR.

The relationship of the molecular aberration at each of the four loci for each patient predicts the order of the loci to be CEN→D15S9→D15S12→D15S11→D15S10→qter (Figure 15.). The model presented here assumes that the fewest number of breaks in the patients' DNA have occurred in each of the PWS patients investigated. It is unlikely that locus D15S10

Table XIII. Summary of results of RFLP studies in 8 PWS patients for alleles at loci D15S12(IR10-1), D15S9(pML34), D15S10(pTD3-21),and D15S11(IR4-3).

Patient	D15S12 IR10-1 ScaI[1]	D15S9 34 ScaI[2]	D15S10 3-21 TaqI[3]	D15S11 IR4-3 RsaI[4]	Haplotype of Normal Chromosome 15
3(NB)	A1	A1	A1	A1*	A1/A1/A1/A1*
2(DS)	A1A3	A1	A1A2	A1A2	Uni
4(LD)	A1	A1	A1	A1	A1/A1/A1/A1
9(AS)	A3	A1	A1	Und	A3/A1/A1/Und
10(AK)	A1	A1	A1	A1	A1/A1/A1/A1
5(RH)	A1	A1	A1	A1/A2	A1/A1/A1/Uni
6(EB)	A1	A1	A1	A1	A1/A1/A1/A1
1(MO)	A2	A1	A1A2	A1A2	Uni

[1]Allele sizes:A1=17.5kb, A2=16.0kb, A3=12.5kb
[2]Allele sizes:A1=6.5kb, A2=6.3kb
3Allele sizes:A1=9.0kb, A2=8.2kb
[4]Allele sizes:A1=1.2kb, A2=1.0kb
Uni=uninformative
Und=undetermined

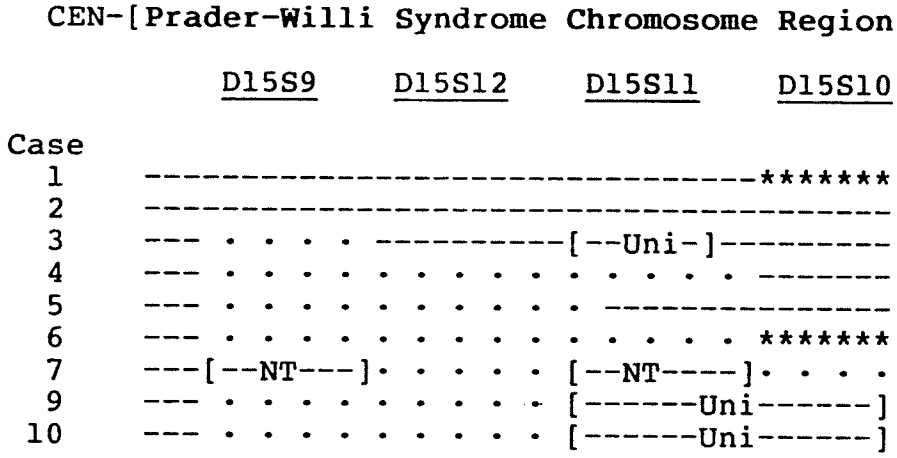


Figure 15. Diagramatical representation of the molecular aberration of the four loci studied for each PWS patient and a possible ordering of the four loci within the PWCR. Dosage for locus D15S11 was determined by RFLP studies only.
Legends: Normal= ---
Duplication= *****
Deletion= . . .
NT= Not tested
Uni= uninformative as determined by RFLP studies

lies between D15S12 and D15S9 as D15S10 is not deleted in patient #5 while both other loci are deleted. If D15S10 were to be positioned between D15S12 and D15S9 two interstitial deletions would be required to account for the findings in this patient. Locus D15S11 is deleted in patient #6; therefore, if D15S12 is positioned between D15S9 and D15S10, then D15S11 must be distal to D15S9 and proximal to D15S10. The fact that Patient #5 does not have locus D15S11 deleted places D15S11 between D15S12 and D15S10. Patient 3 with an unbalanced translocation 45,XX,-5,-15,+t(5;15)(p15.2;q12) has locus D15S9 deleted but not locus D15S10 or D15S12. This places D15S9 most proximal to the centromere followed by D15S12, D15S11, and D15S10. This unusual cytogenetic rearrangement (patient #3) affords a unique opportunity to order the loci. The ordering of the four loci D15S9, D15S10, D15S11, D15S12 within the PWCR has been hypothesized but the physical and linkage distance between the four loci is at present unknown.

