MOLECULAR CYTOGENETIC STUDIES IN ANGELMAN SYNDROME

Helen Louise Gilbert

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Institute of Child Health
30 Guilford Street
London
WC1N 1EH.
ABSTRACT

Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are distinct neurogenetic disorders caused by the loss of function of distinct but closely linked genes on chromosome 15q11-q13, a region subject to genomic imprinting. AS and PWS most commonly arise from a de novo deletion of 15q11-q13. These deletions are maternal in origin in AS and paternal in origin in PWS.

AS patients were analysed in order to find atypical deletions, which would define a critical region for the AS gene. One AS individual who had previously been identified with an apparent maternal deletion around the LS6-1 (D15S113) locus and normal DNA methylation imprints, initially appeared deleted for an LS6-1 positive cosmid by fluorescence in situ hybridisation (FISH). Further studies with overlapping cosmid and phage clones, single copy fragments and microsatellite markers, showed that this individual was not deleted for the D15S113 locus and that the original LS6-1 result was due to non-amplification of the maternal allele. A second AS individual was shown by FISH and microsatellite analysis to have an atypical deletion of 15q11-q13 such that the results obtained with PW71B (D15S63) and SNRPN appeared to conflict. A third individual who did not have the characteristic features of AS, but who had a maternally inherited deletion of 15q which included the D15S113 locus was also studied. FISH studies showed that the centromeric breakpoint was contained within a 520 kb yeast artificial chromosome (YAC) which maps between TD3-21 (D15S10) and LS6-1 (D15S113).

AS individuals with no apparent maternal deletion of 15q11-q13, aberrant DNA methylation imprints and biparental inheritance of chromosome 15 were studied. Haplotypes were constructed in these individuals and their families, to localise a 'methylation control' locus. These studies were in agreement with a methylation control locus on chromosome 15q11-q13. Prenatal diagnosis was attempted in one family. The fetus had inherited the same critical maternal chromosome 15q11-q13 segment as the two affected siblings for the alleles tested, but had normal DNA methylation imprints. The mother was believed to be mosaic.

FISH studies using phage clones obtained from the region on chromosome 15 designated as the 'imprinting centre' were carried out in these AS individuals, but were found to be unsuitable for diagnosing deletions of this region.
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<td>BSA</td>
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<td>BWS</td>
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<td>CCD</td>
<td>Charged coupled device</td>
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<td>DAPI</td>
<td>4′-6-diamidino-2-phenylindole</td>
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<td>Standard saline citrate</td>
</tr>
<tr>
<td>UPD</td>
<td>Uniparental disomy</td>
</tr>
<tr>
<td>WT</td>
<td>Wilms’ tumour</td>
</tr>
</tbody>
</table>
1.0 Introduction
1.1 Angelman syndrome (AS) and Prader-Willi syndrome (PWS)

Angelman syndrome was first described in 1965. Three unrelated children with mental retardation and a similar facial and behavioural phenotype were reported (Angelman, 1965). He called them 'puppet children' and soon the syndrome became known as 'happy puppet syndrome' (Bower and Jeavons, 1967). The majority of parents and professionals considered this term to be derogatory and the name 'Angelman syndrome' is now preferentially used. AS has an incidence of 1 in 20 000 (Clayton-Smith and Pembrey, 1992).

Prader-Willi syndrome was first described in 1956 based on the findings present in nine original patients (Prader et al., 1956). PWS is the commonest syndromal cause of obesity and has an incidence of 1/10 000 - 1/30 000 live births (Cassidy, 1984).

1.1.1 Clinical description of AS

AS is a neurogenetic disorder characterised by severe mental retardation and delayed motor milestones. The frequency of the clinical features associated with AS, as summarised by Clayton-Smith and Pembrey (1992), are shown in table 1. Pregnancy is usually uneventful although the babies tend to weigh 200-300g less than their normal siblings. Feeding problems are common in the neonatal period due to difficulties with sucking or persistent regurgitation of feeds. The babies are often tremulous on handling and during the first months of life, jerky movements become apparent (Clayton-Smith and Pembrey, 1992). Truncal hypotonia is common and hypertonicity is usually present in the limbs. A clinical diagnosis of AS is based on a combination of the physical appearance and the behavioural phenotype. Where dysmorphic features are not apparent, a clinical diagnosis can often be made based on the characteristic behavioural phenotype alone.

The dysmorphic facial features associated with AS are not usually apparent during infancy but tend to evolve during the first five years of life. In 90% of AS individuals the skull is brachycephalic and in 98% of AS individuals, the head circumference falls below the 50th centile and often below the 25th centile. The mouth is wide and smiling with wide spaced teeth and a protruding tongue. The chin is pointed and prominent and there is usually a thin upper lip and midfacial hypoplasia. Hypopigmentation of the eyes, hair and skin is observed in 65% of AS individuals and the eyes are often blue and
<table>
<thead>
<tr>
<th>CLINICAL FEATURES</th>
<th>PERCENTAGE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delayed motor milestones</td>
<td>100</td>
</tr>
<tr>
<td>Ataxia</td>
<td>100</td>
</tr>
<tr>
<td>Absent speech &lt; 3 words</td>
<td>98</td>
</tr>
<tr>
<td>OFC &lt; 50th centile</td>
<td>98</td>
</tr>
<tr>
<td>Pointed chin / Prognathism</td>
<td>95</td>
</tr>
<tr>
<td>Truncal hypotonia</td>
<td>90</td>
</tr>
<tr>
<td>Brachycephaly</td>
<td>90</td>
</tr>
<tr>
<td>Blue eyes</td>
<td>88</td>
</tr>
<tr>
<td>Limb hypertonia</td>
<td>85</td>
</tr>
<tr>
<td>Hyperreflexia</td>
<td>85</td>
</tr>
<tr>
<td>Seizures</td>
<td>80</td>
</tr>
<tr>
<td>Macrostomia</td>
<td>75</td>
</tr>
<tr>
<td>Feeding problems</td>
<td>75</td>
</tr>
<tr>
<td>Blonde hair</td>
<td>65</td>
</tr>
<tr>
<td>Widely spaced teeth</td>
<td>60</td>
</tr>
<tr>
<td>Strabismus</td>
<td>40</td>
</tr>
<tr>
<td>Bowed primary dentition</td>
<td>35</td>
</tr>
<tr>
<td>Occipital groove</td>
<td>35</td>
</tr>
<tr>
<td>Cerebral atrophy on CT scan</td>
<td>30</td>
</tr>
<tr>
<td>OFC &lt; 3rd centile</td>
<td>25</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1. Frequency of the clinical features associated with AS.

The gait in AS individuals is ataxic and stiff legged. Figure 1 shows a child with the characteristic facial features and hypopigmentation observed in AS.

AS individuals have a happy sociable disposition, laughing frequently and sometimes inappropriately. The laughter is always provoked and when excited there is a tendency to flap the hands. They also show a love of water, reflections and noises (Clayton-Smith, 1992). In a study of 82 AS individuals (Clayton-Smith, 1993), 90% had a sleep disorder with the need for sleep reduced. This was most apparent between 2 and 6 years, but tended to improve with age. No individual spoke more than 6 words. Most spoke 1-3 words and 30% of individuals had no speech. The majority of individuals...
Figure 1. A child with Angelman syndrome showing the characteristic hypopigmentation of the hair and eyes, the wide smiling mouth with wide spaced teeth and protruding tongue.
were able to communicate using sign language or other gestures and 20% were able to use Makaton sign language. 80% of AS patients suffer from a seizure disorder, the onset of which is generally between 18 and 24 months, the first of which is usually precipitated by a fever. A characteristic seizure pattern is observed, with severe bouts of uncontrollable seizures lasting 3-4 weeks interspersed with seizure free periods (Clayton-Smith, 1992). The electroencephalogram (EEG) findings are characteristic, the most consistent of which is posterior slow wave activity with discharges, facilitated by or only seen on passive eye closure. More than one EEG is often required to demonstrate the typical changes. These changes are seen to some extent in most AS individuals, although they are age dependent and become less florid in older children (Boyd et al., 1988).

The clinical profile of AS changes with age, as AS individuals grow older, they tend to calm down and have less bursts of laughter although they retain their happy disposition. The EEG findings are not as obvious and seizures become less frequent (Buntinx et al., 1995).

1.1.1 Clinical description of PWS

PWS is characterised by psychomotor and growth retardation, neonatal and infantile hypotonia, characteristic facies including dolichocephaly, narrow bifrontal diameter, almond shaped eyes and a small mouth with down turned corners, short stature, small hands and feet, hypopigmentation, mild to moderate mental retardation, behavioural problems and hypothalamic dysfunction causing hypogonadism and hyperphagia with obesity (Butler et al., 1986). Obesity is the most prominent physical characteristic of PWS individuals and this can lead to hypertension, cardiovascular compromise and diabetes mellitus (Butler, 1990).

The clinical features of PWS can be quite variable between individuals and a diagnostic scoring system was designed to aid diagnosis (Holm et al., 1993). Table 2 is adapted from the two scoring categories of diagnostic criteria for PWS as proposed by Holm et al., (1993) and illustrates the criteria required for a diagnosis of PWS. Major criteria are scored at one point and minor criteria are scored at one half point. The supportive criteria are not included in the scoring system and are therefore not shown in the table. The scoring system is adjusted depending on the age of the patient as the PWS phenotype varies with age. Fewer symptoms are demonstrated in children aged three years and under, compared with older children and adults. Only
five points are therefore required for a diagnosis, with four criteria from the major group. A score of eight or above is required in the older age group, with at least five criteria from the major group.

<table>
<thead>
<tr>
<th>MAJOR CRITERIA</th>
<th>CLINICAL FEATURES</th>
</tr>
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<tbody>
<tr>
<td>Neonatal and infantile hypotonia with poor suck</td>
<td></td>
</tr>
<tr>
<td>Feeding problems in infancy and failure to thrive</td>
<td></td>
</tr>
<tr>
<td>Excessive weight gain between 1-6 years of age</td>
<td></td>
</tr>
<tr>
<td>Characteristic facial features (3 or more required)</td>
<td></td>
</tr>
<tr>
<td>Hypogonadism</td>
<td></td>
</tr>
<tr>
<td>Global developmental delay at 6 years and younger, mild to moderate mental retardation or learning problems in older children</td>
<td></td>
</tr>
<tr>
<td>Hyperphagia / food foraging / food obsession</td>
<td></td>
</tr>
<tr>
<td>Cytogenetic / molecular abnormality or maternal disomy</td>
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</tbody>
</table>

| MINOR CRITERIA                                       |                                                                                |
|------------------------------------------------------|                                                                               |
| Decreased fetal movement or infantile lethargy       |                                                                                |
| Characteristic behavioural problems (5 or more required) |                                                                                |
| Sleep disturbance or sleep apnea                      |                                                                                |
| Short stature for genetic background by age 15        |                                                                                |
| Hypopigmentation (fair skin and eyes compared to family) |                                                                                |
| Small hands (<25th centile) and feet (<10th centile)  |                                                                                |
| Narrow hands with straight ulnar border              |                                                                                |
| Eye abnormalities (esotropia, myopia)                 |                                                                                |
| Thick viscous saliva                                  |                                                                                |
| Speech articulation defects                           |                                                                                |
| Skin picking                                         |                                                                                |

Table 2. Diagnostic scoring criteria for PWS.

1.2 Genetic mechanisms in AS and PWS

1.2.1 Cytogenetic deletions associated with AS and PWS

The most common cytogenetic abnormality associated with AS and PWS is an interstitial deletion of chromosome 15q11-q13 (Ledbetter et al., 1981; 1982; Butler et al., 1986; Kaplan et al., 1987; Magenis et al., 1987;
A review of cytogenetic findings in PWS using high resolution methods, showed that about 60% of all PWS individuals had an interstitial deletion of 15q11-q13 (Ledbetter et al., 1987). In five different cytogenetic studies of AS, high resolution banding analysis of prometaphase chromosomes looked at 120 individuals with AS. These studies detected an interstitial deletion of the long arm of chromosome 15q11-q13 in 56/120 cases, representing almost half of the AS cases (Kaplan et al., 1987; Magenis et al., 1987; Pembrey et al., 1989; Williams et al., 1989; Chan et al., 1993).

A single human chromosome 15 consists of approximately 1X10^8 base pairs, composing nearly 3% of the total human haploid genome (Mendelsohn et al., 1973; Southern, 1982). Chromosome 15 is an acrocentric chromosome with satellite-rich heterochromatic centromere and stalk regions (Miklos and John, 1979). The heteromorphic nature of chromosome 15q11.2 makes the cytogenetic detection of deletions in AS and PWS difficult due to the size of the region, its variability in homologue condensation and its proximity to the centromere. If AS or PWS is suspected clinically, chromosome analysis should be carried out at no less than the 550 band stage and preferably at the 850 band stage (ISCN, 1995). Figure 2 shows an ideogram of chromosome 15 at the 850 band level.

**Figure 2.** An ideogram of chromosome 15 at the 850 band level.

Cytogenetic polymorphisms of chromosome 15 including variations in the nucleolar organising regions and the size of the short arms, has enabled
cytogeneticists to assess the parental origin of the deleted chromosome 15's in AS and PWS individuals. The deleted chromosome in PWS is paternally derived (Butler and Palmer, 1983; Mattei et al., 1983; Niikawa and Ishikiriyama, 1985; Butler et al., 1986) and maternally derived in AS (Magenis et al., 1990; Williams et al., 1990).

1.2.2 Cytogenetic rearrangements

Chromosomal rearrangements involving 15q11-q13 are observed in a small number of cases of AS and PWS. These abnormalities include translocations, inversions, duplications and small marker chromosome 15's. Reciprocal translocations have been reported in individuals with AS and PWS (Charrow et al., 1983; Butler et al., 1990; Hulten et al., 1991; Chan et al., 1993; Reeve et al., 1993; Reis et al., 1993; Tepperberg et al., 1993; Burke et al., 1996). In the majority of cases, the segregation of these translocations produced affected offspring when the unbalanced forms of the translocations were inherited, resulting in deletions of either the maternal or paternal 15q11-q13 region. In two further cases of AS (Smeets et al., 1992; Smith et al., 1994), inheritance of the translocation in either its balanced or unbalanced form was associated with uniparental paternal disomy (section 1.2.4) and this was the cause of AS in these individuals. Recently, two de novo balanced reciprocal translocations resulting in PWS have been reported (Sun et al., 1996; Schulze et al., 1996). The consequences of these translocations are discussed later (section 5.1). Unlike the other acrocentric chromosomes, chromosome 15 is rarely involved in Robertsonian translocations, although they appear to be over represented in the PWS population (Mattei et al., 1984). Affected offspring with Robertsonian translocations were produced when either one of the chromosome 15's was deleted or as a result of uniparental disomy (Nicholls et al., 1989a; Casamassima et al., 1991; Smith et al., 1993; Clayton-Smith et al., 1995).

Inversions interfere with the pairing of homologous chromosomes during meiosis, giving a predisposition to de novo deletions and duplications (Hoo, 1983). Inversions have occasionally been described in the literature as a cause of either AS or PWS. Paternal inheritance of a pericentric inversion with an accompanying de novo paternal deletion of 15q11-q13 resulting in PWS was described in two cases (Kahkonen et al., 1990; Chan et al., 1993). Maternal inheritance of a pericentric inversion with an accompanying de novo maternal deletion of 15q11-q13 resulting in AS was reported in the cousin of
the PWS individual described by Chan et al., (1993) and in a further case of AS (Pembrey et al., 1989; Webb et al., 1992).

Duplications of the 15q11-q13 region as a cause of PWS have been well documented (de France et al., 1984; Berry et al., 1987; Pettigrew et al., 1987). A duplication and triplication in the 15q11-q13 region, resulting in clinical features with similarities to AS have also been described (Clayton-Smith et al., 1993; Schinzel et al., 1994a). Molecular analysis in the first case confirmed a de novo duplication of the 15q11-q13 region. In the second case, FISH analysis with cosmids and YACs from the AS/PWS region showed that there was an intrachromosomal triplication of the 15q11-q13 region, with the middle repeat being inverted. Molecular analysis of microsatellite polymorphisms within the 15q11-q13 region showed inheritance of one paternal allele and two different maternal alleles.

Small supernumerary marker chromosomes are estimated to occur in 1.5 per 1000 livebirths of which 50% may be inv dup (15) (Buckton et al., 1985; Webb, 1994). The phenotype of individuals who carry these marker chromosomes varies considerably from apparently normal to those who are severely mentally retarded. This may either be due to the size of the marker or mosaicism. The presence of supernumerary inv dup (15) marker chromosomes in only a small percentage of mitoses might be expected to result in a less severe phenotype (Mignon et al., 1996). In patients with no demonstrated mosaicism, clinical severity has been related to the size and molecular content of the markers (Nicholls et al., 1989b; Leana-Cox and Schwartz, 1993; Robinson et al., 1993a; Cheng et al., 1994; Grammatico et al., 1994; Leana-Cox et al., 1994; Crolla et al., 1995; Mignon et al., 1996). These markers have been associated with AS and PWS (Ridler et al., 1971; Michaelson et al., 1979; Fujita et al., 1980; Wisniewski et al., 1980; Ledbetter et al., 1982; Mattei et al., 1983; Goh et al., 1984; Robinson et al., 1993b; Spinner et al., 1995). A case of AS and two cases of PWS associated with a supernumerary inv dup (15), in which molecular studies showed uniparental paternal disomy in the AS patient and uniparental maternal disomy in the two PWS patients, were reported (Robinson et al., 1993b; Cheng et al., 1994). Uniparental disomy (section 1.2.4) is therefore the most likely cause of the phenotype in these cases. In a further case of AS associated with a supernumerary inv dup (15) (Spinner et al., 1995), molecular studies demonstrated that one of the apparently normal chromosome 15's was deleted and this is the most likely cause of the AS phenotype in this case.
1.2.3 Deletions detected by molecular probes

Chromosome 15q11-q13 markers have been isolated from recombinant phage libraries enriched for the DNA of flow sorted supernumary marker chromosome 15's (Donlon et al., 1986), hybrid cell lines (Tassett et al., 1988) and from microdissected 15q11-q13 chromosomal regions (Buiting et al., 1990). The development of probes for the 15q11-q13 region initially enabled more reliable and routine detection of deletions in AS and PWS individuals than routine cytogenetic analysis, as well as determining the extent of the deletions and their parental origin. Southern blot analysis and dosage blot hybridisation analysis of DNA from AS and PWS individuals, probed with the cloned DNA segments, showed that similar molecular deletions involving 15q11.2 were encountered in both syndromes (Donlon et al., 1988). Five PWS individuals and two AS individuals were shown to be deleted for the same five DNA probes (pML34, IR4-3R, pTD189-1, TD3-21 and pIR10-1). The probe order (figure 3) was determined by deletion analysis in PWS individuals with cytogenetic deletions and a PWS individual with a translocation (Tantravahi et al., 1989).

Restriction fragment length polymorphisms (RFLPs) were found for all five of these probes, p34 (D15S9), pIR4-3R (D15S11), p189-1 (D15S13), p3-21 (D15S10), pIR10-1 (D15S12) and for an additional probe pIR39 (D15S18), enabling the assessment of copy number and for informative RFLPs, the parental origin of the deletion (Nicholls et al., 1989b). RFLP analysis with these probes, confirmed earlier cytogenetic findings that the deletions in PWS were paternal in origin and maternal in origin in AS (Knoll et al., 1989; Clayton-Smith et al., 1992a). A role for genomic imprinting, in which gene expression is dependent on the parent from whom the gene was inherited, was proposed.

Knoll et al., (1990) identified three molecular classes in AS individuals (figure 3). Class I and class II show a deletion of chromosome 15q11-q13 specific DNA markers 34 (D15S9), IR4-3R (D15S11), 189-1 (D15S13), 3-21 (D15S10) and IR10-1 (D15S12), with class I also deleted for the proximal marker IR39d, a sub-fragment of IR39 (D15S18) (Donlon et al., 1986; Nicholls et al., 1989b). In class III, no deletion was detected. The class I and II deletion breakpoints have been further defined (Christian et al., 1995). The proximal breakpoint of the class I deletions lies proximal to D15S541/D15S542, while the proximal breakpoint of the class II deletions lies between D15S541/D15S542 and D15S543 (figure 6).
Microdissection of the PWS region identified 11 microclones (Buiting et al., 1990). Three of these clones MN6 (D15S76), MN7 (D15S62) and PW71 (D15S63) were shown to be deleted in three PWS individuals. The exact order of the 15q11-q13 markers, relative to each other has been difficult to assess by deletion analysis alone as the majority of these markers are deleted in most AS and PWS individuals.

Figure 3. Schematic representation of the three molecular classes observed in AS.

The common deletions observed in AS and PWS encompass a physical distance of approximately 4 Mb. FISH analysis with YACs in AS and PWS patients showed that the same YACs were deleted in the interval from IR4-3R (D15S11) to GABRB3 (Kuwano et al., 1992). On the proximal side, 10/10 breakpoints fell within a single ML34 (D15S9) YAC and 8/9 distal breakpoints clustered within a single IR10-1 (D15S12) YAC. These common deletion breakpoints suggest recombination 'hotspots'.

Molecular studies have shown that 70-75% of AS individuals can be accounted for by a deletion of 15q11-q13 (Beuten et al., 1993; Chan et al., 1993; Saitoh et al., 1994). In two independent studies, DNA from a total of 69 PWS individuals was analysed and 47 of these PWS individuals showed a molecular deletion of 15q11-q13 (Robinson et al., 1991; Webb et al., 1995). These molecular deletions account for 68% of individuals with PWS.
1.2.4 Uniparental disomy (UPD) and chromosome 15

Nicholls et al., (1989a) identified two PWS patients in whom DNA markers showed that both copies of chromosome 15 were inherited from the mother and none were inherited from the father. One of the PWS patients had inherited a balanced Robertsonian translocation involving chromosome 15 and analysis of his DNA showed the presence of two maternal alleles for the probes 3-21 (D15S10) and CMW-1 (D15S24), but no paternal alleles had been inherited at either of these loci. This individual therefore demonstrates maternal heterodisomy for at least the critical region of chromosome 15 which is involved in the aetiology of PWS. Analysis of DNA from the second PWS patient showed that he had inherited a maternal allele for probes 34 (D15S9) and IR39d (D15S18) but he had not inherited a paternal allele at either of these loci. These two loci are flanked by IR4-3R (D15S11) and IR10-1 (D15S12), which were heterozygous in the proband. While paternal inheritance at these two loci could not be excluded, as the mother and father were heterozygous for the same alleles, heterodisomy, which is the inheritance of two non identical chromosomal segments from only one parent, rather than isodisomy was suggested.

Maternal UPD for chromosome 15 has since been shown to occur in 20-25% of PWS individuals and molecular analysis has shown that the majority of these cases represent maternal heterodisomy (Robinson et al., 1991; Mascari et al., 1992; Mutirangura et al., 1993a). The majority of PWS individuals can therefore be accounted for either by a molecular deletion of 15q11-q13, an unbalanced chromosomal rearrangement involving chromosome 15 which results in a deletion of the 15q11-q13 region, or uniparental maternal disomy.

PWS individuals with UPD 15 mat do not have the hypopigmentation that is commonly seen in PWS individuals with a deletion of chromosome 15 (Mascari et al., 1992), suggesting that hypopigmentation may result from a gene dosage effect rather than an imprinted gene. This appears to be the only phenotypic difference between PWS deletion and disomy cases. The observation of maternal UPD in PWS without additional phenotypic abnormalities, suggests the presence of a paternally imprinted gene(s) on chromosome 15 is limited to the PWS region.

Uniparental paternal disomy (15) in which both copies of chromosome 15 are inherited from the father and none are inherited from the mother, was demonstrated in two AS individuals (Malcolm et al., 1991) with the probes.
cMS620 (D15S86) (Armour et al., 1990) and CMW-1 (D15S24), which is a multiallelic or VNTR probe (Rich et al., 1988). Further cases of UPD 15 pat resulting in AS have subsequently been described and molecular analysis has demonstrated that the majority of cases show complete paternal isodisomy (Malcolm et al., 1991; Nicholls et al., 1992; Mutirangura et al., 1993a; Robinson et al., 1993c). Uniparental paternal disomy is a minor mechanism in AS accounting for approximately 5% of cases (Knoll et al., 1991; Chan et al., 1993). The observation of paternal UPD without additional phenotypic effects in AS, suggests that there is a maternally imprinted gene on chromosome 15 limited to the AS region and there are no other genes located on chromosome 15 outside the AS/PWS region that are imprinted.

It has been suggested that the AS phenotype due to paternal UPD may be milder (Bottani et al., 1994). Two unrelated females with paternal isodisomy for the entire chromosome 15 had late onset mild seizures, their gross motor functions were largely undisturbed as they grew older and in particular there was no severe ataxia. One of the girls did not show the characteristic AS facial features and in addition, neither individual showed the commonly observed hypopigmentation of the skin, hair and eyes. A further AS individual with UPD 15 pat and a milder phenotype confirms these findings (Gillessen-Kaesbach et al., 1995).

A maternal and paternal age effect have been observed in uniparental maternal disomy in PWS and uniparental paternal disomy in AS (Robinson et al., 1991; Mascari et al., 1992; Robinson et al., 1993d; Christian et al., 1996). An increase in the frequency of nondisjunction events has been observed in trisomy 21 and other aneuploid conditions and is associated with advanced maternal age (Hassold and Jacobs, 1984; Hassold and Chiu, 1985). Approximately 78% of maternal nondisjunction events leading to trisomy 21 are meiosis I errors, whereas the majority of paternal errors were attributed to either meiosis II or mitotic events (Antonarakis et al., 1993). An error at meiosis I has been observed in approximately 82% of maternal nondisjunction events leading to UPD (15), whereas most cases of UPD 15 pat involve errors at meiosis II or mitotic errors (Robinson et al., 1993c). An association between paternal age and nondisjunction has been difficult to assess due to the smaller numbers of paternally derived trisomy cases. There is a high frequency of trisomy for chromosome 15 (Hassold and Jacobs, 1984) and there is evidence that some UPD 15 mat cases arise as a trisomic fertilisation with subsequent loss of the paternal chromosome. Confined placental mosaicism occurs in approximately 2% of all CVS samples.
and may arise through correction of a trisomy (Kalousek et al., 1991). Lineage confined mosaicism arises when aneuploid embryos are rescued through post-zygotic mutation in either placental or embryonic progenitors. When the trisomie chromosome is lost in the trophoblast, a viable non-mosaic infant is produced (Kalousek et al., 1989). If a similar mutation occurs in the embryonic progenitor cell, a diploid fetus develops supported by a trisomic placenta. One third of these diploid fetuses are expected to have UPD for the chromosome pair involved in the original trisomy (Hall 1990; Engel and Delozier-Blanchet, 1991). Trisomic rescue has been documented in at least three cases of PWS (Cassidy et al., 1992; Purvis-Smith et al., 1992; Morichon-Delvallez et al., 1993). Trisomy 15 was reported in CVS samples in each of these cases and follow-up studies on amniotic fluid indicated a normal karyotype. However, following birth, a diagnosis of UPD 15 mat resulting in PWS was made. Molecular studies for UPD are therefore warranted in cases of mosaic trisomy 15 in prenatal CVS samples (Christian et al., 1996).

Cases of UPD 15 pat are often associated with complete isodisomy suggesting a monosomy 15 conception followed by a duplication of a single paternal chromosome 15 or by long arm isochromosome formation (Freeman et al., 1993; Mutirangura et al., 1993a; Robinson et al., 1993c). This type of somatic selection has also been observed in two cases of isodisomy for chromosome 21, in which a constitutional abnormality of chromosome 21 was present at birth, was lost and replaced by a duplicated copy of the normal 21 later in life (Petersen et al., 1992). These findings can also be explained by gamete complementation or trisomic rescue but would require a meiosis II nondisjunction event.

The frequency of aneuploidy in human oocytes has been estimated as 18-19% with an excess of D-group chromosomes aneuploid in oocytes. The frequency of aneuploidy in spermatocytes is estimated as 3-4% (Martin et al., 1991), suggesting that maternal errors are more likely. The observation of a low frequency of UPD 15 pat in AS supports the finding that paternal nondisjunction is not as common as maternal nondisjunction (or a fetus with UPD 15 pat may be less viable in utero).

1.2.5 Autosomal dominant inheritance of AS modified by imprinting

Amongst individuals with AS, 20-25% of cases remain in which there is biparental inheritance of markers from 15q11-q13 and no detectable deletion. Evidence of autosomal dominant inheritance of AS was provided by a study of
5 familial cases which showed that the affected siblings inherited the same maternal chromosome 15 alleles, whereas random paternal chromosome 15 alleles were inherited (Clayton-Smith et al., 1992b). This was dubbed 'dominant inheritance of small mutations modified by parental imprinting'. The recurrence risk in these families is 50%, based on the inheritance of the maternal chromosome only.

Two pedigrees were identified in which multiple cases of AS arose only after maternal transmission, with asymptomatic transmission through male ancestors. These pedigrees indicate that the loci responsible for AS and PWS, although closely linked, are distinct (Wagstaff et al., 1992; Meijers-Heijboer et al., 1992). The strongest evidence for dominant inheritance of AS modified by genomic imprinting comes from a Japanese family with three affected siblings (Hamabe et al., 1991). The three affected siblings, their unaffected mother and the maternal grandfather all carried a small deletion encompassing the TD3-21 (D15S10) and LS6-1 (D15S113) loci (Saitoh et al., 1992), with the distal breakpoint within intron 3 of the GABR33 gene (Greger et al., 1993). The deletion is harmless when transmitted through the male germline but results in AS when transmitted maternally, showing that the AS and PWS regions on chromosome 15q11-q13 are distinct. This strongly supports the hypothesis that an AS gene(s) is imprinted and distinct from the PWS gene(s) and in familial cases of AS, it is likely that a small rearrangement or point mutation in a single AS gene is responsible for the phenotype.

1.2.6 DNA methylation imprints and imprintor mutations

Parent of origin differences in DNA methylation have been observed at several loci on chromosome 15. A DNA methylation imprint was first discovered at the D15S9 locus which encodes a novel zinc finger protein (ZNF127) in the AS/PWS region (Driscoll et al., 1992). Altered methylation patterns were subsequently observed at PW71 (D15S63) which is associated with an endogenous retroviral solo LTR sequence (Dittrich et al., 1992) and at the SNRPN gene (Özçelik et al., 1992) which maps 130kb downstream of PW71 on 15q11-q13. Parent of origin specific DNA methylation was observed within intron 5 and exon alpha of SNRPN (Glenn et al., 1993a; Sutcliffe et al., 1994).

Hind III/Hpa II digested genomic DNA probed with PW71 detects a 6.6 kb band (due to methylation of a Hpa II site on the maternal chromosome) which
represents the maternal methylation imprint and a 4.7 kb band which represents the paternal methylation imprint (Dittrich et al., 1993). Normal individuals have both maternal and paternal methylation imprints, whereas AS deletion or UPD cases have a paternal methylation imprint (4.7 kb band) only (figure 4), lacking the 6.6 kb maternal band and PWS or UPD cases have a maternal methylation imprint (6.6 kb band) only, lacking the 4.7 kb paternal band.

Figure 4. Hind III/Hpa II digested genomic DNA hybridised with the probe PW71B showing 1) a normal control, 2) an AS individual, 3) a PWS individual, 4) a normal 21 week fetus and an AS imprinter mutation family.

Exon alpha is a newly defined 5' exon of SNRPN that contains a differentially methylated CpG island (Sutcliffe et al., 1994). A 4.2 kb Xba I fragment was isolated which contained exon alpha. This fragment was shown to contain three Not I sites, Not I is a methylation sensitive enzyme and analysis of a normal maternal chromosome (PWS deletion or UPD) produces an intact 4.2 kb band, consistent with the methylation of the three Not I sites on the maternal chromosome, while analysis of a paternal chromosome (AS deletion or UPD), produces a pattern (4 bands of 3.0, 0.9, 0.15 and 0.1 kb) consistent with complete absence of methylation at the three Not I sites. Intron 5 of SNRPN contains Hha I and Hpa II sites which are methylated on the paternal chromosome and unmethylated on the maternal chromosome in most cells (Glenn et al., 1993a).
Most AS individuals with biparental inheritance (non deletion / non UPD) show the same methylation patterns as normal individuals. However, a sub-set of AS and PWS individuals with neither deletions nor disomy have been identified in which the methylation pattern is altered (Glenn et al., 1993b; Reis et al., 1994; Sutcliffe et al., 1994; Bueting et al., 1994; Beuten et al., 1996; J. Buxton, pers. comm.). These individuals are said to have 'imprinter mutations'.

The methylation patterns in 15q11-q13 appear to be correlated with the expression status of the PWS gene(s) and AS gene(s). The observation of a maternal methylation imprint on a paternal chromosome and of a paternal methylation imprint on a maternal chromosome, suggests that the PWS and AS genes become silenced by the imprinting process in individuals with an imprinter mutation. A defect in the imprinting process may result from a mutation that acts either in cis or in trans on the AS and PWS genes. Figure 5 shows a schematic representation of the differential DNA methylation patterns observed on chromosome 15q11-q13.

1.3 Chromosome 15 and genomic imprinting

The strongest evidence that genes in the 15q11-q13 region are subject to imprinting, came from a family in whom segregation of the unbalanced form of a translocation between chromosomes 15 and 22 led to AS when transmitted maternally and PWS when transmitted paternally (Hulten et al., 1991). This translocation therefore excludes the possibility that some other factor, such as the unmasking of an autosomal recessive disorder due to homozygosity, dictates the AS and PWS phenotypes.

1.3.1 The AS and PWS critical regions

Translocations and smaller deletions have narrowed the AS and PWS critical regions within the 4 Mb deletion common to both disorders. The deletion described by Saitoh et al., (1992) narrows both critical regions. The proximal deletion breakpoint is defined by probe PB11 (D15S174), which lies distal to SNRPN, between PAR-1 and PAR-4 and defines the proximal boundary of the AS region. The distal breakpoint lies within intron 3 of the GABRB3 gene (Greger et al., 1993) and defines the distal boundary of the AS region. The AS critical region as defined by this family is shown in figure 6.
Figure 5. A schematic representation of the DNA methylation imprints in a normal individual and an AS individual with an imprintor mutation at PW71 (D15S63), SNRPN (exon alpha) and SNRPN (intron 5).
The proximal breakpoint for the PWS critical region was defined by a PWS individual with an atypical deletion (Robinson et al., 1991). This PWS individual was not deleted for the D15S9, D15S11 and D15S13 loci but was deleted for loci at D15S63, SNRPN, D15S10, D15S12 and D15S24 (Robinson et al., 1991; Özçelik et al., 1992), suggesting a location between D15S13 and D15S174 for the PWS critical region. However, it is now understood that PWS in this individual could also be explained by loss of imprinting control elements and it is probable that the PWS critical region lies within the 1.5 Mb domain from D15S9 to D15S174, since more than one gene is likely to be involved in the aetiology of PWS.

1.3.2 Identification of genes from the AS / PWS critical region

The minimum estimated size of the 15q11-q13 region is approximately 4 Mb and could contain more than 100 genes. The establishment of probe order by FISH analysis in interphase nuclei (Kuwano et al., 1992; Knoll et al., 1993) and the development of a YAC contig (figure 6) of the 15q11-q13 region (Mutirangura et al., 1993b), has enabled the construction of a physical map of the AS/PWS region. The YAC contig spans approximately 3.5 Mb of the estimated 4 Mb 15q11-q13 region, encompassing loci between D15S9 and GABRA5. It provides an essential source for generating new polymorphic DNA markers such as microsatellite markers and for mapping or cloning candidate genes for PWS and AS. Figure 7 shows the position of the microsatellite markers isolated from 15q11-q13 and figure 8 shows the genes isolated from the 15q11-q13 region.

GABRB3 was the first gene to be mapped to the AS/PWS region on chromosome 15 (Wagstaff et al., 1991). GABRB3 encodes the β3 subunit of the GABA receptor. GABA is the major inhibitory neurotransmitter in the mammalian brain, where it acts at GABA<sub>α</sub> receptors, which are ligand-gated chloride channels. Possible physiological roles for GABA include the suppression of seizure activity (Taylor, 1988; Meldrum 1989). If GABRB3 is to be a candidate gene for AS, then it should only be expressed from the maternal chromosome. GABRB3 is not imprinted in mouse brain (Cattanach et al., 1992; Nicholls et al., 1993) and mice with deletions of central chromosome 7 which shows synteny with human chromosome 15q11-q13, are phenotypically normal, expressing equal levels of GABRB3 from the maternal and paternal chromosomes. The GABRA5 gene was mapped distal
Figure 6. Physical map of chromosome 15q11-q13 including a 4Mb YAC contig (Mutirangura et al., 1993). The jagged lines represent the common deletion breakpoints found in AS and PWS. The two classes of proximal deletions are indicated by I and II and the arrow illustrates the AS critical region (Saitoh et al., 1992).
Figure 7. Physical map of chromosome 15q11-q13 indicating the position of microsatellite markers isolated from the AS/PWS region on chromosome 15 (Malcolm and Donlon, 1994; S. Christian and R. Trent, pers. comm.).
Figure 8. Physical map of 15q11-13 indicating the position of genes isolated from the AS/PWS critical region (Wagstaff et al., 1991; Glenn et al., 1993; Knoll et al., 1993; Greger et al., in press; Malcolm and Donlon, 1994; Ozcelik et al., 1992; Wevrick et al., 1994; Sutcliffe et al., 1994; Nakao et al., 1994) and the patterns of allele-specific replication (Knoll et al., 1994).
to GABRβ3 (Knoll et al., 1993) and encodes an alpha subunit of the GABRβ3 receptor and more recently GABRG3 was mapped distal to GABRA5 (Greger et al., 1995). Gabra5 and Gabrg3 are not imprinted in mouse brain (Culiat et al., 1995) and in mouse compound-deletion heterozygotes not expressing either Gabra5 or Gabrg3, no obvious neurological phenotype was observed. It is therefore unlikely that either Gabra5 or Gabrg3 contribute to AS in humans.

The first set of DNA probes generated by chromosome flow sorting and microdissection were subsequently used to identify the ZNF127 gene (Glenn et al., 1993b), the P gene (Gardner et al., 1992) and the MN7 genes (Buiting et al., 1992). ZNF127 encodes a novel zinc finger protein and is expressed from the paternal allele only (Glenn et al., 1993b). To date, ZNF127 has not been fully characterised. DNA clones derived from the pink-eyed dilution locus (p) on mouse chromosome 7 identified loci that are linked to human 15q11-q13 (Gardner et al., 1992). Disruption of a gene isolated from the mouse p locus was shown to cause hypopigmentation, which is a clinical feature observed in the majority of cases of AS and PWS. The human homologue is the P gene which is involved in melanin biosynthesis in melanocytes and was shown to be responsible for the hypopigmentation seen in AS and PWS, as well as for tyrosinase-positive (type II) oculocutaneous albinism (Rinchik et al., 1993; Lee et al., 1994). This gene is not imprinted but remains the only gene which directly correlates to a phenotypic feature observed in AS and PWS. MN7 is a putative multiple gene family in 15q11-13 and 16p11.2 (Buiting et al., 1992) of unknown function. MN7 maps outside of the critical region although the presence of multiple copies of repeat sequences may somehow contribute to the instability of this region.

The SNRPN gene was identified by mapping the SmN protein which it encodes, to somatic cell hybrids (Özçelik et al., 1992). Two loci were identified, one which was concordant with human chromosome 15pter-q14 and one which was concordant with human chromosome 6pter-p21. The latter locus was intronless, consistent with a retroposed pseudo-gene (gene symbol SNRPNP1). The SNRPN gene is abundantly expressed in brain and heart and encodes a protein, SmN, that is associated with small nuclear ribonucleoprotein particles (snRNPs). These snRNP particles are found in spliceosomes and are essential for pre-mRNA processing (Steitz et al., 1987; Zieve et al., 1990). It is possible that faulty splicing of neuron specific messages plays a role in the pathogenesis of PWS. SNRPN was shown to
be expressed from the paternal chromosome only (Cattanach et al., 1992; Glenn et al., 1993a).

IPW (imprinted gene in the PWS region) was isolated by direct cDNA selection using YACs which formed a contig encompassing the PWS deletion interval on chromosome 15 (Wevrick et al., 1994). IPW is spliced and polyadenylated but its longest open reading frame only codes for 45 amino acids, suggesting that it functions as an RNA. IPW is located about 150 kb distal of SNRPN and is widely expressed in fetal and adult tissues. IPW was shown to be expressed from the paternal chromosome only.

Two further paternally expressed sequences, PAR-5 and PAR-1 were identified from the PWS region by screening fetal brain cDNA libraries with genomic probes (Sutcliffe et al., 1994). PAR-5 and PAR-1 were detected in most adult and fetal tissues. These two sequences mapped distal to SNRPN within 300kb. Two further sequences, PAR-4 and PAR-7 were found to be expressed in human fetal and adult tissues (Sutcliffe et al., 1994). Sequence analysis of PAR-5 and PAR-1 identified no significant homology at a protein or nucleotide level, they do not have significant open reading frames and showed continuity with genomic DNA. Northern analysis indicated that these clones represent different sized transcripts with different expression patterns, some of the transcripts being quite large and heterogeneous. These transcripts could be derived from large 3' untranslated regions of protein coding mRNA, could represent illegitimate transcripts or hnRNA sequences or they could be analogous to the non-coding imprinted transcripts such as IPW (Wevrick et al., 1994), H19 (Brannan et al., 1990) and XIST (Brown et al., 1992).