C. Mode of inheritance of PWS.

An autosomal recessive mode of the PWS has not been ruled out as 10%(1/9) of the PWS patients investigated have no detectable cytogenetic or molecular aberration of 15q11-13. The theory that a disruption of a normal allele allows the expression of an otherwise recessive locus is supported by the eight out of nine PWS patients with loci deleted. The contradiction that some of the time a deletion of 15q11-

12 should not present as the PWS phenotype because the unaffected allele should be normal could be explained by ascertainment bias. Population surveys (Buckton et al., 1980; Hamerton et al., 1975)) have not reported deletions of the 15q11-12 region in normal individuals but the detection of a deletion of the proximal region requires special scrutiny (Labidi & Cassidy, 1986) and would probably not be detected in general survey work.

The hypothesis that a dominant mode of inheritance is evoked by disruption of 15q11-12 by translocation, deletion, or inversion (Fraccaro et al., 1983; Mattei et al.,1983) is supported by the molecular findings. Eight of the nine PWS patients were found to have at least one locus either deleted or duplicated. Only one patient had no disruption yet detected but lack of a disruption can not be ruled out at this time as only a small region of the PWCR has been analyzed.

The possibility that PWS is a contiguous gene syndrome (Schmickel, 1986) resulting from the disruption of a sequence of genes within the PWCR is not refuted by the molecular findings. The PWS patients are a heterogeneous group with only one locus deleted in some patients, two or three in others, and where there is more than one locus deleted the combination of deleted loci vary from patient to patient. One patient had a deletion-duplication, and one patient had an apparent duplication of one locus. This

molecular heterogeneity in the PWS patients is consistent with the cytogenetic and clinical heterogeneity seen among PWS patients. Cytogenetic and clinical heterogeneity is a consistent feature of contiguous gene syndromes (Schmickel, 1986).

Fraccaro et al. (1983) predict that the karyotypically normal PWS patients may have a submicroscopic chromosome aberration and thus present with the PWS phenotype by the same mechanism as those PWS patients who have a cytogenetically visible deletion or duplication. In the present study patient #1 who has an apparently normal karyotype has a molecular duplication; patient #10 who also has an apparently normal karyotype has two loci deleted. Therefore both patients could present with PWS as the result of a disruption of 15q11-12 similiar to those PWS patients having a cytogenetically visible chromosome abnormality. Patient #2 showed no abnormality at any of the four loci tested. It is not possible therefore to determine whether patient #2 has an undetected molecular disruption or represents those PWS patients that present by another mechanism.

It is difficult to imagine that a disruption spanning such a large distance molecularly could involve only one gene locus. Rather it seems more probable that PWS is caused by a disruption of homeostasis caused by imbalance of a number of genes rather than by a single "PWS" gene.

The clinical manifestations of PWS patients are more similiar to the clinical manifestations of aneuploid syndromes rather than to a single gene disorder with a single biochemical pathway altering a specific enzyme or specific pathway. Like aneuploid chromosome disorders PWS patients may present with recognizable clinical features because the genetic information contained in the chromosome segment either is presented in too many or too few copies. A reasonable hypothesis is that an excess or diminished amount of specific genetic material distorts particular biochemical pathways that are involved in the development of a number of tissues. Knowledge of the genetic information contained in the critical region 15q11-13 should provide clues to the biochemical aspects of development of a variety of tissues, especially the pathways involved in energy expenditure, deposition of fat tissue and obesity.