Two further fragments were isolated from the AS/PWS region by screening human cDNA libraries with genomic DNA fragments isolated from cosmid and phage clones. These fragments were identified by using radiolabelled cDNA probes synthesised from poly(A) +RNA of mouse tissues (Nakao et al., 1994). One gene, the ubiquitin protein ligase gene UBE3A, previously known as E6-AP, was found to encode the E6-associated protein which interacts with the E6 protein of human papilloma virus. UBE3A was expressed in heart, liver and skeletal muscle and to a lesser extent in brain, placenta, lung and kidney. The second gene, PAR-2, has not been characterised but was shown to be widely expressed. UBE3A and PAR-2 were expressed in fibroblasts and lymphoblasts from individuals with AS and PWS and are therefore not imprinted.
1.3.3 Imprinting assays

Imprinting analysis of the murine homologue (*Snrpn*) was studied using two different methods. An RNase protection assay of RNA derived from interspecific mouse crosses (Leff et al., 1992) showed *Snrpn* to be expressed from the paternal chromosome only. In the second method, maternal duplications for the central region of mouse chromosome 7 were generated by crossing mice with the Is1CtX-autosome translocation in which the region is inserted into the X chromosome (Cattanach et al., 1992). Maternal duplication of central mouse chromosome 7 produced an early postnatal lethality possibly associated with a reduced suckling activity, suggesting a mouse model for PWS. *Snrpn* expression was not detected in mice with a maternal duplication, but was detected in normal controls. Functional imprinting of the human SNRPN gene was demonstrated using reverse transcription followed by the polymerase chain reaction (RT-PCR) (Glenn et al., 1993a). No expression was observed in cultured skin fibroblasts or lymphoblasts of PWS individuals but was found in all AS individuals and normal controls examined. These findings indicate that SNRPN is only expressed from the paternally derived chromosome 15 in humans. Analysis of maternal genomic DNA and cDNA synthesised from human fetal brain and heart RNA and from adult cerebral cortex also showed monoallelic paternal expression of human SNRPN (Reed and Leff, 1994). A sequence polymorphism was identified in expressed portions of SNRPN and was used to follow allele specific expression. A functional SNRPN gene product is absent in PWS and this may lead to the developmental and neuroendocrine abnormalities observed in PWS.

*IPW* showed expression exclusively from the paternal allele in lymphoblasts, fibroblasts and fetal tissues (Wevrick et al., 1994). Imprinting analysis of *IPW* was studied by preparing a Northern blot from total lymphoblast RNA from normal individuals and from AS and PWS individuals with known deletions of 15q11-q13. No expression was observed in the PWS cell lines, whereas normal and AS cell lines expressed similar amounts of message. The imprinted status of *IPW* was also studied by using a sequence polymorphism within the *IPW* cDNA and studying the inheritance of the alleles.

*IPW* codes for a spliced polyadenylated messenger RNA as is the case for H19 (Brannan et al., 1990) and XIST (Brown et al., 1992). Studies of XIST have shown that a polyadenylated, spliced, non-translated mRNA can have profound effects on the expression of many other genes and it is possible that
IPW may have a direct role in the imprinting process of genes on 15q11-q13. IPW could also have a similar role to that proposed for H19. It has been shown that H19 is involved in the regulation of Igf2 expression in the mouse (Leighton et al., 1995) and it is possible that IPW is involved in the regulation of other imprinted genes in the 15q11-q13 region.

The PAR-5 and PAR-1 transcripts were shown to be paternally expressed. RNA was isolated from cultured cells derived from AS and PWS individuals who were deleted for the 15q11-q13 region and the patterns of expression of the two transcripts in these individuals were studied by RT-PCR. Expression of PAR-5 and PAR-1 was observed in normal individuals and AS individuals but expression was not observed in PWS individuals. PAR-4 and PAR-7 were not sufficiently expressed in either cultured fibroblasts or lymphoblasts of AS and PWS individuals to determine their imprinted status (Sutcliffe et al., 1994).

1.3.4 Imprinted domains

Studies of one familial case of PWS (family O) and one sporadic case of PWS (patient E) demonstrated paternal deletions of 5-60 kb which removed the newly described 5' exon of SNRPN (exon alpha). In family O, a deletion was detected by FISH analysis with the SNRPN cosmid but not with the overlapping centromeric cosmid or cosmids containing the PAR-5, PAR-7 and PAR-1 transcripts on the telomeric side. FISH analysis of patient E demonstrated a deletion of the SNRPN cosmid and the overlapping centromeric cosmid. The regions corresponding to the next overlapping centromeric cosmid and the telomeric cosmid containing PAR-5 were not deleted. Total cellular RNA was extracted from lymphoblasts from these PWS individuals (Sutcliffe et al., 1994) and the expression of SNRPN, PAR-5 and PAR-1 was studied. The expression of SNRPN, PAR-5 and PAR-1 was undetectable in these PWS individuals, suggesting loss of one or more elements from a common region involved in the control of transcriptional activity of these genes and this is in agreement with a domain structure on chromosome 15q11-q13. It was proposed that a cis acting, paternally sensing, imprinting control region was localised close to SNRPN (exon alpha). The transmission of the deletion in family O is benign when transmitted maternally but paternal expression causes PWS, suggesting that the deletion removes a controlling element that is not necessary for normal maternal expression.
A temporal and spatial association has been observed at imprinted domains on 15q11-q13 in human T-lymphocytes (LaSalle and Lalande, 1996). This association only occurred during the late S phase of the cell cycle. When cells from AS and PWS patients were studied, they were shown to be deficient in association, which suggests that normal imprinting involves mutual recognition and preferential association of maternal and paternal chromosome 15’s. These results imply that trans-acting elements could be involved in the regulation of imprinting in somatic cells.

1.3.5 Replication domains

Normally genes replicate synchronously during S phase in the cell cycle. However, all known imprinted genes in humans and the mouse replicate asynchronously, with the paternal allele usually replicating before the maternal allele (Kitsberg et al., 1993).

Asynchronous replicating domains (figure 8) have been observed on chromosome 15 by comparing the replication timing of the R-band in 15q11.2 to X chromosome bands in an unsynchronised peripheral lymphocyte population and by using a FISH assay with 15q11-q13 specific probes on interphase lymphocytes and lymphoblasts (Izumikawa et al., 1991; Kitsberg et al., 1993; Knoll et al., 1994; LaSalle and Lalande, 1995). Asynchronous replication is visualised as a single hybridisation signal on one chromosome (singlet) or a double hybridisation signal on the other chromosome (doublet). At D15S63, D15S10 and GABRB3, the paternal chromosome replicates before the maternal chromosome and at GABRA5, the maternal chromosome replicates first. Asynchronous replication was also confirmed in amniocytes and skin fibroblasts (Lin et al., 1995). The 50-60 kb region contained in the intragenic region between GABR33 and GABRA5 is different from the surrounding replication domain by allele specificity and kinetics of replication (LaSalle and Lalande, 1995), suggesting that its boundaries define a chromatin structure that is differentially regulated on the maternal and paternal chromosomes.

Replication asynchrony has subsequently been observed for a 750 kb region, including the SNRPN gene (Gunaratne et al., 1995), in both lymphocytes and neuroblasts. The first SNRPN allele to replicate was shown to switch from a late replicating region in lymphocytes, to an early replicating region in neuroblasts. Analysis of replication timing patterns in lymphoblasts from a PWS family with a microdeletion of the 5’ end of SNRPN (family O), showed
that the deletion resulted in a change of replication timing when the deletion was inherited paternally, but did not influence replication timing when it was inherited maternally. The replication of the maternal and paternal PWS domains appears to therefore be under the control of distinct and independent control regions.

1.3.6 Imprinting control elements

During the course of this work, a sub-set of AS and PWS individuals who have apparently normal chromosomes of biparental origin, but aberrant DNA methylation patterns at several loci within chromosome 15q11-q13 were identified (section 1.2.6). Microdeletions were subsequently identified in AS and PWS families using probes from a 160 kb phage contig including PW71 (D15S63) and SNRPN (Buiting et al., 1995; Saitoh et al., 1996). These microdeletions impair the imprinting process on chromosome 15q11-q13 and cause either AS or PWS. It has been proposed that the deletions affect a single genetic element that the authors termed the 15q11-q13 imprinting centre (IC). Mutations of the IC can be transmitted silently through the germline of one sex, but appear to block the resetting of the imprint in the germline of the opposite sex. The families discussed in this thesis were included in the work by Saitoh et al., (1996).

1.4 Evidence for genomic imprinting

The phenomenon of imprinting was first described in the homopteran scale insect, Sciara (Crouse, 1960). Embryos triploid for the X chromosome were described, having two copies of the paternally derived X and a single copy of the maternally derived X. At the eight-cell stage they inactivate one or both paternal copies, whereas the maternal X chromosome is always retained in the active form. Crouse proposed that the chromosome which passes through the male germ line acquires an imprint that results in behaviour exactly opposite to the imprint conferred on the same chromosome in the female germ line.

Genomic imprinting is defined as the differential modification of the maternal and paternal contributions to the zygote, resulting in the differential expression of parental alleles. It describes those parental dependent traits in which both the male and female alleles are present but function unequally in the embryo (Barlow, 1995). It has been suggested that the maternal and
paternal genomes have different epigenetic information imprinted on their chromosomes and this results in different roles for the maternal and paternal genomes in development (Reik, 1989). Evidence for the existence of genomic imprinting in mammals has been provided by experimental observations in mice and observations of human genetic disorders (Hall, 1990).

1.4.1 Pronuclear transplantation

Mouse embryos have been created by exchanging pronuclei between eggs such that the embryo contained either two maternally derived pronuclei or two paternally derived pronuclei (Barton et al., 1984; Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Surani et al., 1984). Neither the androgenetic (diploid paternal) or the gynogenetic (diploid maternal) embryos develop to term. The androgenetic embryos showed relatively normal development of the extra-embryonic placental tissues, whereas embryonic development was poor. Conversely, the gynogenetic embryos showed good embryonic development to the early somite stages, but development of the extra-embryonic membranes and placenta was poor. It can be concluded from this that both the maternal and paternal chromosomes are necessary for normal development (Solter, 1988).

1.4.2 Complete hydatidiform moles and ovarian teratomas

Complete hydatidiform mole is the product of an abnormal pregnancy with grossly swollen chorionic villi, but without an embryo, cord or amniotic membrane. Histologically, the villi are characterised by advanced hyperplasia and anaplasia of the trophoblast, oedema of the stroma and the absence of fetal capillaries (Vassilakos et al., 1977). The majority of complete moles have a 46, XX chromosome complement and are mostly composed of trophoblastic tissue. The study of chromosomal polymorphisms, has shown that complete moles are homozygous and androgenetic in origin (Kajii and Ohama, 1977; Lawler et al., 1979; Jacobs et al., 1980; Lawler et al., 1982a; Jacobs et al., 1982a). Occasionally some complete moles have a 46, XY chromosome complement which has been shown to be the consequence of fertilisation of an ‘anucleate’ egg by two spermatozoa (Ohama et al., 1981). In contrast, ovarian teratomas are embryonic tumours with tissues derived from the three embryonic germ layers and these differentiate into a broad
spectrum of somatic tissues but they do not contain placental tissue. Ovarian teratomas have a normal 46, XX karyotype (Galton and Benirschke, 1959; Corfman and Richart, 1964) and have been shown to be gynogenetic in origin (Linder et al., 1975).

1.4.3 Human triploidy

The differential effect of the paternal versus the maternal genetic contribution on development has also been observed in studies of human triploidy. Triploidy is associated with two distinct placental and fetal phenotypes, partial mole (type I) and non molar morphology (type II) (Szulman et al., 1981; McFadden and Kalousek, 1991). Type I is associated with a large characteristic cystic placenta with partial molar changes and normal fetal growth. The development of a partial hydatidiform mole correlates with the extra chromosome set being paternal in origin (Jacobs et al., 1982b; Lawler et al., 1982b; McFadden and Kalousek, 1991). Type II is associated with a small under developed placenta without cystic changes (nonmolar). The fetuses are markedly growth retarded with pronounced wasting of the body. Nonmolar morphology correlates with the extra chromosome set being maternal in origin (Jacobs et al., 1982b; McFadden et al., 1993). These observations suggest that a maternal contribution may be required for early embryonic development whereas a paternal genetic contribution may be required for the development of the membranes and placenta.

1.4.4 Analysis of transgene expression

Genes can be inserted into the mouse genome and the expression of the inserted gene observed in different tissues and generations (Reik et al., 1987; Surani et al., 1988). Studies of transgene expression indicated that the expression of about one quarter of these transgenes was dependent on the parental origin of the gene and depending on whether the gene was transmitted maternally or paternally, different phenotypic effects were observed (Hadchouel et al., 1987; Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987; Sasaki et al., 1991). Expression of the transgene was also shown to be tightly correlated with allele specific DNA methylation. Low levels of expression were associated with high levels of methylation and this was the first observation that methylation may be involved in the imprinting process. The observation of imprinting at transgenic loci may not always be a
reflection of the true situation, due to the potential for unpredictable effects at the chromosomal site of insertion on subsequent gene expression. The transgene DNA may also be modified by the zygotic DNA methyltransferase before or after its insertion into the chromosome.

1.4.5 Uniparental disomy

UPD occurs in a diploid organism when both homologues of a chromosome pair originate from the same parent (Engel, 1980). In imprinted regions of the genome, uniparental disomy results in the observation of different phenotypic effects depending on whether both sets or a part of each chromosome, are inherited maternally or paternally. Two forms of UPD are observed, isodisomy, where two identical copies of a gene or chromosome are inherited from one parent and heterodisomy, where two different copies of a gene or chromosome are inherited from one parent. Heterodisomy and isodisomy are not mutually exclusive phenomena and they will occur for different parts of the same chromosome pair whenever normal meiotic I recombination occurs prior to the nondisjunction event. Heterodisomy in the centromeric region indicates the occurrence of a nondisjunction event at meiosis I, whereas isodisomy in the centromeric region indicates either a nondisjunction error at meiosis II or a postzygotic event (Spence et al., 1988).

1.4.5.1 UPD in the mouse

Evidence of genomic imprinting came from UPD studies in mice (Cattanach and Kirk, 1985; Searle and Beechey, 1985; Beechey and Searle, 1987). Mice carrying Robertsonian or reciprocal translocations that exhibit high rates of non-disjunction, were used to produce offspring that had received both copies of a particular chromosomal region from only one parent. A recent review of these studies (Cattanach et al., 1995), describes ten chromosomal regions of six murine autosomes that are subject to parental imprints. The murine autosomes which are subject to these parental imprints are chromosomes 2, 6, 7, 11, 12 and 17. Four of these ten regions harbour known genes that are parentally imprinted. These regions show homology to regions on human chromosomes (table 3).

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Table 3. Murine autosomes with parental imprints and the homologous human chromosome.

1.4.5.2 UPD as a mechanism for human genetic disorders

UPD has been identified for 15 autosomes and the X chromosome (Ledbetter and Engel., 1995). Maternal UPD was diagnosed in the majority of UPD cases while the remainder were diagnosed as paternal UPD (Kalousek and Barrett, 1994). UPD has been recognised as a cause of human genetic disease as a result of (a) parental imprinting of genes on the chromosome of uniparental origin, (b) homozygosity for recessive gene alleles that map to the chromosome of uniparental origin and (c) a reflection of placental trisomy. UPD is evaluated by using polymorphic DNA markers and chromosome specific alpha satellite probes. Investigation of UPD should be considered in cases of an apparent new mutation leading to a recessive disorder and in cases of females affected with X-linked recessive disorders. Imprinting effects have been established with certainty for 4 human chromosomes that have homology to murine chromosomes (which have been shown to have significant phenotypic effects in mice with UPD for these chromosomes).

UPD and chromosome 7

The first reported case of UPD in a human individual was a female with short stature and cystic fibrosis (CF) (Spence et al., 1988). The CF phenotype was due to the inheritance of both copies of the mutant CF allele from her mother. Subsequently, two further cases confirmed the association of UPD 7 mat with short stature (Voss et al., 1989; Spotila et al., 1992). A fourth case of UPD 7 mat was reported in a child with short stature who on prenatal diagnosis was shown to have an isochromosome 7p and an isochromosome 7q (Eggerding
et al., 1994). Molecular analysis demonstrated that both chromosome 7p's were paternal in origin and both 7q's were maternal in origin. Isodisomy for chromosome 7q was demonstrated in all of these cases. Therefore, the observation of short stature could either be due to the unmasking of an autosomal recessive mutation due to homozygosity or the presence of an imprinted gene. The presence of one or more imprinted growth related genes on chromosome 7 was substantiated by the report of an individual with prenatal and postnatal growth retardation who showed maternal heterodisomy for chromosome 7 markers (Langlois et al., 1995). This finding rules out the unmasking of an autosomal recessive mutation and lends strong support to the hypothesis of imprinted gene(s) on chromosome 7. Further evidence for an imprinted gene regulating growth on chromosome 7 has come from the finding of UPD 7 mat in Silver-Russell syndrome (SRS) (Kotzot et al., 1995). Paternal isodisomy for chromosome 7 was reported in an individual with normal growth. No intrauterine growth retardation was reported (Högland et al., 1994), implying that paternal isodisomy for human chromosome 7 may have no phenotypic effect on growth.

**UPD and chromosome 11**

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth syndrome (BWS) and is characterised by exomphalos, macroglossia, visceromegaly, gigantism and a pre-disposition to embryonal tumours such as Wilms' tumour (WT) of the kidney, adrenocortical carcinoma, hepatoblastoma, rhabdomyosarcoma and occasionally pancreatic tumour and neuroblastoma (Wiedemann, 1964; Beckwith, 1969; Wiedemann, 1983). The BWS gene has been localised to 11p15.5 through linkage studies in families with the disease (Koufos et al., 1989; Ping et al., 1989). Familial BWS occurs in approximately 15% of patients and the phenotype is only observed after passage of the disease gene through the maternal germline (Lubinsky et al., 1974; Koufos et al., 1989; Ping et al., 1989; Brown et al., 1990). Most frequently BWS occurs sporadically and chromosomal abnormalities have been observed in some of these cases. These include partial trisomies, where the duplicated segment is always paternal in origin (Brown et al., 1992) and maternally inherited balanced translocations and inversions (Weksberg et al., 1993). In approximately 20-25% of cases, paternal disomy for 11p has been demonstrated (Henry et al., 1991; Grundy et al., 1991; Nystrom et al., 1992; Slatter et al., 1994). In all but one case (Grundy et al., 1991), UPD 11 pat does not involve the whole chromosome but is confined to distal 11p.
Somatic mosaicism and isodisomy was demonstrated for the 11p paternal alleles. This indicates that a mitotic recombination event must have occurred after fertilisation (Henry et al., 1993; Slatter et al., 1994; Bischoff et al., 1995). These observations imply that either paternally expressed genes with growth enhancing activities or maternally expressed genes with growth suppressing activities are located on 11p15.5. Three imprinted genes have been identified on 11p15.5 in humans. Insulin-like growth factor 2 (IGF2), a growth promoting gene, was found to be paternally expressed (Giannoukakis et al., 1993; Ohlsson et al., 1993). Embryonal tumours show loss of imprinting and biallelic expression of IGF2 (Ogawa et al., 1993; Rainier et al., 1993; Weksberg et al., 1993). H19 lies within 100 kb of IGF2 (Zemel et al., 1992) and was shown to be maternally expressed (Zhang et al., 1993). It has been shown that 5-10% of BWS patients have biparental chromosomal contributions and parental patterns of methylation and expression of IGF2 and H19 on the maternal chromosome implying the existence of imprintor mutations (Reik et al., 1995). p57KIP2 is a cyclin-dependent kinase inhibitor that causes G1 arrest (Matsuoka et al., 1995; Lee et al., 1995). p57KIP2 is preferentially expressed from the maternal allele and biallelic expression has been observed in some embryonal tumours (Matsuoka et al., 1996). Recently, mutations of p57KIP2 have been described in two patients with BWS and it is therefore a strong candidate for involvement in the aetiology of BWS (Hatada et al., 1996). These three genes are part of a 450-500 kb domain similar to that observed on 15q11-q13 and it is likely that other imprinted genes may lie within this domain. BWS patients with UPD can be diagnosed by analysing the allelic methylation patterns of the H19 and IGF2 genes (Reik et al., 1994).

**UPD and chromosome 14**

Phenotypic similarities have been observed in cases of UPD 14 mat, including short stature, precocious puberty, arrested hydrocephalus, small hands and feet, scoliosis, hyper-extensible joints, delayed motor and / or mental development and recurrent otitis media (Temple et al., 1991; Pentao et al., 1992; Antonarakis et al., 1993; Healey et al., 1994; Tomkins et al., 1996). These findings are suggestive of paternally expressed genes on chromosome 14. Evidence of maternally expressed genes on chromosome 14 has come from a report of UPD 14 pat in a child with severe mental retardation and a seizure disorder (Wang et al., 1991). Molecular analysis demonstrated heterodisomy for chromosome 14, implying that the clinical
phenotype may be associated with imprinting and not a reduction to homozygosity of recessive alleles.

**UPD and other chromosomes**
Possible imprinting effects have been suggested in an additional six UPD types (2 mat, 6 pat, 16 mat, 20 pat and XX pat) (reviewed in Ledbetter and Engel, 1995), although there is insufficient data at present to determine whether imprinting effects are present and whether UPD poses a significant phenotypic risk beyond that associated with residual trisomy and autosomal recessive disease. UPD is not always associated with an abnormal phenotype. Maternal UPD for chromosome 13 (Slater et al., 1994; Stallard et al., 1995) and chromosome 22 (Schinzel et al., 1994) and paternal UPD for chromosome 21 (Blouin et al., 1993) have been reported in individuals with a normal phenotype.

**1.4.4.3 Mechanisms leading to UPD**

Four mechanisms have been proposed which can result in UPD (Spence et al., 1988; Engel and Delozier-Blanchet, 1991). Trisomic rescue and monosomic conception have been discussed previously (section 1.2.4). The other mechanisms that have been proposed to explain the occurrence of UPD include gamete complementation and somatic recombination. Gamete complementation involves the fertilisation of a nullisomic gamete with a gamete disomic for the same chromosome, resulting in a diploid individual. Gamete complementation has been shown for some but not all chromosomes in mice and is dependent on which parental gamete is nullisomic or disomic (Lyon, 1983; Searle and Beechey, 1985). UPD may also result from mitotic errors after presumed crossing-over or gene conversion in somatic cells (somatic recombination). This results in UPD for a portion of a chromosome (partial isodisomy) and has been documented in cases of UPD 11 pat. Gamete complementation and trisomic rescue predict some degree of heterozygosity along the length of the chromosome involved and are impossible to distinguish from each other unless the trisomic cell line is observed in a mosaic state. Gamete complementation can not be demonstrated as it can not be distinguished from trisomic rescue and therefore remains a hypothetical mechanism leading to UPD.
1.4.6 Other human genetic disorders involving imprinting

Some human cancers are associated with the preferential loss of a parental chromosome. These chromosomes presumably contain imprinted tumour suppressor genes and loss of the expressing copy results in loss of growth suppression and subsequently tumour development. In at least one familial tumour syndrome, the inherited paraganglioma syndrome, the phenotype is only manifested after paternal transmission of the disease gene (van der Mey et al., 1989). The disease which is characterised by bilateral carotid body tumours is transmitted in an autosomal dominant manner and it has been postulated that the gene which accounts for this syndrome on 11q23-qter, is a paternally expressed dominant oncogene (Heutink et al., 1992). Recently, fine mapping studies have localised the disease region to 11q13.1 (Mariman et al., 1995).

1.5 Characteristics of imprinted genes

17 imprinted genes have been reported to date and of these the majority are paternally expressed (table 4). Characteristics which have been noted in several systems and previously discussed include imprinted domains (sections 1.3.4), replication domains (section 1.3.5), methylation (section 1.2.6) and non-translated imprinted RNA (section 1.3.3). Other common features that have been suggested include direct repeats, intron exon structure and sex-specific recombination rates. Imprinted DNA sequences appear to contain or are closely associated with regions rich in direct repeats. The repeats range from 25-120 bp and are unique to the respective imprinted region (Leighton et al., 1995; Neumann et al., 1995). These direct repeats have been found in all imprinted genes analysed to date and are arrayed in a head to tail fashion, they are present in variable copies and are occasionally accompanied by inverted repeats. These repeats are evolutionarily conserved, suggesting that they could be important (Pfeifer and Tilghman, 1994; Neumann et al., 1995; Smrzka et al., 1995). Direct tandem repeats have also been found at the 5' end of the \textit{Xist} gene (Pfeifer and Tilghman, 1994). These direct repeats are potentially able to form secondary structures and it has been proposed that repetitive elements in genomic DNA induce gene silencing by pairing and heterochromatin formation (Dorer and Henikoff, 1994).
### Table 4. Mammalian genes that exhibit parent of origin specific expression.

<table>
<thead>
<tr>
<th>HUMAN</th>
<th>MOUSE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENE</td>
<td>LOCATION</td>
<td>EXPRESSION</td>
</tr>
<tr>
<td>WT1</td>
<td>11p</td>
<td>Maternal</td>
</tr>
<tr>
<td>INS</td>
<td>11p</td>
<td>Not Tested</td>
</tr>
<tr>
<td>IGF2</td>
<td>11p</td>
<td>Paternal</td>
</tr>
<tr>
<td>H19</td>
<td>11p</td>
<td>Maternal</td>
</tr>
<tr>
<td>p57kip2</td>
<td>11p</td>
<td>Maternal</td>
</tr>
<tr>
<td>MASH2</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>SNRPN</td>
<td>15q</td>
<td>Paternal</td>
</tr>
<tr>
<td>ZNF127</td>
<td>15q</td>
<td>Not Tested</td>
</tr>
<tr>
<td>PAR-1</td>
<td>15q</td>
<td>Paternal</td>
</tr>
<tr>
<td>PAR-5</td>
<td>15q</td>
<td>Paternal</td>
</tr>
<tr>
<td>IPW</td>
<td>15q</td>
<td>Paternal</td>
</tr>
<tr>
<td>Igf2r/M6P</td>
<td>6q25-q27</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Mas</td>
<td>6q25.3-q26</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Peg1/Mest</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Peg 3</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Grf 1</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>U2afbp-rs</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
</tbody>
</table>
It has been suggested that the intron exon structure of imprinted genes was different to a control group of non imprinted genes (Hurst et al., 1996). Controlling for the total exon size, the total amount of intronic DNA in imprinted genes was one fifth that of the control group. This difference is due to two separate effects, the average intron size in the imprinted genes is significantly smaller than in the control group and the number of introns per kb of exon is significantly lower for imprinted genes than the control group. These findings correspond with the results of a previous study which showed that on average, genes in GC-rich chromosomal regions have a low ratio of intronic DNA to coding DNA (Duret et al., 1995). Imprinted genes tend to be localised to these GC-rich regions (Ellsworth et al., 1994).

In both the 15q11-q13 region and the IGF2-H19 region on chromosome 11, higher rates of male recombination to female recombination have been observed (Paldi et al., 1995; Robinson and Lalande, 1995). It has been hypothesised that differences in male / female recombination rates may be associated with differences in gene activity of the two sexes during meiosis and specifically, regions activated during the imprinting process are potential sites for recombination (Thomas and Rothstein, 1991). A link between chromatin confirmation and meiotic recombination in humans has also been suggested (Handel and Hunt, 1992) and it is possible that differences in chromatin structure established during meiosis could be involved in the marking of a region as maternal or paternal prior to fertilisation. Therefore, the meiotic recombination associated with imprinted regions could reflect an initial sex-specific imprinted state. The reasons for the associations between imprinted genes, high GC content, small intron size and sex-specific recombination are presently unclear.

1.6 Mechanisms of genomic imprinting

Imprinted genes must be recognised in the parental gametes and marked in an inheritable manner to allow the cells of the offspring to identify and independently regulate each parental allele (Forejt and Gregorova, 1992; Allen and Reik, 1992). Two models have been proposed to explain the epigenetic modifications which bring about monoallelic expression (Efstratiadis, 1994).
1.6.1 DNA methylation

An attractive model which is relatively simple is that an enzyme introduces a
covalent DNA modification (imprint), to the DNA sequence (imprinting box)
that is modified by the imprint. Allele specific DNA methylation represents a
good candidate for the primary mark to distinguish the individual parental
alleles at the molecular level and appears to be a characteristic of imprinted
gene domains, represents a mechanism for the regulation of gene
expression, is a heritable and reversible modification process and can be
reset in the germ line (Brandeis et al., 1993).

Evidence that DNA methylation may be the primary gametic imprint is
supported by the observation that CpG residues located in an intronic region
(region 2) of the maternally expressed Igf2r gene (Stöger et al., 1993) and
sequences within the imprinted RSVIgmyc transgene (Chaillet et al., 1991)
are initially unmethylated in sperm, yet modified in the mature oocyte. The
maternal allele then retains these methyl moieties throughout pre-implantation
development, suggesting that DNA modification can originate in the gametes.
As these methylation patterns survive the massive genome wide
demethylation that occurs in the morula, this implies that they are specifically
recognised at this stage. This is further supported by recent work on the
upstream region of H19 (Tremblay et al., 1995) and the expression of Xist in
mouse pre-implantation embryos (Norris et al., 1994; Ariel et al., 1995;
Zucotti and Monk, 1995). The pattern of differential methylation in the 5'
portion of H19 has been shown to be established in the gametes and a subset
is maintained in the pre-implantation embryo. This is sufficient to confer
monoallelic expression to the gene in blastocysts, implying that the paternal
methylation of the far 5' region is the mark that distinguishes the two parental
alleles (Tremblay, 1995). CpG islands at the 5' end of the Xist gene become
demethylated in the perinatal prospermatogonia and remain demethylated
throughout pre-implantation development (Ariel et al., 1995; Zucotti and
Monk, 1995). This is consistent with the hypothesis that male gamete specific
hypomethylation is important for the imprinting of the paternal X chromosome
in the extraembryonic ectoderm and trophectoderm, which only express the
paternal Xist allele, whereas the maternal allele is methylated. Later in
development, when the imprint is erased and X inactivation becomes random,
the repressed Xist allele on the active X chromosome is fully methylated,
whereas the expressed gene on the inactive X chromosome is hypomethylated (Norris et al., 1994).
DNA methylation could also be a response to a primary mark, functioning to maintain differential gene expression (Pfeifer and Tilghman, 1994) although in the case of the imprinted \textit{U2abp-rs} gene, imprinted expression precedes methylation differences (Hatada et al., 1995). In this case, methylation appears to be a secondary consequence of imprinting. The gamete derived signal could be DNA methylation, but it is likely to be complex and modifier genes of DNA methylation or chromatin may also be involved in the imprinting process. The products of these genes may protect the imprint from erasure by a demethylase enzyme or they may act as activators and repressors such that the chromatin configuration and condensation is altered at imprinted alleles (Izumikawa et al., 1991; Barlow, 1994). If methyl moities are to serve as the gamete derived signal of allelic identity, rather than just maintaining methylation, then imprinted genes must be protected from the wave of demethylation that occurs in the morula, possibly at certain demethylation resistant sites in the genome. It has been shown that Sp1 elements protect CpG islands from de novo methylation in the adenine phosphoribosyltransferase (APRT) gene (Brandeis et al., 1994), although there is no evidence of Sp1 elements acting on imprinted genes.

1.6.2 DNA binding proteins

Other models for the gamete derived signal conferring allelic identity include DNA binding proteins such as the Drosophila Polycomb proteins (Pirrotta, 1995). A DNA binding protein could bind to the imprinting box and this would protect the imprinting box of one allele from methylation following fertilisation, while allowing methylation of the other allele. Developmental changes occurring in methylation during early embryogenesis can also be accommodated by this model (Kafri et al., 1992; Razin and Cedar, 1993) and alleviates the need to postulate that the differential allelic methylation patterns of imprinted genes should be detectable from the germline onwards (Brandeis et al., 1993). Whatever determines the imprint, the imprinting process is predicted to have an epigenetic component, the 'imprint' which marks one parental chromosome and a genetic component, the 'imprinting box' which is modified by the imprint.
1.7 Positional cloning

The technique of positional cloning locates the gene responsible for a genetic disorder solely on the basis of its map position. The identification of patients with visible cytogenetic rearrangements can greatly assist with assigning a disease gene locus. The locus for Duchenne Muscular Dystrophy (DMD) was assigned to Xp21 through the observation of affected females with X; autosome translocations (reviewed in Boyd et al., 1986). If there is no obvious cytogenetic rearrangement, then the disease gene must be localised solely by genetic linkage analysis in families segregating for the disease phenotype.

Once a disease locus has been assigned, the identification of patients with atypical deletions, translocations or the observation of recombination events in families segregating for the disease phenotype can greatly facilitate the search for a causative gene. The cloning of translocation breakpoints led to the isolation of the gene for Menkes disease (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993) and the identification of microdeletions in patients with Retinitis Pigmentosa defined a small candidate region from which the RGPR gene was isolated (Meindl et al., 1996). Genes that have been identified for disorders in which chromosomal rearrangements have not been observed, include the gene for Cystic Fibrosis (Riordan et al., 1989) and more recently, the gene causing familial breast ovarian cancer, BRCA-1 (Miki et al., 1994).

In order to search a particular chromosomal region for potential coding sequences, it is necessary to clone the region in question into an appropriate vector. The ability to clone large fragments of DNA in YACs (Burke et al., 1987), has greatly advanced the physical mapping of the region that surrounds a disease locus. This region is known as the critical disease region and a series of overlapping YAC clones, a contig, can be constructed over the entire region of interest. A long range physical map can be developed and physical distances along a chromosome can be measured by restriction mapping of cloned YAC DNA or by analysing YACs hybridised to stretched DNA fibres (Section 1.8.2.4). Recognition sites for rare cutting enzymes which cut at unmethylated CpG nucleotides (HTF islands) (Bird et al., 1986) can be marked on the map and delineation of genomic rearrangements in patients can either be determined using the YACs for FISH analysis (Heitz et al., 1991; Tümer et al., 1992) (Section 1.8.2) or by using restriction fragments from the YAC as probes. For more detailed mapping, YAC clones are often
represented by contigs of overlapping bacteriophage and cosmid clones. This is accomplished by partial restriction enzyme digest of the YAC, subcloning into bacteriophage and cosmid vectors and identifying human positive clones (Monaco et al., 1994) or by directly hybridising YAC inserts purified from the yeast host chromosomes to gridded chromosome specific libraries (Baxendale et al., 1991).

1.7.1 Approaches to cloning genes

The majority of eukaryotic genes consist of short coding sequences, the exons, which are separated by intervening sequences, the introns (reviewed by Breathnach and Chambon, 1981; Brennan and Hochgeschwender, 1995). Coding regions are generally conserved single copy sequences that are preceded by CpG islands. The identification of expressed sequences can be achieved by a number of different techniques. The more traditional techniques involve screening expressed sequence libraries with evolutionary conserved sequences or DNA fragments containing CpG islands. The identification of conserved DNA fragments in cosmids led to the isolation of the gene for DMD (Monaco et al., 1986).

A more recent technique for obtaining DNA fragments that contain CpG islands is HTF island rescue. This technique is based on the observation that up to 60% of promoters, especially those of housekeeping genes are associated with unmethylated CpG dinucleotides (HTF islands) (Bird, 1986). The DNA surrounding HTF islands is therefore enriched for the first exons of genes. HTF island rescue is achieved by cutting YACs or cosmids from a critical region with an enzyme that recognises CpG and the products are then used to screen libraries of expressed sequences (Valdes et al., 1994; John et al., 1994).

Methods which depend on screening libraries with genomic fragments such as cDNA selection (Lovett et al., 1991; Parimoo et al., 1991), depend on using a library from a tissue at a stage in which the gene expressed. Exon amplification (Buckler et al., 1991) enables large genomic regions to be screened for coding sequences independent of the time or tissue of expression. The technique of exon amplification was instrumental in identifying the glycerol kinase and Huntington’s disease genes (Walker et al., 1993; The Huntington’s Disease Collaborative Research Group, 1993) and the genes for X-linked agammaglobulinaemia and Wiskott Aldrich Syndrome (Vetrie et al., 1993; Derry et al., 1994) were isolated using cDNA selection.
Expressed sequences can also be identified in cloned genomic DNA after direct nucleotide sequencing, to find potential coding sequences by computer analysis. These have proved useful in identifying the genes for adrenoleukodystrophy and diastrophic dysplasia (Mosser et al., 1993; Hästbacka et al., 1994). Two frequently used computer programmes are BLASTX (Gish and States 1993), which translates nucleotide sequences and searches a protein database for regions of homology and GRAIL (Uberbacher and Mural 1991), which uses a multi-sensor / neural network approach to find genes in DNA sequences.

Large scale efforts to sequence ESTs (Adams et al., 1991, 1992) have been encouraged by the human genome project. These are entered on to a database dbEST (Khan et al., 1992). Matching a sequence from a genomic region with an expressed sequence confirms the presence of a transcribed sequence. Once all these ESTs have been mapped to YACs, this technique should prove to be even more powerful (Berry et al., 1995).

1.8 Techniques

1.8.1 Microsatellite analysis

Polymorphisms in DNA structure provide the basis of genetic analysis. Microsatellites can be defined as relatively short (< 100 bp) runs of tandemly repeated DNA with repeat lengths of 6 bp or less. The number of repeats within a specific block of repeats is often highly variable (Weber, 1990). Microsatellites occur frequently and randomly in all eukaryotic DNAs examined, except yeast (Hamada et al., 1982; Litt and Luty, 1989; Stallings et al., 1991). It is almost always possible to find microsatellite markers in a 50-100 kb DNA sequence (Beckmann and Weber, 1992), although the distribution of these markers is not always even.

Most microsatellite polymorphisms are based upon dinucleotide repeats, particularly (CA)n . (GT)n repeats. Other microsatellites such as (A)n . (T)n and (AAAT)n . (TTTA)n have also been found to exhibit length polymorphisms (Tautz, 1989; Economou et al., 1990; Zuliani and Hobbs, 1990). Microsatellites with ≥ 3 bp may become more important as PCR artefacts are reduced compared to those with dinucleotides.

The power of microsatellites lies in their informativeness and the fact that they can be efficiently analysed by PCR rather than Southern based analysis. Primers are designed to anneal to single copy DNA flanking the repeats and
the amplification products which vary in length between individuals are resolved on polyacrylamide gels.

1.8.2 Fluorescence *in situ* hybridisation

Gall and Pardue (1969) and John et al., (1969) pioneered the concept of applying molecular hybridisation directly to cytological material. Over the following decade, applications of the technique tended to be restricted to sequences that were highly represented. In 1981, three groups demonstrated the possibility of localising single copy sequences cloned from individual genes by isotopic *in situ* hybridisation to metaphase chromosomes using $^{125}$I- or $^3$H-labelled probes (Gerhard et al., 1981; Harper et al., 1981; Malcolm et al., 1981). There are however, a number of significant limitations with autoradiography. It is insensitive, lacks the high topological resolution required for detailed regional mapping and is time consuming. To overcome the limitations of autoradiography, non-isotopic detection techniques were developed.

FISH utilises the same principles as radioactive *in situ* hybridisation. Target sequences in biological specimens are detected by nucleic acid probes labelled with a fluorochrome rather than with radioactivity. Fluorochromes are molecules that are excited by light of a particular wavelength and then re-emit the light at a longer wavelength. This is termed fluorescence. Stokes law governs the relationship between the wavelength of the exciting light and that of the emitted light and states that the light re-emitted by a substance is always of a longer wavelength and lower intensity than the exciting light. A fluorescence emission spectrum is established by plotting fluorescence intensity versus the wavelength (in nanometers) or wave number (in cm$^{-1}$). Different fluorochromes are distinguished by their absorption and emission spectra and table 5 shows the excitation and emission wavelengths for fluorochromes that are commonly used as conjugates to nucleotides or antibodies and as DNA stains for chromosomes (Lichter and Cremer, 1992; Schwarzacher and Heslop-Harrison, 1994).

1.8.2.1 Fluorescence microscopy

There are two types of microscope available for fluorescence microscopy, depending on the positioning of the light source. The transmitted light fluorescence microscope uses a condenser to focus the exciting light on to
the specimen and the emitted fluorescence is collected by an objective lens. Almost all of the exciting light enters the objective and high quality barrier filters are required to remove this light to prevent the emitted fluorescence from being masked. The reflected light microscope (epifluorescence) relies on the use of a chromatic beam splitter or dichroic mirror. These mirrors have an interference coating which reflects light shorter than a certain wavelength and transmits light of a longer wavelength. These mirrors act as both excitation and barrier filters, but another barrier filter is usually required to remove residual exciting light (Monk, 1992).