In order to understand how the presence of an excess or disruption of genetic materials within this region leads to PWS it will be necessary to map the gene or genes in the PWCR that are responsible for the disorder, to identify the proteins corresponding to these genetic loci, and to understand the metabolic effects on cells of different tissues at different stages of development of diminished or altered gene products.

IX. FUTURE INVESTIGATIONS-WHAT NEXT?

In order to further clarify the etiology of PWS one approach will be to saturate the large PWCR locus with a large number of cloned DNA segments. In order to investigate larger areas than are possible with conventional probes that span only several kilobases at a time, large fragments isolated by field-inversion gel electrophoresis (FIGE) could be cloned into yeast cells using the yeast artificial chromosome vector (Burke, 1987). Enrichment for the region of interest could be accomplished by use of a cell line which is trisomic or tetrasomic for 15q11-13 as a source of DNA for the cloning experiment. This method depends on the availability of a starting point for the walk through the PWCR and a method of determining the direction of the walk. The starting point could be locus D15S10(pTD3-21) which is not deleted in translocation patient #3 while two other loci are. This patient could serve as a means of moving towards the centromere from the breakpoint 15q12. This approach is limited as all others have been to this point in that densitometry scans of autoradiographs are very tedious and time consuming and RFLP studies are not always informative.

A more direct approach for the detection of a deletion at a locus is to have a system which allows the analysis of only the aberrant chromosome 15. This could be accomplished by utilizing cell lines derived from the patients in this

study whose molecular aberrations are different, to be used to make individual hybrid cell lines containing the aberrant human chromosome 15. Thus dosage analysis would not depend on densitometric scans which require standardization of many parameters but would depend on the presence or absence of a locus as evidenced by the presence of a hybridization band. Seawright et al. (1988) report analysis of WAGR (Wilms' tumors, aniridia, genitourinary abnormalities, and mental retardation) deletions of chromosome 11 using mouse-human somatic cell hybrids prepared from six WAGR patients. A selection system was developed in which the deleted chromosome 11 homolog was segregated from the normal chromosome 11.

These hybrid cell lines could also be used to clone segments specific to the deletion region in the PWS patient from which the cell line was produced using the PERT technique (Kunkel et al., 1985b). The lymphoblast cell line (GM6246 available from the Human Genetic Mutant Cell Repository, Camden, NJ) tetrasomic for the region 15q11-13 could be used as the source of DNA sequences to be cloned in the reassociation of sequences from the deleted region of the patient's deleted chromosome 15. An excess of DNA from the hybrid cell line containing a deleted chromosome 15 would serve as the driver DNA.

Another approach is to screen a chromosome 15 library using oligomers useful for detecting variable number tandem

repeat markers (VNTR's)(Nakumura et al., 1987). The region 15q11-12 is rich in repetitive DNA and from other screening attempts more clones were found to map to 15q11-12 if they were repetitive than if screened for unique sequences (Donlon et al., 1986). This method not only is more likely to select for DNA sequences from the proximal region of 15q but the probes would more likely be informative for patient and family studies. Nakamura et al.(1987) report that VNTR markers have a higher frequency of multiple allele loci which make them more informative for linkage. The use of VNTR's in the analysis of the PWCR in PWS patients would be more likely to detect molecular rearrangements and parental orgin of the aberrant chromosome could more easily be assigned. Also, there is a higher probability of a probe being polymorphic if it is in close proximity to repetitive sequences (Aldridge et al.,1984). VNTR markers might also provide clues as to the type of sequences present in this region and to the mechanisms that presents such a hot spot of recombination.

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XII. APPENDIX

A. Stock solutions and buffers.

Activation buffer
10mM Tris-HCl(pH:7.5)
5mM $MgCl_2$
1mg BSA/ml

10% Blotto
10g Nonfat powdered milk
100ml sterile deionized water
0.2% sodium azide

DNAse
DNAse is stored in 0.01N HCl at -20°C at a concentration of 1mg/ml. To activate, dilute an aliquot(50ul) into 450ul of Activation buffer and hold on ice for 2hrs. Store at 20°C in 10ul aliquots. For nick translation, 1ul is placed in 750ul of Activation buffer.