<table>
<thead>
<tr>
<th>FLUOROCHROME</th>
<th>EXCITATION (nm)</th>
<th>EMISSION (nm)</th>
<th>FLUORESCENCE COLOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin (AMCA)^a</td>
<td>350</td>
<td>450</td>
<td>Blue</td>
</tr>
<tr>
<td>Fluorescein (FITC)^b</td>
<td>495</td>
<td>515</td>
<td>Green</td>
</tr>
<tr>
<td>Rhodamine 600 (TRITC)^c</td>
<td>575</td>
<td>600</td>
<td>Red</td>
</tr>
<tr>
<td>DAPI^d</td>
<td>355</td>
<td>450</td>
<td>Blue</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>340, 530</td>
<td>615</td>
<td>Red</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>455</td>
<td>495</td>
<td>Yellow-Green</td>
</tr>
<tr>
<td>Chromomycin A3</td>
<td>430</td>
<td>570</td>
<td>Yellow</td>
</tr>
<tr>
<td>Hoescht 33258</td>
<td>356</td>
<td>465</td>
<td>Blue</td>
</tr>
</tbody>
</table>

Table 5. Fluorochrome absorption and emission spectra, (a) 7-amino-4-methyl-coumarin-3-acetic acid, (b) Fluorescein isothiocyanate, (c) Tetramethyl rhodamine isothiocyanate and (d) 4'-6-diamidino-2-phenylindole.

The most important aspect of fluorescence microscopy is the use of optical filters. The excitation filters select the optimum wavelength of light for excitation of the fluorochrome and the barrier filters suppress the excess exciting light and select out the emission wavelengths of the fluorochrome. Dual or triple band pass filters allow the simultaneous detection of two or more fluorochromes (Nederlof et al., 1989, 1990). However, the changing of filter sets generally results in image shift when the signals are recorded and this becomes important when the geometric relationship of two differentially stained sites has to be recorded such as in two colour mapping experiments. These filters also reduce the amount of light reaching the camera and some low intensity signals are not detectable.
Fine localisation of signals, visualisation of weak signals and multiple colours and ratio imaging are facilitated by digital imaging microscopy (Ried et al., 1992a; Baldini and Lindsay, 1994; Coppey-Moisan et al., 1994). Digitised images can be generated by using specialised camera systems, the most sensitive of which is the cooled charged coupled device (CCD). In this system, the filters are distinct for each fluorochrome. The filters are moved by a computer controlled filter wheel located near the arc lamp. There is no movement of the filters between the sample and the camera and the images can be collected in sequence. The CCD camera collects the colour specific images, one image for DAPI, one image for FITC and one image for rhodamine. These images are then pseudocoloured and merged to produce a 24 bit colour image.

### 1.8.2.2 Hapten labelling of nucleic acids.

Two types of non isotopic labelling procedures have been developed, the direct method and the indirect method.

In the direct method, the reporter molecule (hapten) is bound directly to the nucleic acid probe so that formed hybrids can be visualised immediately after their hybridisation to the nucleic acid. The first reported use of the direct method was in the terminal fluorochrome labelling of RNA probes (Baumann et al., 1980). More recently, fluorescein, rhodamine and coumarin derivatives of d(UTP) have been synthesised using allylamine-dUTP as the key intermediate compound (Dirks et al., 1991; Wiegant et al., 1991; Ried et al., 1992a; Wiegant et al., 1993). This method is less sensitive than the indirect method and is therefore suitable for the detection of relatively large targets such as the alpha satellite DNAs, although cosmid sized targets can be visualised directly using epifluorescence microscopy.

Indirect procedures require the chemical or enzymatic introduction of a reporter molecule (hapten) into the probe. The hapten should be accessible to antibodies for the detection of the probe. Due to their high sensitivity, biotin and digoxigenin are the most frequently used reporter molecules. Biotin (vitamin H of B<sub>2</sub> complex) binds to streptavidin which is a secreted protein of Streptomyces avidinii with high affinity. Digoxigenin is a steroid isolated from digitalis plants (Digitalis purpurea and Digitalis lanata). The leaves and blossom of these plants are the only natural source of digoxigenin and binding of the anti-DIG antibody in other biological material does not occur. Non-isotopic labelling is carried out by the use of a biotinylated or digoxigenin
modified nucleotide containing a carbon spacer arm which increases the availability of the hapten (Brigati et al., 1983).

1.8.2.3 Principles of FISH

There are several different factors which can influence the efficiency of a FISH protocol. These include sample preparation, pre-treatments, probe preparation, denaturation of probe and target DNA, hybridisation, post-hybridisation washes and immunocytochemistry and fluorescence detection. FISH is performed on metaphase chromosomes, interphase nuclei and DNA fibres. To preserve morphology, the sample must be fixed and slides are prepared using conventional methanol / acetic acid fixed samples. It is possible to use the slides without pre-treatment although an RNase treatment serves to remove endogenous RNA and may improve the signal to noise ratio in hybridisations to DNA targets.

Probe preparation

One of the oldest and most popular methods for the labelling of DNA is nick translation (Rigby et al., 1977). Subsequently, the incorporation of biotin and digoxigenin into DNA by nick translation was reported (Langer et al., 1981; Kessler et al., 1990). Nick translation involves the simultaneous action of pancreatic deoxyribonuclease (DNase I) and E. coli DNA polymerase I holoenzyme (Pol I) on double stranded DNA. DNase I is a double strand specific endonuclease hydrolysing phosphodiester bonds in DNA chains, resulting in short oligonucleotides characterised by 5' phosphate groups. The generated free phosphorylated 5' ends within the nicks trigger the action of Pol I. The 5'-3' exonuclease activity of Pol I progressively removes nucleotides from the phosphorylated 5' end, while the 5'-3' polymerase activity of Pol I synchronously adds new nucleotides to the opposite free 3'-hydroxyl ends of the nick, incorporating the hapten modified nucleotide. The ratio of DNase I to DNA polymerase I is important in order to achieve an efficient labelling and to get a suitable probe size distribution, ideally 200-400 base pairs.

cDNAs consist of single copy sequences, whereas cloned genomic DNA fragments contain interspersed repetitive sequences such as SINES (small interspersed repetitive elements such as Alu elements) and LINES (large interspersed repetitive elements such as L1 elements). When these repetitive elements are present in the probe, application of a FISH protocol results in
hybridisation signals distributed over the whole chromosome complement due to the presence of these elements throughout the genome. These repetitive elements can be eliminated from participating in the *in situ* hybridisation reaction by adding sufficient unlabelled competitor DNA (either total genomic DNA or its Cot-1 fraction) to the probe. This approach was first described for Southern filter hybridisation and then implemented for *in situ* hybridisation (Landegent et al., 1987). This approach proved particularly useful for *in situ* hybridisation with DNA isolated from chromosome specific libraries (CIS hybridisation) (Pinkel et al., 1988; Cremer et al., 1988; Lichter et al., 1988a) and for the rapid mapping of cosmid and YAC DNAs (Kievets et al., 1990; Lichter et al., 1990; Wada et al., 1990).

**Hybridisation**

Hybridisation depends on the ability of denatured DNA to reanneal with complementary strands in an environment just below their melting point (Tm). The Tm is the temperature at which half the DNA is present in a single stranded denatured form. Tm and renaturation (hybridisation) of DNA are primarily influenced by temperature, pH, the concentration of monovalent ions and the presence of organic solvents. The maximum rate of renaturation of DNA is at a temperature of 25°C. The stability of the DNA is directly dependent on the GC content and the higher the molar ratio of GC pairs in the DNA, the higher the melting point Tm. The rate of hybridisation is relatively independent of pH in the range 5 to 9. Higher pH can be utilised to increase the stringency of the hybridisation. Monovalent cations such as sodium ions interact electrostatically with nucleic acids particularly with the phosphate groups so that the electrostatic repulsion between the two strands of the duplex decreases with increasing salt concentration and the stability of the hybrid is increased. Low sodium concentrations effect the Tm as well as the rate of hybridisation.

Organic solvents reduce the thermal stability of double stranded poly nucleotides, so that hybridisations can be performed at lower temperatures. DNA denatures in 0.1-0.2M sodium ions at 90-100°C implying that for *in situ* hybridisation to microscopic preparations, probes must be hybridised at 65-75°C for prolonged periods. However, the melting temperature of DNA:DNA hybrids can be reduced in a linear fashion by using formamide. Hybridisations can then be performed at 30-45°C with 50% formamide present in the hybridisation mixture. Additional hybridisation variables include
probe concentration, probe length, the inclusion of dextran sulphate and the extent of the mismatch between the probe and its target.

Post-hybridisation washes
Labelled probe can hybridise non-specifically to sequences which bear homology but are not entirely homologous to the probe sequence. These hybrids are less stable than perfectly matched hybrids. They can be dissociated by performing washes of various stringencies. The stringency of the washes can be manipulated by varying the formamide concentration, the salt concentration and the temperature.

Immunocytochemistry and fluorescence detection
Immunocytochemical procedures generally involve the detection of nucleic acid probes labelled with biotin or digoxigenin. A blocking step is used prior to the immunological procedure to remove background. Biotin detection is achieved through the use of avidin conjugated to a fluorochrome, usually FITC (green) but avidin-AMCA (blue) and avidin-rhodamine (red) can also be used. Similarly, digoxigenin is detected by anti-digoxigenin antibody conjugated to a fluorochrome such as FITC or rhodamine.

Signal amplification is often required to detect fluorescence signals and this technique is based on the binding of antibodies to the previously used detection reagent (avidin or antibody) (Pinkel et al., 1988). The procedure can either be direct, using one layer of fluorescently labelled antibodies or indirect, using one layer of antibodies conjugated with a reporter group and a second layer of fluorescent reagents directed against the reporter groups such as in the biotin-avidin-FITC system. A schematic illustration of the signal amplification procedure is shown in figure 9.

A detection principle for indirect FISH methods using only one or two antibody layers, which permits ultra-sensitive FISH has recently been developed (Raap et al., 1995). Signal amplification is increased 1000 fold over standard FISH techniques, enabling the detection of short unique sequences in situ. The method is based on the use of fluorochrome or biotin labelled tyramides as peroxidase substrates to generate and deposit many fluorochrome or biotin molecules close to the in situ bound antibody-peroxidase conjugate(s). These may be directly evaluated under the fluorescence microscope or after incubation with a streptavidin conjugate.
Figure 9. A schematic illustration of the signal amplification procedure.
1.8.2.4 Applications of FISH

Chromosomal abnormalities are a leading cause of genetic disease including congenital disorders and acquired diseases such as cancer. The introduction of banding techniques (Caspersson et al., 1968; Yunis et al., 1976) enabled each human chromosome to be individually recognised and provides the major basis upon which chromosomal abnormalities are identified. Rearrangements and small deletions causing specific syndromes were identified, including deletions involving 15q11-q13 in AS and PWS (section 1.2.1) and deletions involving 17p13.3 in Miller-Dieker syndrome (MDS) which is associated with type I lissencephaly (Dobyns et al., 1991). The deletions in these and other microdeletion syndromes are frequently at the limits of resolution for routine cytogenetic analysis and are only detected in a proportion of cases. The detection of chromosome aberrations using FISH was first described by Pinkel et al., (1986). FISH has proven to be versatile because of its direct nature and is now frequently used in the cytogenetic laboratory to improve on the analysis of metaphase chromosomes.

The development of chromosomal in situ suppression (CISS) hybridisation with flow sorted libraries (Cremer et al., 1988; Lichter et al., 1988a, 1988b; Pinkel et al., 1988) provided a powerful tool for the rapid analysis of human chromosome aberrations. Chromosome painting works efficiently for the confirmation and refinement of the diagnosis of abnormal karyotypes in cases where banding analysis can provide an indication of which chromosome libraries to use. However, it will not detect small deletions and other subtle rearrangements. The microdeletion syndromes such as MDS can be rapidly and efficiently diagnosed by using probes specific to the commonly deleted region itself. Commercially available probes (Oncor and Vysis) are available for the diagnosis of these microdeletion syndromes and these include probes for identifying the D17S379 locus in MDS, the D17S258 locus in Smith-Magenis syndrome, the D7S472 locus in Williams syndrome, the D22S75 locus in DiGeorge syndrome, the D5S23 chromosome 5p15.2 probe for detecting deletions in Cri Du Chat syndrome and the D4S96 chromosome 4p16.3 probe for detecting deletions in Wolf-Hirschhorn syndrome. These probes can also be used to identify cryptic translocations in the parents of affected children (Kuwano et al., 1991; Ledbetter, 1992), which may be the cause of the partial monosomy observed in these children.

In the case of small rearrangements or chromosomes containing additional material of unknown origin, the only approach is to try each library or region
specific probe in turn which is both time consuming and expensive. A technique which overcomes this problem is reverse chromosome painting (Carter et al., 1992; Rack et al., 1993). This technique generates the probe from the aberrant chromosome itself. The aberrant chromosomes are flow sorted from peripheral blood cultures or cell lines. The sorted chromosomes are then amplified and labelled enzymatically using a degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) protocol (Telenius et al., 1992). The probe is hybridised back to normal metaphase spreads and its origin determined.

FISH can also be used for the detection of numerical aberrations using centromere specific probes or libraries and is particularly useful for identifying the chromosome number of the major contributors to aneuploidy (13, 18, 21, X and Y) in the interphase nuclei of chorionic villi and amniotic fluid cells. This is an important contribution to the speed and analytical potential of prenatal cytogenetics (Ward et al., 1993; Philip et al., 1995). The development of a complete set of human telomeric probes (National Institutes of Health and Institute of Molecular Medicine collaboration, 1996) will also provide a 10-fold improvement in deletion detection sensitivity compared with high resolution cytogenetics (2-3 Mb resolution) and will facilitate the detection of cryptic rearrangements. Chromosomal rearrangements can also be detected in the interphase nuclei and this approach has been adopted in the identification of the duplication in Charcot-Marie-Tooth disease (CMT1a) (Lupski et al., 1991). FISH to interphase nuclei also allows probes to be ordered with approximately 50 kb resolution in positional cloning studies (Brandriff et al., 1991; van den Engh et al., 1992).

Recently, the introduction of multi-colour FISH has enabled each individual chromosome to be discriminated (Speicher et al., 1996; Schröck et al., 1996). The technique developed by Speicher et al., (1996) uses a 'combinatorial' or 'ratio-labelling' strategy (Nederlof et al., 1989, 1990; Dauwerse et al., 1992; Ried et al., 1992a) to label probes in a multiplex fashion. A pool of human chromosome painting probes was each labelled with a different fluorochrome combination and hybridised to metaphase chromosomes. Epifluorescence filter sets and computer software was developed to visualise each of the different probes. Both simple and complex chromosomal rearrangements could be detected rapidly and unequivocally. Multiplex FISH will be applicable to the identification of numerical abnormalities, as well as many structural rearrangements and could eventually be used as a screening test for these abnormalities.
The analysis of solid tumour and other cancer tissues has been greatly facilitated by the development of comparative genome hybridisation (CGH) (Kallioniemi et al., 1992). CGH can be used to detect and map deletions or amplifications (Speicher et al., 1993; du Manoir et al., 1993). This technique involves the combined hybridisation to a normal metaphase spread of equal amounts of test and reference DNA labelled with different haptens and detected with different fluorochromes such as FITC (green) or TRITC (red). Regions with equimolar DNA appear yellow and regions that are under or over represented DNA appear red or green.

FISH is also a powerful tool in research and has been applied to positional cloning strategies using a technique known as FiberFISH (Wiegant et al., 1992; Parra and Windle, 1993; Fidlerova et al., 1994, Houseal et al., 1994; Heiskanen et al., 1994). This technique uses extended single DNA fibres and contributes to the creation and refinement of physical maps of cosmid contigs because of its wide resolution range (1-400 kb), its ability to accurately size gaps and overlaps and its reproducibility. YAC overlaps, the assessment of the linear continuity of YACs in deletion prone regions, the sizing of uncloned gaps between adjacent contigs and the rapid mapping of cosmids and cDNAs along cloned YACs can be determined using this technique. Long range continuity over several hundreds of kilobases in large genes has been shown in linear bar code maps of 300-400 kb regions for the human thyroglobulin and dystrophin genes (Florijn et al., 1995) and this has enabled deletion breakpoints to be mapped in DMD.

1.9 Objectives of this project

At the start of this project, the AS critical region was defined by a Japanese family with a small inherited deletion encompassing the D15S10, D15S113 and GABRB3 loci (Saitoh et al., 1992). This deletion was approximately 1.5 Mb and defined the critical region for an AS gene. The aim of this study was to (a) identify AS patients with atypical deletions and (b) identify recombination events in families segregating for the AS disease phenotype. Identifying such patients would narrow the region within chromosome 15q11-q13 that needs to be screened for an AS gene. The technique of FISH was chosen to screen AS patients for deletions and it's suitability as a diagnostic test for detecting deletions in these patients was assessed.
2.0 Methods
2.1 Materials

2.1.1 Cell lines

Cell line (3731) was transformed as part of the service of the European collection of animal cell cultures (ECACC), Porton Down. The sample had been collected as part of the study carried out by Jill Clayton-Smith (1992).

Cell lines from AS patients (6468, A.W., B.M., M.D. and M.S.) were transformed in house by Mr. Paul Rutland.

Cell lines (B.S. and D.H.) were provided by Dr. Ron C. Michaelis.

2.1.2 Probes

Chromosome 15 cosmid probe MR-60 maps to 15q26.1 and was provided by Dr. Roger Schultz.

Probe pVAW409R3a is a 1.4 kb ECOR I/BamH I single copy fragment of plasmid probe pVAW409R3 (Raeymaekers et al., 1991) which has been assigned as D17S122.

Lambda 48.25X (exon alpha) is a 4.2kb Xba I fragment cloned into the Xba I site of bluescript. The vector for phage clones 48.8, 48.3 and 48.25 is Lambda Dash II and the bacterial host is XL1-Blue MRA (Stratagene). These were provided by Dr. Karin Buiting.

The vector for phage clones 31, 64, 150, 4 and 30 is Lambda FIX II and the bacterial host is XL1-Blue MRA (Stratagene). These were provided by Dr. Ron Trent.

Yeast artificial chromosomes, 378A12, 142A2, 23OH12, B23OE3 and 132D4 were obtained from the Human Genome Centre at Baylor College of Medicine, Houston, Texas. 378A12, 142A2, 23OH12 and 132D4 were isolated from the CEPH library and B230E3 was isolated from the St. Louis library. The vector is pYAC4 and the strain is AB1380.

2.1.3 Primers
Primers were either synthesized in house on an Applied Biosystems 381A DNA synthesizer by Mr. Paul Rutland or they were synthesized by Pharmacia. Primer sequences and loci are shown in tables 6 and 7.

2.2 Chemicals

All chemicals, with the exception of those listed below or otherwise stated were obtained from either Sigma or Gibco-BRL.

Accugel™ was supplied by National Diagnostics, UK.

Agar was supplied by Difco Laboratories and Sea Kem LE agarose was supplied by Flowgen.

Alpha $^{35}$S-dATP (1000 Ci/mm mol), $^{32}$P-dATP (3000 Ci/mm mol) and Hybond-N/N+ were supplied by Amersham International plc.

Analar grade acetic acid and methanol were supplied by Merck.

Avidin fluorescein isothiocyanate (FITC) and biotinylated anti-avidin were supplied by Vector laboratories limited.

Citifluor was obtained from Citifluor Ltd.

Hexadeoxyribonucleotides, 2' Deoxynucleoside 5' Triphosphate dATP, dGTP, dCTP, dTTP and G50 medium grade Sephadex were obtained from Pharmacia.

Klenow fragment was obtained from Promega.

Restriction enzymes and their respective buffers were supplied by Northumbria Biologicals Ltd. with the exception of Xba I and Not I which were supplied by Promega.

Sequenase kit version 2.0 was obtained from United States Biochemical.

Taq polymerase and reaction buffers were obtained from Bioline.
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Table 6. Microsatellite markers from the AS/PWS region on chromosome 15q11-q13 (Lindeman et al., 1991; Bowcock, 1992; Mutirangura et al., 1992a, 1992b 1993a; Malcolm and Donlon 1994; Trent et al., 1995; S. Christian, pers. comm.).
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**Table 7.** Microsatellite markers from chromosome 7 (Zielenski et al., 1991; Weissenbach, 1992).
2.3 Solutions

2.3.1 General solutions

TE: 10mM Tris (tris (hydroxymethyl) aminomethane)
1mM EDTA
pH adjusted to 7.6 with HCl

20XSSC: 2.9M NaCl
0.3M Sodium citrate
pH adjusted to 7.0 with NaOH

5XTBE: 0.089M Tris
0.089M boric acid
2mM EDTA (pH 8.0)

10XTAE: 40mM Tris-acetate
1mM EDTA (pH 8.0)

Loading buffer: 50% glycerol
10mM EDTA
0.05% bromophenol blue
0.05% xylene cyanol

Xylene cyanol loading buffer: 50% glycerol
10mM EDTA
0.05% xylene cyanol

2.3.2 Media solutions

LB (Luria-Bertani) Medium: 10g/l Bactotryptone
5g/l Bacto-yeast extract
10g/l NaCl

LB agar: Add 15g bacto-agar to 1 litre of LB broth
before autoclaving
YPD medium:  
10g/l yeast extract  
20g/l peptone  
After autoclaving, add 10mls 20% filter sterilised dextrose

NZY broth:  
5g/l NaCl  
2g/l MgSO₄·7H₂O  
5g/l yeast extract  
10g/l NZ amine (casein hydrolysate)

NZY bottom agar:  
1 litre of NZY broth with 15g of bacto-agar added before autoclaving.

NZY top agarose:  
As NZY broth with 0.7% Sea Kem LE agarose.

Tissue culture media  
1XRPMI 1640 media  
10% heat activated fetal calf serum  
200mM glutamine  
50mg/ml penicillin and streptomycin or gentamicin

2.3.3 DNA extraction solutions

2.3.3.1 DNA extraction solutions for blood samples and cell lines

Nuclei Lysis Buffer:  
10mM Tris (pH 8.0)  
400mM NaCl  
2mM NaCl

Proteinase K Solution:  
2mM EDTA  
1% SDS  
2mg/ml Proteinase K

2.3.3.2 DNA extraction solutions for cosmids
Solution I: 50mM Glucose
10mM EDTA (pH 8.0)
25mM Tris-HCl (pH 8.0)
100μg RNase/ml

Solution II: 0.2M NaOH
1% SDS

Solution III: 3.0M Potassium acetate pH 5.5 at 4°C

2.3.3.3 DNA extraction solutions for yeast

CiDIS:
2% Triton-X
1% SDS
100mM NaCl
10mM Tris/HCl (pH 8.0)
1mMEDTA

2.3.3.4 DNA extraction solutions for bacteriophage

SM buffer: 5.8g NaCl
2.0g MgSO₄.7H₂O
50ml 1M Tris-Cl (pH 7.5)
5ml 2% gelatin
distilled water to 1 litre

Nuclease solution: 50mg DNase I
50mg RNase A
30mM Sodium acetate (pH 6.8)
10ml 50% glycerol

2.3.4 Southern blotting solutions

Oligolabelling solution (OLB):

Solution O: 1.25mM Tris-Cl (pH 8.0)
0.125M MgCl₂
Solution A:  
1 ml Solution O  
18 μl B-Mercaptoethanol  
5 μl 0.1 M dATP for ³²P incorporation  
5 μl 0.1 M dGTP  
5 μl 0.1 M dTTP

Solution B:  
2.5 M HEPES  
pH adjusted to 6.6 with 4 M NaOH

Solution C:  
random hexanucleotides in TE (90 OD units/ml)

Solutions A:B:C mixed in the ratio 100:250:150

PEG hybridisation solution:  
5X SSC  
10% PEG  
1% SDS  
1.0 mM EDTA  
100 μg/ml denatured sonicated salmon sperm

Denaturing solution:  
1.5 M NaCl  
0.5 M NaOH

Neutralising solution:  
0.4 M NaOH

2.3.5 Solutions for microsatellite analysis

6% Polyacrylamide gel mix:  
6% polyacrylamide (19:1 acrylamide : bisacrylamide)  
7 M urea  
1XTBE

Formamide dye:  
95% deionised formamide  
20 mM EDTA  
0.05% bromophenol blue  
0.05% xylene cyanol ff

2.3.6 Chromosome preparation solutions
Fixative: 1 part glacial acetic acid
3 parts methanol

2.3.7 FISH solutions

Hybridisation buffer: 50% deionised formamide (IBI)
10% dextran sulphate (autoclaved)
2XSSC (autoclaved)
Store at -20°C

Blocking buffer: 3% BSA
4XSSC
0.1% Tween-20

FITC dilution buffer: 1% BSA
4XSSC
0.1% Tween-20

The blocking buffer and FITC dilution buffer were clarified by spinning for 5 minutes at 13,000g. Only the supernatant was used.

Anti-Digoxigenin Rhodamine: Dilute stock 1 in 200 with FITC dilution buffer.

Avidin-FITC (Vector labs): Dilute stock 1 in 400 with FITC dilution buffer.

Biotinylated anti-Avidin: Dilute stock 1 in 100 with FITC dilution buffer.

DAPI /Antifade: Dilute DAPI (0.2mg/ml) 1 in 100 with Citifluor.

2.4 Methods

2.4.1 Preparation of DNA
2.4.1.1 Extraction of human DNA from blood

Blood samples were collected in 10ml plastic tubes containing EDTA and stored at -70°C. EDTA tubes were defrosted by removing the lids and inverting inside 50ml Falcon tubes. Ice cold water was added to each sample to a final volume of 50ml. The red blood cells were mixed by inversion to lyse them and spun at 900g at 4°C for 20 minutes. The supernatant was discarded and the Falcon tube inverted to remove the lysed blood cells, leaving the white nuclear pellet. 25ml of 0.1% Nonidet P-40 was added to wash the pellet and the Falcon tubes were spun at 900g at 4°C for 20 minutes. The supernatant was discarded and the pellet resuspended in 3ml of cold nuclei lysis buffer. 200μl of 10% SDS and 600μl of fresh Proteinase K (2mg/ml) were added and the samples were incubated at 60°C for one hour or overnight at 37°C. Protein was precipitated by adding 1ml of saturated ammonium acetate solution, shaking vigorously for 15 seconds and then allowing to stand at room temperature for 15 minutes. Following centrifugation at 900g for 20 minutes at room temperature, the supernatant was poured into a clean tube. The DNA was precipitated by adding 2 volumes of absolute ethanol and spooled out on the tip of a sealed pasteur pipette. DNA was dissolved in 500μl of TE and stored at -20°C.

The concentration of the extracted DNA was determined by measuring the absorbance at 260nm on a Pharmacia GeneQuant spectrophotometer. The purity of the extracted DNA was assessed by measuring the ratio of absorbance 260nm/280nm. A high degree of purity was indicated by a ratio of between 1.8 and 2.0.

2.4.1.2 Extraction of human DNA from cell lines

10mls of an actively growing cell line were taken and spun at 900g for 20 minutes at 4°C. 600μl of nuclei lysis buffer was added to the cell pellet which was then vortexed to resuspend it completely. 40μl of 10% SDS and 120μl of fresh proteinase K solution (2mg/ml) were added to the resuspended cell pellet and the samples were incubated at 60°C for one hour or overnight at 37°C. Protein was precipitated by adding 200μl of saturated ammonium acetate solution, shaking vigorously for 15 seconds and then allowing to stand for 15 minutes at room temperature. The samples were spun at 900g for 20 minutes at room temperature and the supernatant was poured into a clean tube. 2 volumes of absolute ethanol was added to precipitate the DNA, which
was spooled out on the tip of a sealed glass pasteur pipette. The DNA was resuspended in 200µl of TE and the quality and quantity of DNA was determined spectrophotometrically (OD260/280).

2.4.1.3 Alkali lysis extraction of cosmid and plasmid DNA

For small scale preparation, 10ml of LB broth containing the appropriate antibiotic was inoculated with a single colony of bacteria and grown in a shaking incubator at 37°C overnight. The cells were spun at 20,000g for 10 minutes and resuspended in 100µl of solution 1. 200µl of fresh solution 2 was added and then placed on ice for 5 minutes after gentle inversion. 150µl of solution 3 was added, vortexed and placed on ice for 5 minutes. The protein and genomic DNA were removed by spinning at 20,000g for 10 minutes at 4°C. The supernatant was transferred to a clean tube and an equal volume of phenol/chloroform (pH 8) was added. This was mixed well and spun at 20,000g for 5 minutes. The upper aqueous phase was carefully removed to a clean tube and an equal volume of chloroform/isoamylalcohol (1:24) added. After spinning at 20,000g for 5 minutes, the upper aqueous phase was removed to a clean tube. To precipitate the DNA, 2 volumes of cold ethanol was added and placed at -70°C for 30 minutes. After spinning at 20,000g for 5 minutes, the DNA was washed with 70% ethanol and resuspended in 50µl of TE.

2.4.1.4 Extraction of total yeast DNA

5mls of YPD media was inoculated with 1 yeast colony and incubated at 30°C in a shaking incubator for 48 hours. The culture was centrifuged at 900g for 5 minutes and the pellet resuspended in 500µl of distilled water. The resuspended pellet was transferred to a clean 1.5ml tube and spun in a bench microfuge at 13,000g for 10 seconds. The supernatant was removed and 200µl of CiDIS added to the pellet. 200µl of phenol/chloroform (pH 8) and 0.35g of acid washed beads (Sigma 710 - 1180µ) were added, vortexed vigorously for 2.5 minutes and 200µl of distilled water added. This was spun at 13,000g for 5 minutes and the aqueous layer removed to a clean 1.5ml tube. 50µg of RNase A was added and incubated at 37°C for 10 minutes. 1ml of ethanol and 10µl of 7.5M ammonium acetate was added to precipitate the DNA and spun at 13,000g for 5 minutes. The pellet was resuspended in 100µl of distilled water.
2.4.1.5 Extraction of bacteriophage DNA

10mls of NZY supplemented with 0.2% maltose and 10mM MgSO₄ was inoculated with 1 colony of the appropriate host bacteria, taken from an NZY plate. The culture was shaken overnight at 37°C and grown to an OD₆₀₀ of 0.2. The cells were spun for 10 minutes at 4000g and resuspended in 10mM MgSO₄ to a final OD of 1.0. These were stored at 4°C. Tenfold serial dilutions of the bacteriophage stocks were prepared in SM and 100μl of each dilution was transferred to a sterile tube. 100μl of plating bacteria were added, vortexed and incubated at 37°C for 30 minutes. 3mls of melted (45°C) NZY top agarose containing 0.2% maltose and 10mM MgSO₄ was added, mixed gently and poured onto the centre of a plate containing hardened bottom NZY agar supplemented with 0.2% maltose and 10mM MgSO₄. The plate was swirled gently to ensure an even distribution of agarose and when the top agarose had hardened the plates were inverted and incubated at 37°C for 6-12 hours until the plaques had become confluent.

The following calculation is used to obtain the bacteriophage titre:

\[
\text{Nos. of Plaques/Plate} \times \frac{1}{\text{Plate dilution}} \times \frac{1}{\text{Volume phage/Plate}} = \text{p.f.u./ml}
\]

Isolated plaques were obtained by stabbing the chosen plaques with a pasteur pipette equipped with a rubber bulb and gently drawing up the plaque and the underlying agarose/agar. The plaques were placed in 1ml of SM containing one drop of chloroform and left at room temperature for 2 hours to allow the bacteriophage particles to diffuse out of the agarose/agar. 10μl-100μl of eluted phage was added to 500μl of host cells and adsorbed at 37°C for 30 minutes. The pre-adsorbed phage were added to 37mls of NZY media supplemented with 0.2% maltose and 10mM MgSO₄ in a 250ml culture flask and grown in a shaking incubator for 12-15 hours at 37°C or until complete lysis was obtained (usually between 12-15 hours). The cultures were transferred to 50ml Falcon tubes containing 100μl of chloroform and mixed gently. 370μl of nuclease solution was added and the Falcon tubes incubated at 37°C for 30 minutes. 2.1g NaCl was added and the Falcon tubes inverted to dissolve the NaCl. The solution was transferred to centrifuge tubes and spun at 6000g for 20 minutes at 4°C. The supernatant was transferred to clean tubes containing 3.7g of PEG (6-8000). This was
dissolved gently and left on ice for 1 hour. The tubes were spun at 6,000g for 20 minutes at 4°C and the phage pellet resuspended in 500μl of SM on a rotating wheel at 4°C for 1 hour. The solution was transferred to a 1.5ml tube and 500μl of chloroform added. This was mixed gently and spun for 5 minutes to separate the phases. The aqueous phase was transferred to a clean tube and 20μl of 0.5M EDTA, 5μl of 20% SDS and 10μl of Proteinase K (2.5mg/ml) added. This was incubated at 65°C for 30 minutes and an equal volume of phenol/chloroform (pH8.0) added. 170μl of 6M ammonium acetate was added to the final aqueous phase and the DNA precipitated with 700μl of isopropanol. The DNA was placed on ice for 15 minutes, then spun at 13,000g for 15 minutes at 4°C. The pellet was rinsed in 70% ethanol and resuspended in 500μl of TE.

2.4.2 Cell culture

2.4.2.1 Maintenance of lymphoblastoid cell lines

Cells were grown in the growth medium RPMI 1640 supplemented with L-glutamine, heat inactivated fetal calf serum, penicillin and streptomycin. The media was pre-warmed to 37°C prior to use. Cultures were grown at 37°C under 5% CO₂ in 50ml tissue culture flasks. After 3-4 days the culture was split by removing half the volume of cells and replacing it with an equal volume of growth medium. Once the cell line had become established, aliquots were removed for freezing.

2.4.2.2 Storage of cell line stocks

A 12ml sample of the growing cell medium was pipetted into a 15ml centrifuge tube and centrifuged at 900g for 5 minutes at room temperature. The supernatant was removed except for the last 0.5ml into which the pellet was resuspended. The tube was placed on ice and approximately 1ml of chilled culture was aliquoted into three 1.8ml labelled and dated Nalgene cryotubes. An equal volume of chilled 20% dimethylsulphoxide was added in droplets and the cryotubes were shaken to ensure mixing. The tubes were then returned to ice. Initially the cultures were chilled to -70°C over several days, before being transferred to liquid nitrogen for permanent storage. To grow cell lines from frozen stocks, the cells were centrifuged for 5 minutes at 900g and the pellet resuspended in 10ml of RPMI medium. 5mls of this
solution was transferred to two small tissue culture flasks, then placed in in a 37°C incubator under 5% CO₂.

2.4.3 Preparation of metaphase chromosomes

2.4.3.1 Preparation of blood lymphocyte chromosomes

Cultures were set-up in a class 1 microbiological safety cabinet. 5.0ml RPMI containing 0.05ml phytohaemagglutinin (PHA) was inoculated with either 0.2ml whole blood or 0.1ml baby blood. Cultures were incubated at a 20° angle at 37°C for 48 or 72 hours. On the morning prior to harvesting, 0.1ml thymidine solution (final concentration 0.3mg/ml) was added to the synchronised cultures, mixed and the cultures incubated overnight. The thymidine block was released after 24 hours by adding 0.1ml 2-deoxycytidine solution (final concentration 10μM). The cultures were mixed and incubated for a further 4.5 hours at 37°C. Either 45 minutes (long colcemid) or 10 minutes (short colcemid) before harvest, 0.1ml colcemid solution per 5ml culture was added and incubated at 37°C. The cultures were spun at 400g for 5 minutes and the supernatant carefully removed. 5mls of pre-warmed 0.075M KCl (37°C) was added and the cultures incubated at 37°C for 5 minutes. The cultures were spun at 400g for 5 minutes and the supernatant removed. The cells were fixed using a whirlimix to keep the cell pellet moving and 2.0mls of fixative was added dropwise to avoid cell clumping. A further 3.0mls of fixative was added and the cells spun at 400g for 5 minutes. After the supernatant had been removed, the pellet was resuspended in a further 5.0mls of fixative and this was repeated twice. After the final fixation, the pellet was resuspended in 1.0ml of fixative and slides prepared. Slides used for chromosome preparations were cleaned thoroughly with fixative to ensure good spreading of the cells. A single drop of cell suspension was dropped onto the slide from a height of 10cms. A drop of fixative was applied to this and the slide allowed to dry. Slides were left to age overnight on a heating block and stored for upto three weeks in a protective box or at -20°C. The fixed cell suspension was stored at -20°C.

2.4.3.2 Preparation of lymphoblastoid cell line chromosomes
At least 10mls of a vigorously growing culture was transferred to fresh RPMI containing 20% fetal calf serum and incubated at 37°C overnight. 100µl/10ml culture of colcemid was added, swirled gently to mix and incubated at 37°C for 1 hour. The cells were transferred to 10 ml Falcon tubes and spun at 400g for 5 minutes. The cells were resuspended in 10mls of 0.075M KCl and incubated at 37°C for 5 minutes. The cells were spun at 400g for 5 minutes and the supernatant removed. The cells were fixed slowly by adding the fixative dropwise. A total of 5mls fixative was added and the cells spun at 400g for 5 minutes. This was repeated twice more. Slides were prepared as described in section 2.4.3.1 and the fixed suspension stored at -20°C.

2.4.4 Fluorescence in situ hybridisation

Two different FISH methods were used and these were based on a method proposed by Lichter et al., (1988). The essential differences between methods 1 and 2 are shown in table 8. In method 2, the overall stringency of the FISH experiment was increased by raising the temperature of the 50% formamide/2XSSC washes from 42°C to 45°C, altering the SSC concentration in the second set of washes from 1XSSC to 0.1XSSC and increasing the temperature of these washes from 42°C to 60°C. The blocking buffer and FITC dilution buffer were spun in method 2 to remove debris which may increase the background.

2.4.4.1 Labelling of probes by nick translation

Probes for FISH studies were labelled with either biotin or digoxigenin. Total yeast DNA (10µg-20µg) was digested with Cfo I for 4-6 hours at 37°C, to provide a smaller template for the enzymes in the nick translation reaction. An equal volume of phenol/chloroform was added to the digested DNA and the DNA precipitated with 0.1X volume sodium acetate (pH 5.2) and 2.5X volume 100% ethanol. The DNA was redissolved in 10-20µl of TE (pH 7.6). Cosmid and phage DNA probes were not digested prior to labelling.

A) Labelling of probes with biotin

Method 1
<table>
<thead>
<tr>
<th>EXPERIMENTAL COMPONENT</th>
<th>EXPERIMENTAL CONDITIONS</th>
<th>EXPERIMENTAL CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>METHOD 1</td>
<td>METHOD 2</td>
</tr>
<tr>
<td>HYBRIDISATION BUFFER</td>
<td>50% FORMAMIDE</td>
<td>50% FORMAMIDE</td>
</tr>
<tr>
<td></td>
<td>10% DEXTRAN SULPHATE</td>
<td>10% DEXTRAN SULPHATE</td>
</tr>
<tr>
<td></td>
<td>2XSSC</td>
<td>2XSSC</td>
</tr>
<tr>
<td></td>
<td>1% TWEEN-20</td>
<td></td>
</tr>
<tr>
<td>NICK TRANSLATION</td>
<td>1μg DNA</td>
<td>2μg DNA</td>
</tr>
<tr>
<td></td>
<td>50μg E. COLI tRNA</td>
<td>20μg SALMON SPERM DNA</td>
</tr>
<tr>
<td></td>
<td>50μg SALMON SPERM DNA</td>
<td>20μg COT-1</td>
</tr>
<tr>
<td></td>
<td>3μg COT-1</td>
<td></td>
</tr>
<tr>
<td>HYBRIDISATION</td>
<td>1μg PROBE</td>
<td>400ng PROBE</td>
</tr>
<tr>
<td>50% FORMAMIDE/2XSSC WASHES</td>
<td>42°C</td>
<td>45°C</td>
</tr>
<tr>
<td>4XSSC/0.1% TWEEN-20 WASHES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSC WASHES</td>
<td>1XSSC / 42°C</td>
<td>0.1XSSC / 60°C</td>
</tr>
<tr>
<td>BLOCKING BUFFER AND FITC</td>
<td>BUFFERS NOT SPUN</td>
<td>BUFFERS SPUN TO REMOVE DEBRIS</td>
</tr>
<tr>
<td>DILUTION BUFFER</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 8.** Principal differences between FISH method 1 and method 2.
Probes for FISH studies were labelled with biotin-14-dATP by nick translation using the Gibco BRL Bionick kit. To 1μg of probe DNA, 5μl of dNTP mix containing 0.1mM biotin-14-dATP, 0.1mM dATP and 0.2mM dCTP, dGTP and dTTP and 5μl of enzyme mix containing 0.5 units/μl DNA Polymerase and 0.0075 units/μl DNase I were added. Distilled water was added to the labelling mixture to give a final volume of 45μl. The labelling mixture was incubated at 16°C for 90 minutes and the reaction stopped by the addition of 5μl 300mM EDTA. After the incubation step, 3μg Cot-1 DNA, 50μg E. Coli tRNA, 50μg sonicated salmon sperm DNA, 0.1X volume 3M sodium acetate (pH 5.6) and 2.0X volume 100% ethanol were added to the labelling reaction to precipitate the labelled probe. The probe was precipitated at -20°C overnight and after spinning at 15,000g for 10 minutes, the supernatant was carefully removed and the pellet redissolved in 50μl of hybridisation buffer (section 2.3.7). Probes were stored at -20°C and 1μg of the nick translated product was used in each hybridisation.