Hybridization solution
47% formamide
10% dextran sulfate
3X SSPE
1% SDS
0.5% Blotto
+Sperm DNA to final concentration 200ug/ML

LB broth(1l)
10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl
pH:7.5

5x Nick Translation Kit
500mM Tris-HCl(pH:7.5)
500ugml BSA
50mM $MgCl_2$
50mM ß-ME
25uM dGTP
25uM dTTP

20% SDS(1l)
200g Sodium dodecyl sulfate
(BioRad electrophoreses purity reagent)
pH:7.4

20x SSC(4l)
701.3g NaCl
352.9g NaCitrate
pH:7.0

20x SSPE(1l) (Maniatis et al., p.447, 1982)
174g NaCl
27.6g NaH_2PO_4
7.4g EDTA
pH:7.4
Sterilize by autoclaving.

5x Stop buffer
50mm EDTA
50% glycerol

10X Tris Acetate(4l)
193.6g TRIS
45.7mls
29.8G Na_2EDTA
pH:8.1

B. Mathematical calculations used for normalization of densitometry readings.

Experimental values used in calculation of dosage of locus D15S12:

Normal Control	Peak area of Chr. 15 locus	Peak area of Chr. X locus	Experimental Ratio(chr15/chr.X)	Expected Ratio
1(F)	0.812	6.08	0.133	1.0
2(F)	0.615	4.37	0.141	1.0
3(M)	0.883	2.80	0.315	2.0

Each control was tested versus the other two controls to verify that normal dosage was determined. The expected ratio for females is 1.0 because we are comparing 2 chr15s/2 chrXs; for males the expected ratio is 2.0(2 chr 15s/1 chrXs). The following calculations were performed to give the normalized ratios above to be used to analyze a patient's ratio:

control 1: experimental ratio=0.133; expected ratio=1.0
control 2: experimental ratio=0.141; expected ratio=1.0
control 3: experimental ratio=0.315; expected ratio=2.0

To determine the dosage for control 1, the experimental ratios of controls 2 and 3 were used as standards, where Normal dosage for control 2 is 1.0=0.141 and for control 3 Normal dosage is 2.0=0.315. Therefore, the mean dosage(D) for control 1 is:

$$\frac{0.133}{d} = \frac{0.141}{1.0} \qquad \frac{0.133}{d} = \frac{0.315}{2.0}$$

$$d = 0.94 \qquad d = 0.90$$

Therefore $D = 0.92(\pm 0.03)$

The mean dosage(D) for control 2 is:

$$\frac{0.141}{d} = \frac{0.133}{1.0} \qquad \frac{0.141}{d} = \frac{0.315}{2.0}$$

$d = 0.94$ $\quad$ $d = 0.90$

Therefore $D = 0.92(\pm 0.03)$

The mean dosage(D) for control 3 is:

$$\frac{0.315}{d} = \frac{0.141}{1.0} \qquad \frac{0.315}{d} = \frac{0.133}{1.0}$$

$d = 2.23$ $\quad$ $d = 2.36$

Therefore $D = 2.30(\pm 09)$

To determine a patient's mean dosage(D), the same calculations were performed using each of the three controls' experimental ratios as a standard. For example, patient #4 had an experimental ratio of 0.14. His expected ratio is 2.0. Comparing each of the controls ratio versus the patients, his mean dosage (D) calculated to be:

$$\frac{0.14}{d} = \frac{0.141}{1.0} \qquad \frac{0.14}{d} = \frac{0.133}{1.0} \qquad \frac{0.14}{d} = \frac{0.315}{2.0}$$

Therefore, D = 0.89; this locus is interpreted as being deleted.