Method 2
Probes were labelled with biotin-14-dATP by nick translation using the Gibco BRL Bionick kit as for method 1, with the following changes. Probe DNA (2μg) was added to 10μl dNTP mix containing biotin 14-dATP and 10μl DNase I/DNA polymerase I mix. Distilled water was added to give a final volume of 90μl. The labelling reaction was incubated at 16°C for 3 hours and then stopped by the addition of 10μl 300mM EDTA. A 10μl aliquot was removed and run on a 2% agarose gel to estimate the fragment size before continuing. The optimal size range is between 200 bp and 500 bp. 20μl Cot-1 (1μg/μl), 2μl sheared Herring sperm DNA (10μg/μl), 78μl distilled water, 20μl 3M sodium acetate (pH 5.2) and 500μl 100% ethanol were added to the labelling mixture. The labelled probe was precipitated at -70°C for 30 minutes and spun for 10 minutes at 15,000g. The pellet was rinsed twice with 70% ethanol and redissolved in 100μl of hybridisation buffer to give a final concentration of 20ng/μl. The probe was left at 37°C overnight to allow the pellet to completely dissolve. Probes were stored at -20°C and 200ng - 400ng probe/slide was used in each hybridisation.

B) Labelling of probes with digoxigenin
Probes DNA (0.1-2μg) was labelled using the Boehringer Mannheim digoxigenin labelling kit. 1 volume of 0.4mM digoxigenin-11-dUTP
(Boehringer Mannheim) was added to 2 volumes of 0.4mM dTTP and 3 volumes of 0.4mM dATP, dCTP and dGTP. 10μl of the dNTP mix was added to 0.1-2μg of probe DNA. 2μl of nick translation buffer was added to the labelling mixture and the volume made up to 18μl with sterile distilled water. 2μl of enzyme mix containing DNA polymerase I and DNase I was added and the labelling mixture was incubated at 15°C for 90 minutes. The reaction was stopped by the addition of 2μl of 0.2M EDTA (pH 8.0). The labelled probe was precipitated at -70°C for 30 minutes following the addition of 20μl Cot-1 (1μg/μl), 2μl sheared Herring sperm DNA (10μg/μl), 58μl distilled water, 10μl 3M sodium acetate (pH 5.2) and 250μl 100% ethanol to the labelling mixture. The labelled probe was prepared and stored as described in method 2 above.

2.4.4.2 In situ hybridisation and detection of labelled probe

Method 1
Slides (Chance propper Ltd.) were prepared at least one day before hybridisation. The probe was denatured at 70°C for 5 minutes and then left at 37°C for 1-3 hours to reanneal. Simultaneously, the denaturation solution containing 70% deionised formamide/2XSSC was heated to 70°C. Slides were placed in the denaturation solution for 2 minutes and then dehydrated for 2 minutes in 70%, 85 % and 100% ice cold ethanol.

The probe was pipetted onto the slide and a coverslip (Chance propper Ltd.) gently lowered into place. The edges were sealed with Cow Gum rubber solution (Cow Proofings Ltd.) and the slides placed in a moist container, which was incubated at 37°C overnight.

After hybridisation, the coverslips were removed and the slides were washed three times in 50% formamide/2XSSC at 42°C for 5 minutes, followed by three washes in 1XSSC at 42°C for 5 minutes.

200μl of blocking buffer (4XSSC; 0.05% Tween-20; 3% BSA) was applied to each slide and covered with a 50 X 22mm coverslip. The slides were incubated at 37°C for 30 minutes. The coverslips were then removed and 200μl of avidin-FITC diluted in blocking buffer (1/200) was applied. The coverslips were replaced and the slides incubated at 37°C for 30 minutes.

The slides were then washed in 4XSSC/0.1% Tween-20 three times at 42°C for 5 minutes each.

The avidin-FITC signal can be amplified by incubating the slides in biotinylated anti-avidin diluted in blocking buffer (1/100) for 30 minutes at 37°C, washing and re-detecting with avidin-FITC as above. The slides were
mounted in 2μg/ml DAPI/Antifade and placed at 4°C for 30 minutes before viewing.

**Method 2**

Slides were either prepared at least one day prior to hybridisation or if they were prepared on the day of hybridisation, then they were incubated in 2XSSC at 37°C for 1 hour, followed by dehydration in 70%, 80% and 95% ethanol for 2 minutes. The ethanol was pre-chilled to -20°C. The slides were allowed to air dry and placed on a hot plate until ready to use.

The 70% formamide/2XSSC denaturation solution was heated to 70°C, increasing the temperature by 1°C per slide. The slides were denatured for 2 minutes in the denaturation solution and dehydrated for 2 minutes in 70%, 80%, 90% and 100% ice-cold ethanol. At the same time, the probe was denatured for 10 minutes at 70°C and incubated at 37°C for 10 minutes to enable the probe to reanneal. The probe was pipetted onto the denatured slide and a coverslip gently lowered into place. The edges were sealed with Cow Gum rubber solution and the slides incubated overnight at 37°C for cosmids and phage probes and for 48 hours for YAC probes.

After hybridisation, the rubber cement was gently removed with a pair of forceps. The slides were placed in a coplin jar containing 4XSSC/0.1% Tween-20 wash solution to remove the coverslips. The slides were then washed three times in 50% formamide/2XSSC at 45°C for 5 minutes each, followed by three washes in 0.1XSSC at 60°C for 5 minutes.

200μl of blocking buffer was applied to each slide and covered with a 50 X 22mm coverslip. The slides were incubated at 37°C for 20 minutes. The coverslips were removed following incubation and 200μl of Avidin-FITC was applied. The coverslips were replaced and the slides incubated at 37°C for a further 20 minutes. The slides were then washed in 4XSSC / 0.1% Tween-20 three times at 45°C for 2 minutes each.

The avidin-FITC signal can be amplified by incubating the slides in biotinylated anti-avidin for 20 minutes at 37°C, washing and re-detecting with avidin-FITC as above. The slides were mounted in 2μg/ml DAPI/Antifade and placed at 4°C for 30 minutes before viewing.

### 2.4.4.3 Visualisation of the fluorescent signal and image capture

FISH analysis was performed on a Zeiss Axioskop microscope connected to a Photometrics series 200 cooled charged-coupled device (CCD) camera
system. The images were captured and analysed on a Power Macintosh 8100/80AV using the Smart Capture software (Digital Scientific). A series of images were captured with the appropriate filters (these were selected depending on the fluorochromes used in each FISH experiment) and a 24 bit colour image was produced. Automatic capture selects the optimum exposure time and exposure times were generally set to automatic. After the images were captured they were normalised, which allows a percentage of the lowest pixels to be black and a percentage of the highest pixels to be the maximum brightness that can be displayed on the computer. Values were set for each colour component.

Captured images were printed on a Mitsubishi sublimination colour printer using A4-SPW printing paper (Southern). The printing of captured images requires manipulation of the colours which have been applied to the images by the Smart Capture programme, to enable visualisation of the DAPI stained chromosomes above the black background. The photographs presented in this thesis were generated by using the Join programme to lighten the pseudocoloured blue DAPI images. The green FITC images and the red Rhodamine images were also manipulated to provide the optimum contrast. Join composes up to eight greyscale images to form a single colour image. The contribution to each image can be adjusted in both depth and hue.

2.4.5 Polymerase chain reaction

25-100ng of DNA were amplified in the polymerase chain reaction (PCR). The reaction mix consisted of 10X buffer containing 160mM (NH₄)₂SO₄, 670mM Tris-HCl (pH 8.8) at 25°C, 25 picomoles of each primer, 0.2mM each of dATP, dGTP, dTTP and dCTP, magnesium ions (the concentration of which had been determined by titration) and 1.5-2.5 units of Taq polymerase. Reactions were overlaid with 50µl of mineral oil to prevent evaporation and amplified on a Techne PHC-2 water cooled thermal cycler.

For PCR amplified CA(n) repeats, it was necessary to incorporate ³²P-dCTP. The concentrations of the nucleotides were 0.2mM dATP, dGTP, dTTP and 0.1mM dCTP. 0.1µl of ³²P-dCTP (3000 Ci/mmol, 1mCi = 100µl) was added to each sample.

Conditions for thermal cycling consisted of an initial denaturation at 95°C for 5 minutes, the reaction was held at 85°C while the Taq polymerase was added (hot start), then 25-30 cycles were carried out at the annealing temperature of the primer for 1 minute, followed by an extension step at 72°C for 1 minute.
and a denaturation step at 95°C for 1 minute. A final extension step at 72°C for 10 minutes was carried out.

2.4.6 Restriction endonuclease analysis

Human, plasmid, phage and cosmid DNA were digested with the appropriate restriction enzyme. Digests of human DNA were carried out in a total volume of 40μl. 3-5μg of human DNA was added to 4μl of the appropriate restriction enzyme buffer and 10-20 units of restriction enzyme. The volume was made up to 40μl with distilled water. The restriction endonuclease digests were incubated at 37°C for either 4-6 hours or overnight. Cosmid, plasmid and phage DNA for electrophoresis were digested in a total volume of 20μl. DNA was added to 2μl of the appropriate restriction enzyme buffer and 10-20 units of enzyme.

2.4.7 Electrophoresis

2.4.7.1 Separation of restriction enzyme fragments

An agarose gel, the percentage of which was determined by the size of the fragments to be separated, was prepared in 1XTAE, brought to boiling point in a microwave oven to dissolve the agarose and allowed to cool to 60°C prior to pouring. Ethidium bromide (final concentration 0.15 mg/ml) was added to the cooled agarose to enable the visualisation of the DNA. The ends of a 20X25 cm BRL gel tray were sealed with tape. A 20 well comb was inserted into the agarose and the gel allowed to set at room temperature for 1-2 hours. A volume of 10μl of loading dye was added to each 40μl digest and the total volume loaded into a well. Lambda DNA (1μg) digested with either BstE II or Hind III was used as a molecular marker. Gels were electrophoresed at 50 Volts for 16-24 hours and photographed on an ultraviolet (UV) light transilluminator against a fluorescent ruler, to mark the relative positions of the bands. Smaller volumes of restriction enzyme digests were separated on a 11 cm x 13.5 cm NBL gel tray and electrophoresed at 100 Volts for 2 hours.

2.4.7.2 Separation of insert from cosmid and plasmid vectors
The vector was digested with the appropriate restriction enzymes and electrophoresed as described in section 2.4.7.1. A UV transilluminator was used to visualise the appropriate fragment, which was cut from the gel in a slice with a scalpel. The gel slice was transferred to a 0.5ml tube which had been plugged with glass wool and with a hole punctured in its base. This tube was then placed inside a 1.5ml eppendorf tube and the DNA eluted by centrifugation at 13,000g in a bench microfuge for 10 minutes. The concentration of the eluted DNA was determined spectrophotometrically.

2.4.7.3 Preparation of single copy probe from cosmid and plasmid vectors

Single copy fragments were obtained by digesting the vector with the appropriate restriction enzyme and separating the digested fragments by agarose gel electrophoresis as described in section 2.4.7.1. The gel was photographed prior to Southern blotting (section 2.4.8) and the blotted DNA was then hybridised with radio-labelled total human DNA. The bands which are observed on the X-ray film (X-OMAT-AR5, Kodak) contain repeat sequences, therefore bands which are not visible on the film but are visible on the original photograph should be free of repeat sequences.

2.4.7.4 Separation of PCR products in agarose gels

A 2% agarose solution in 1XTBE buffer containing ethidium bromide (final concentration 0.15 mg/ml) was prepared and poured into a sealed 11 cm x 13.5 cm NBL gel tray. After setting, the gel was placed in an electrophoresis tank, covered with 1XTBE buffer and 5μl of PCR amplified DNA sample loaded. The gel was electrophoresed at 10 volts/cm for 30-60 minutes. Band sizes were determined by comparison with a 100 bp ladder (BRL) which was also loaded onto the gel at the same time as the PCR product.

2.4.7.5 Separation of microsatellite polymorphisms by polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels were used for the analysis of PCR amplified CA(n) repeats. A large (42x33cm) BRL glass plate and a small (39x33cm) BRL glass plate were washed in detergent, rinsed and dried. The plates were wiped with 70% ethanol and the smaller of the two plates was coated with a
siliconising fluid (Sigmacote, Sigma). This prevents the gel from adhering to the small plate and ensures that it remains bound to the large plate when the two plates are separated. The gel mould was formed by placing the large plate on a flat surface, arranging two 0.4mm spacers (0.4mm) down the edges and placing the small plate with the siliconised surface facing inwards, on top. The two plates and spacers were carefully aligned and clipped in place with bulldog binder clips.

A denaturing 6% (v/v) polyacrylamide gel (section 2.3.5) was caste. To 90μl of gel mix, 540μl of 10% ammonium persulphate and 60μl of TEMED were added to catalyse cross linking in the liquid gel. The gel mix was gently poured between the two plates which were placed horizontally, using a 50ml syringe. Two 25 well sharkstooth combs were inserted into the gel, with the flat edge approximately 1cm into the gel and clipped in place. The gel was left to polymerise for 2 hours.

The gel was then placed in a vertical BRL tank and clamped into place. The upper and lower reservoirs were filled with 1XTBE (500mls in each). The sharkstooth combs were removed from the gel and the wells were washed thoroughly with 1XTBE to remove any traces of urea prior to inserting the sharkstooth combs. The sharkstooth combs were inverted so that the teeth just penetrated the gel to form wells. The wells were rinsed again. The sequencing apparatus was connected to a Consort E734 power pack and the gel pre-run at 60W for 30 minutes. The wells were washed out with 1XTBE prior to loading. An 8μl aliquot of each PCR product was added to 2μl of formamide dye and the samples were denatured at 94°C for 5 minutes and then placed on ice. 4μl of each sample was loaded onto the gel and run at 60W for 2-3 hours, depending on the size of the PCR product.

After electrophoresis was complete, the gel assembly was removed from the tank and the smaller plate was levered away. The polyacrylamide gel was blotted onto a piece of 3MM Whatman paper, covered with clingfilm and dried on an ATTA Gel Drying Processor AE-3700. The dried gel was exposed to X-ray film overnight at room temperature.

2.4.8 Southern blotting and hybridisation

The DNA digests were separated as described in section 2.4.7.1. The gel was denatured in denaturing solution (section 2.3.4) for 30 minutes on a shaking platform. The denatured gel was inverted and placed on a sponge in
a photographic tray. The sponge was covered with 3MM Whatman paper pre-soaked in 20XSSC, which acts as a wick.

A piece of charged nylon membrane (Hybond N+) was cut to the exact size of the gel, soaked in 2XSSC and placed on top of the gel. 2 pieces of 3MM Whatman paper pre-soaked in 2XSSC were placed on top of the Hybond N+ and air bubbles were removed by gently rolling a 10ml plastic pipette over the surface. Clingfilm was placed around all four sides of the gel to prevent evaporation and three paper towels were unfolded and laid out flat over the gel. Paper towels were then placed on the gel to a height of approximately 10cms to draw the 20XSSC and therefore, the DNA onto the filter.

After 16 hours the filter was removed from the gel and the position of the wells marked with a permanent marker. DNA was fixed to the filter by soaking in 0.4M NaOH for 30 minutes and neutralised by rinsing in 2XSSC until the pH of the SSC was 7.0. The filters were blotted to remove excess liquid and wrapped in clingfilm until required.

### 2.4.8.1 Oligolabelling of DNA

Plasmid or insert DNA was diluted in TE to a concentration of 10ng/μl and a total of 50ng of probe was labelled per reaction. The probe was placed in a 1.5ml tube with a pierced lid to avoid a build up of pressure while heating. The probe was denatured for 5 minutes in a boiling water bath and placed on ice to prevent renaturation. To this was added 10μl of OLB, 1μl BSA (10mg/ml), 3μl of alpha^{32}P-dCTP (3000Ci/mmol, 1mCi = 100μl) and 2.5 units of Klenow fragment of DNA polymerase 1. The reaction was mixed and left to stand at room temperature for 2-4 hours.

After labelling, unincorporated nucleotides were separated from the labelled DNA by filtration through a Sephadex G-50 column in a 1ml syringe plugged with an autoclaved glass bead. The column was equilibrated with TE. The column was placed in a centrifuge tube and the probe was applied to the column, the elute was discarded. Radio-labelled DNA was eluted by applying an equal volume of TE to the column. The radioactivity of the eluted DNA was determined by counting a 4μl aliquot in a calibrated Bioscan QC2000 bench top beta counter. 1x10^6 dpm of probe per ml of hybridisation solution was added to pre-hybridising filters.

### 2.4.8.2 Hybridisation of ^{32}P labelled DNA to filters
The filter was soaked in 2XSSC, rolled up and placed in a hybridisation bottle (Hybaid) containing 10mls of hybridisation solution. The hybridisation bottle was placed in a 65°C rotary oven (Hybaid) and allowed to pre-hybridise for 2 hours to saturate non-specific sites in the DNA and reduce the level of non-specific binding. After prehybridisation, the radiolabelled probe was denatured in a boiling water bath, placed on ice, then added to the hybridisation bottle containing the filter to give a final concentration of $10^6$ dpm/ml hybridisation solution.

When competitive sequences were present, 100μg of sheared human placental DNA (10mg/ml) per ml of hybridisation solution was added to the filter and pre-hybridised for at least 3 hours. In addition, the probe was boiled for 5 minutes and added to 1.8ml of 300μg of sheared human placental DNA, hybridisation solution and 20XSSC (final concentration 0.5XSSC). This was maintained at 65°C for 90 minutes and then added to the pre-hybridised filter. After hybridisation, the filters were washed in solutions of increasing stringency. The filters were monitored with a beta emission counter until the signal detected was at background level. Generally, the unhybridised probe was removed by washing the filter in 3XSSC/0.1% SDS at room temperature on a shaking platform for 1 hour, followed by washing at 65°C in a shaking water bath in 1XSSC/0.1% SDS, 0.5XSSC/0.1% SDS and 0.1XSSC/0.1% SDS for 30, 15 and 10 minutes respectively, depending on the probe. Filters were dried on 3MM Whatman paper, wrapped in clingfilm and exposed to X-ray film in a cassette backed with an intensifier screen. The cassette was placed at -70°C for 12-16 hours, depending on the intensity of the radioactive signal. The film was developed using a Fuji RGlI X-ray film processor.

### 2.4.8.3 Quantification of radioactivity using a phosphorimager

Phosphorimager screens were erased by exposing them to yellow light for 6 minutes, prior to use. Labelled filters for dosage analysis were placed in a phosphorimager cassette box and exposed to the phosphorimager screen for 24-72 hours. The exposed phosphorimager screen was scanned and the radioactive signal quantified using a phosphorimager (Molecular Dynamics) and the ImageQuant software. Dosage was determined by measuring the ratio of radioactive signals from the test probe and a control probe using the phosphorimager.
2.5 Patients

3731 is the third child of healthy parents. The neonatal period was uneventful but by two years of age global developmental delay was apparent. 3731 has blonde hair, blue eyes with pale fundi, microbrachycephaly, a wide smiling mouth and a prominent chin. Her voluntary movements are jerky and she has never developed any speech. An EEG performed at five years of age showed the slow wave changes characteristic of AS. High resolution chromosome analysis performed by the Northern region genetics advisory service was normal.

13581 presented at clinic at 7 years of age and is the only child of normal parents. Clinical features include developmental delay, especially in the area of speech, a history of seizures and a smiling happy personality. Her mother was 7 weeks pregnant. High resolution chromosome analysis performed by the North East Thames regional cytogenetic service was normal.

B.S has a borderline intellectual deficit whereas D.H. has mild to moderate mental retardation. Neither individual has the characteristics of AS. High resolution chromosome analysis showed a deletion of 15q (Michaelis et al., 1995).

Family R were referred to the molecular genetics unit at ICH when the mother of two siblings with AS was 17 weeks pregnant. Both siblings were mentally retarded, had a speech impairment and a sociable disposition.

Family W were referred to the molecular genetics unit at ICH. The affected male sibling was retarded with a prominent lower jaw and mid facial hypoplasia. He had a history of seizures. The affected female sibling had developmental delay and a protruding tongue. Both siblings had an EEG pattern consistent with a diagnosis of AS.

Patient 6468 was referred to the molecular genetics unit at ICH with global developmental delay. Her clinical features were consistent with a diagnosis of AS. High resolution chromosome analysis performed by the North East Thames regional cytogenetic service was normal.
Patients EA, KC and HR were collected as part of the study by J. Clayton-Smith (1992) and had clinical features consistent with a diagnosis of AS. Patient HR did not have the paroxysms of laughter or seizures that are commonly associated with a diagnosis of AS and high resolution chromosome analysis performed by the Northern region genetics advisory service was normal. High resolution chromosome analysis performed by Dr. Tessa Webb was normal for patient EA. Patient KC was reported as having a deletion of one of the chromosome 15 homologues.
3.0 Characterisation of atypical deletions in AS patients
3.1 Introduction

AS individuals were studied in order to find atypical deletions which would define a critical region for the AS gene. A combination of techniques including FISH using YACs, cosmids and phage, microsatellite analysis, dosage analysis with single copy probes and methylation studies at exon alpha, were used to characterise molecular changes in these patients. The following patients were studied:

An AS individual, 3731, had been identified who failed to inherit a maternal allele for the CA(n) repeat at LS6-1 (D15S113). Other CA(n) repeats in the 15q11-q13 region were either uninformative or indicated biparental inheritance of both alleles. Both normal maternal and paternal DNA methylation imprints were present at ZNF127 (D15S9), PW71 (D15S63) and SNRPN (intron 5) (Buxton et al., 1994).

AS individual 13581 had inherited maternal and paternal alleles for the CA(n) repeats at GABR83 and MS14 (D15S97) (S. Cottrell, pers. comm.). This individual failed to inherit a maternal allele for the CA(n) repeat at LS6-1 (D15S113) (S. Ramsden, pers. comm.) and had normal maternal and paternal DNA methylation imprints at PW71 (D15S63) (T. McKay, pers. comm.).

A child with AS who had inherited an unbalanced form of a reciprocal translocation between chromosomes 4 and 15 was reported (Reis et al., 1993). The unbalanced translocation had been inherited from the mother who carried a balanced form of the translocation 46, XX, t (4; 15)(q35.2; q13). Molecular analysis of DNA from the patient and his mother showed that they were both heterozygous at GABR83. The child had not inherited a maternal allele at the D15S11 locus and dosage analysis showed a deletion of the D15S10 locus in the child. The child was heterozygous for the dinucleotide repeat at D15S113, placing the translocation breakpoint between D15S10 and D15S113 (Greger et al., 1994). The child was therefore monosomic for chromosome 15 proximal to this breakpoint.

A mother and son were reported with a deletion of 15q that included the D15S113 (LS6-1) locus. The son had mild to moderate mental retardation,
whereas the mother had a borderline intellectual deficit. Neither individual had the characteristics of AS (Michaelis et al., 1995).

Further AS individuals in whom no molecular defect had been identified were studied with probes isolated during the course of this work.

3.2 Characterisation of FISH probes

11HE12 is a 260 kb LS6-1 positive YAC which was isolated by PCR screening (Buxton et al., 1994) of the ICI total human library (Anand et al., 1990).

MR-60 is a cosmid which maps to 15q26.1 (R. Schultz, pers. comm.) outside the AS/PWS region on chromosome 15 and could be suitable as a marker for chromosome 15.

3.2.1 Characterisation of cosmid MR-60

To ascertain whether there were any related signals on other chromosomes or any other region of chromosome 15 which would make MR-60 unsuitable as a chromosome 15 marker, it was hybridised to metaphase spreads from an individual with a normal karyotype. MR-60 produced signal on both chromosome 15 homologues in 10/10 metaphases analysed (figure 10). No other signals were observed in these 10 metaphases and MR-60 is therefore a suitable marker for chromosome 15.

3.2.2 Characterisation of YAC 11HE12

The LS6-1 positive YAC 11HE12 was tested for chimerism by hybridisation to metaphase spreads from an individual with a normal karyotype. YAC 11HE12 hybridised to both chromosome 15 homologues in all ten metaphases analysed, the chromosome 15' s were identified by the presence of MR-60 (figure 11). Although YAC 11HE12 was not chimeric, a small degree of cross-hybridisation to the satellite regions of the acrocentric chromosomes was observed in the metaphase spreads analysed (not shown) and this may represent contaminating ribosomal sequences from the yeast DNA.
Figure 10. Chromosome 15 cosmid probe MR-60 hybridised to a metaphase spread from an individual with a normal karyotype (A) and the inverted grey scale image confirming the presence of signal on chromosome 15 (B).
3.3 FISH analysis of individual 3731

3.3.1 YAC 11HE12 analysis

Labelled YAC 11HE12 DNA was hybridised to metaphase spreads obtained from a lymphocyte culture, prepared from a peripheral blood sample from 3731. In 10/10 metaphases analysed, 11HE12 hybridised to both chromosome 15 homologues, indicating that the majority of this YAC was not deleted in this individual (figure 12). The failure of 3731 to inherit a maternal allele for the CA(n) repeat at LS6-1 (D15S113) meant that either (a) a deletion lay within YAC 11HE12 or (b) there is not a deletion at LS6-1 and the CA(n) repeat result was due to non amplification of the maternal allele. Cosmids from either end of the YAC and cosmids containing the LS6-1 locus (Buxton et al., 1994) were studied to test whether a potential deletion was spanned by YAC 11HE12. The 11HE12 LS6-1 positive YAC and the partial cosmid contig derived from it are shown in figure 13.

3.3.2 YAC 11HE12 cosmid analysis

The cosmids were used as probes for FISH analysis of 3731, using method 1 as described in section 2.4.4.

3.3.2.1 End cosmids

FISH analysis was performed on metaphase spreads from 3731 with the two end cosmids. E19 was derived from the centromeric end of the YAC and E16 was derived from the telomeric end. Normally 10 metaphases were analysed, but if the FISH probes detected both metaphases with signal on one chromosome 15 homologue only and metaphases with signal on both chromosome 15 homologues in the same individual, then 20 metaphases were analysed. The FISH results with the two end cosmids are shown in table 9.

End cosmid E19
In 17/20 (85%) metaphases analysed, E19 produced signal on both chromosome 15's from 3731 (table 9). In comparison, E19 produced signal on one chromosome 15 homologue only in 10/10 metaphases analysed from an AS individual with a known deletion encompassing all loci from D15S11.
Figure 11. YAC 11HE12 and chromosome 15 marker MR-60 hybridised to a metaphase spread from an individual with a normal karyotype.

Figure 12. YAC 11HE12 and chromosome 15 marker MR-60 hybridised to a metaphase spread from AS individual 3731.
Figure 13. Long range map of 15q11-q13 indicating the position of YAC 11HE12 and the corresponding partial cosmid contig.
(IR4-3R) to D15S24 (CMW 1) (table 9). E19 is therefore not deleted in 3731. Figure 14 shows the presence of signal from E19 on both chromosome 15 homologues in 3731, compared to a known AS deletion control showing the presence of signal from E19 on one chromosome 15 homologue only.

**End cosmid E16**

E16 produced signal on both chromosome 15's in 10/10 metaphases analysed from 3731, whereas signal was only produced on one chromosome 15 in 10/10 metaphases analysed from the known AS deletion control (table 9). E16 is therefore not deleted in 3731. Figure 15 shows the presence of signal from E16 on both chromosome 15 homologues in 3731, compared to a known AS deletion control showing the presence of signal from E19 on one chromosome 15 homologue only.

### 3.3.2.2 Internal cosmids

The overlapping LS6-1 positive cosmids E24 and E42 were hybridised to metaphase spreads from 3731. Both E24 and E42 produced signal on one chromosome 15 only in 18/20 (90%) metaphases analysed from 3731 (table 10). E24 produced signal on both chromosome 15's in 10/10 metaphases analysed from a normal control (table 10). A normal control for E42 was not tested. These results indicate that E24 and E42 may be deleted in 3731. Figure 16 shows the presence of signal from E24 on one chromosome 15 homologue only in 3731, compared to a normal control showing the presence of signal from E24 on both chromosome 15 homologues.

These results indicate a possible deletion around LS6-1 of less than 200kb, which if confirmed would be the smallest reported deletion in the AS critical region to date. However, two non-deleted cells were observed for both the LS6-1 cosmids in 3731 and this could indicate that either (a) a deletion breakpoint falls within the region common to both of these cosmids or (b) 3731 is a mosaic.
Figure 14. Comparison of a metaphase spread from (A) 3731 and (B) an AS individual with a known deletion of 15q11-q13, probed with the centromeric end cosmid E19 and MR-60. Chromosome 15's marked with arrows.
Figure 15. Comparison of a metaphase spread from (A) 3731 and (B) an AS individual with a known deletion of 15q11-q13, probed with the telomeric end cosmid E16 and MR-60. Chromosome 15's marked with arrows.
Figure 16. Comparison of a metaphase spread from (A) 3731 and (B) an individual with a normal karyotype, probed with the LS6-1 positive cosmid E24 and MR-60. Chromosome 15's marked with arrows.
### Table 9.
Results of FISH analysis with the centromeric and telomeric end cosmids E19 and E16, AS individual 3731 compared to an AS individual with a known deletion encompassing all loci from D15S11 - D15S24.

<table>
<thead>
<tr>
<th>COSMID</th>
<th>37 31</th>
<th>DELETION CONTROL</th>
<th>(D15S11-D15S24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIGNAL ON ONE 15 ONLY</td>
<td>SIGNAL ON BOTH 15'S</td>
<td>SIGNAL ON ONE 15 ONLY</td>
</tr>
<tr>
<td>E19</td>
<td>3 / 20</td>
<td>17 / 20</td>
<td>10 / 10</td>
</tr>
<tr>
<td>E16</td>
<td>-</td>
<td>10 / 10</td>
<td>10 / 10</td>
</tr>
</tbody>
</table>

### Table 10.
Results of FISH analysis with the LS6-1 positive cosmids E24 and E42, AS individual 3731 compared to a normal control. NT-Not Tested.

<table>
<thead>
<tr>
<th>COSMID</th>
<th>37 31</th>
<th>NORMAL</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIGNAL ON ONE 15 ONLY</td>
<td>SIGNAL ON BOTH 15'S</td>
<td>SIGNAL ON ONE 15 ONLY</td>
</tr>
<tr>
<td>E24</td>
<td>18 / 20</td>
<td>2 / 20</td>
<td>-</td>
</tr>
<tr>
<td>E42</td>
<td>18 / 20</td>
<td>2 / 20</td>
<td>NT</td>
</tr>
</tbody>
</table>
3.3.3 Series 200 cosmid analysis

The subcloning of YAC 11HE12 only resulted in a partial cosmid contig. To further characterise the probable deletion in 3731, additional probes were required. A collaboration with Dr. David Ledbetter at the National Institutes of Health, Bethesda was set-up. A complete cosmid contig (series 200) was available for a second 245 kb LS6-1 positive YAC, A229A2. This is part of a YAC contig spanning the AS/PWS region (Mutirangura et al., 1993). This YAC has been shown by restriction mapping to be almost identical to the 11HE12 YAC, although it is positioned proximally (J.Buxton, pers. comm.). Figure 17 illustrates the 245 kb LS6-1 positive YAC A229A2 and the complete cosmid contig derived from it.

Metaphase spreads were prepared from a lymphoblastoid cell line set-up from 3731 and were used in all the following experiments which were carried out at the National Institutes of Health.

FISH analysis was performed on 3731 with a cosmid from the centromeric end of the YAC (cosmid 209) and cosmid 420 from the centromeric end of another LS6-1 positive YAC, 378A12. The position of cosmid 420 is shown in figure 17. These two cosmids overlap and were used to confirm the original E19 result. The LS6-1 positive cosmids 210 and 231 were used as probes to confirm the E24 and E42 results and cosmid 208 from the telomeric end of the YAC was used to confirm the original E16 result. Cosmids 206, 209 and 223 which covered the region which had not previously been analysed, were also used as probes to ascertain the extent of the deletion in 3731.

Method 2 as described in section 2.4.4 was used for all the following FISH experiments. The chromosome 15's were detected with a chromosome 15 centromere probe (D15Z1, Oncor) which was provided ready labelled with digoxigenin-11-dUTP. D15Z1 was detected with anti-digoxigenin conjugated to rhodamine, without amplification. Five metaphases were analysed from the controls and 10-20 metaphases were analysed from 3731. The series 200 cosmid results are shown in table 11.

Cosmid 209 produced signal on both chromosome 15 homologues in 15/15 metaphases analysed from 3731, although 2/15 metaphases showed a
Figure 17. Long range map of 15q11-q13 indicating the position of YAC A229A2, the corresponding series 200 cosmid contig, YAC 378A12 and cosmid 420.
<table>
<thead>
<tr>
<th>COSMID</th>
<th>SIGNAL ON ONE 15 ONLY</th>
<th>SIGNAL ON BOTH 15'S (EQUAL INTENSITY)</th>
<th>SIGNAL ON BOTH 15'S (UNEQUAL INTENSITY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td>8 / 20</td>
<td>6 / 20</td>
<td>6 / 20</td>
</tr>
<tr>
<td>231</td>
<td>3 / 20</td>
<td>9 / 20</td>
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<td>-</td>
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<td>6 / 20</td>
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</tr>
<tr>
<td>223</td>
<td>-</td>
<td>9 / 11</td>
<td>2 / 11</td>
</tr>
</tbody>
</table>

*Table 11.* Results of FISH analysis with the series 200 cosmids derived from YAC A229A2, AS individual 3731.
reduced signal on one of the chromosome 15 homologues compared to the other. Cosmid 420 from the centromeric end of YAC 378A12 was also tested and signal of equal intensity was detected on both chromosome 15 homologues in 10/10 metaphases analysed from 3731 (figure 18). Signal of equal intensity was observed on both chromosome 15 homologues in all 5 metaphases analysed from the normal control. Cosmids 209 and 420 are therefore not deleted in 3731 and confirm the original E19 result.

Figure 18. A metaphase from 3731 probed with cosmid 420 and D15Z1. Chromosome 15's marked with arrows.

Cosmid 206 produced signal of equal intensity on both chromosome 15's in 10/10 metaphases analysed from 3731, while cosmid 223 detected signal of equal intensity on both chromosome 15 homologues in 9/11 (82%) metaphases analysed. Signal of reduced intensity on one chromosome 15 homologue compared to the other was detected in 2/11 (18%) metaphases analysed. In comparison, signal of equal intensity was detected on both chromosome 15 homologues in 5/5 metaphases analysed from the normal controls. No metaphases with signal on one chromosome 15 homologue only were observed for either of these two cosmids in 3731, indicating that these
two cosmids are not deleted and that the apparent deletion in 3731 is confined to the region around the LS6-1 locus.

The LS6-1 positive cosmid 210 (B.Huang, pers. comm.) produced signal on both chromosome 15 homologues in 20/20 metaphases analysed from 3731. However, in 6/20 metaphases analysed (30%), an increased signal on one of the chromosome 15 homologues compared to the other was detected (figure 19). In 14/20 metaphases (70%), signal of equal intensity was detected on both chromosome 15's (figure 19). The normal control showed signal of equal intensity in all 5 metaphases analysed.

The LS6-1 positive cosmid 231 produced signal of equal intensity on both chromosome 15's in 9/20 metaphases analysed (45%) from 3731 (figure 20). A reduced signal on one chromosome 15 homologue compared to the other was detected in 8/20 metaphases analysed (40%) (figure 20) and in 3/20 metaphases analysed (15%), signal was detected on one chromosome 15 homologue only. Signal of equal intensity was detected in all 5 metaphases analysed from the normal control.

Cosmid 208 from the telomeric end of the YAC detected signal of equal intensity in 6/20 metaphases analysed (40%) from 3731 (figure 21). In 6/20 metaphases analysed (30%), a reduced signal was detected on one chromosome 15 homologue compared to the other (figure 22) and in 8/20 metaphases analysed (30%) signal was detected on one chromosome 15 homologue only (figure 23). Signal of equal intensity was detected in all 5 metaphases analysed from the normal control.

Each cosmid from the distal LS6-1 (D15S113) end, which was analysed in 3731, gave a proportion of metaphases which showed positive hybridisation signals of equal intensity on both chromosome 15's and positive hybridisation signals on one chromosome 15 homologue only. Positive hybridisation signals of differing intensities were also detected on one chromosome 15 homologue compared to the other in a proportion of the metaphases. In comparison, the normal controls showed signal of equal intensity in all the metaphases examined. This indicated that 3731 was either (a) mosaic for this region or (b) that her deletion was extremely small as no single cosmid lay completely within the deleted region.
Figure 19. Comparison of metaphases from 3731 probed with cosmid 210 and D15Z1 showing (A) signal of equal intensity on both 15's and (B) signals of differing intensities on both 15's.
Figure 20. Comparison of metaphases from 3731 probed with cosmid 231 and D15Z1 showing (A) signal of equal intensity on both 15's and (B) a deleted chromosome 15.
Figure 21. A metaphase from 3731 probed with cosmid 208 and D15Z1 showing signal of equal intensity on both chromosome 15's.
The presence of cosmid 11E8 and cosmid 208 revealed signal on each of the chromosome 15 homologues in the metaphases shown in Figure 22, whereas the signal was weaker on chromosome 15 homologues in metaphases from the same sample shown in Figure 23.

Figure 22. A metaphase from 3731 probed with cosmid 208 and D15Z1 showing signal of differing intensities on both 15's.

Figure 23. A metaphase from 3731 probed with cosmid 208 and D15Z1 showing a deletion of one of the chromosome 15 homologues.
The presence of metaphases which showed signal of differing intensities on each of the chromosome 15 homologues suggests that the latter theory is the most likely interpretation of the results, with these cosmids being partially deleted. If this is the case, then the deletion in 3731 would be estimated to be less than that of a single cosmid, depending on the overlap between the cosmids.

3.3.4 11HE12 cosmid analysis, method 2

As the results around the LS6-1 (D15S113) locus obtained with the series 200 cosmids differed from the results obtained with the 11HE12 cosmids, the 11HE12 cosmid data was reassessed using method 2. FISH analysis was performed on AS individual 3731 with the LS6-1 positive cosmids E24 and E42 and the telomeric end cosmid E16. In each case, the results were compared to a normal control and a known AS deletion control (table 12).

The LS6-1 cosmid E24 and the end cosmid E16 detected signal on one chromosome 15 homologue only in 3/20 (15%) metaphases analysed from 3731. E24 and E16 detected signals of differing intensities between the two chromosome 15 homologues in 8/20 (40%) metaphases, whereas signals of equal intensities on both 15's were detected in 9/20 (45%) metaphases from 3731. Cosmid E42 detected signals of differing intensities between the two chromosome 15 homologues in 11/20 (55%) metaphases and signals of equal intensities were detected on both 15’s in 8/20 (40%) metaphases analysed from 3731. E42 detected signal on one chromosome 15 homologue only in 1/20 (5%) metaphases analysed from 3731. In comparison, cosmids E24, E42 and E16 detected a greater number of metaphases with signals of equal intensities in the normal control (80%, 70% and 85% respectively) and a smaller number of metaphases with signals of differing intensities (20%, 25% and 15% respectively). One metaphase showing positive hybridisation signals on one chromosome 15 only was observed in the normal control with cosmid E42. A comparison of the percentages of the metaphases showing signal with differing intensities from 3731 versus the normal control is shown in table 13. Figure 24 shows a metaphase from 3731 probed with the LS6-1 positive cosmid E42 showing signals of differing intensities between the two chromosome 15 homologues.
<table>
<thead>
<tr>
<th>CASE</th>
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</tr>
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<tr>
<td></td>
<td>SIGNAL ON BOTH 15'S (UNEQUAL INTENSITY)</td>
<td>3/20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 12. 11HE12 cosmid results obtained with method 2, 3731 compared to a normal control and an AS control and an AS individual deleted for D15S11-D15S24.
Figure 24. A metaphase from 3731 probed with cosmids E42 and MR-60 showing the difference in signal intensities between the two chromosome 15 homologues.
<table>
<thead>
<tr>
<th></th>
<th>COSMID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E24</td>
</tr>
<tr>
<td>3731</td>
<td>40%</td>
</tr>
<tr>
<td>NORMAL</td>
<td>20%</td>
</tr>
</tbody>
</table>

**Table 13.** A comparison of the percentages of metaphases showing signals of differing intensities from 3731 versus a normal control.

Experiments mapping the 11HE12 cosmids to the series 200 cosmids showed cosmid E24 hybridised to cosmids 242, 201 and 210 and cosmid E16 hybridised to cosmids 210, 231 and 208. Cosmid E42 was not mapped (B. Huang, pers. comm.).

The results indicate that 3731 neither fits the pattern of a normal individual nor that of an AS deletion. It is still possible that no single cosmid lies entirely in the deleted region. The presence of one metaphase showing positive hybridisation signals of equal intensity between the two chromosome 15 homologues and 3/20 metaphases with signals of differing intensities in the AS deletion control with cosmid E24, could also indicate the presence of a related sequence elsewhere on chromosome 15q and this could interfere with the detection of a deletion in 3731.

### 3.3.5 A229A2 phage analysis

5 phage clones derived from the 245 kb LS6-1 positive YAC, A229A2, which formed a contig across the LS6-1 (D15S113) locus were provided by Dr. Ron Trent, for higher resolution FISH analysis of AS individual 3731.

The phage clones were 10-15 kb in size compared to 35 kb for the cosmid clones. If there was a sequence contained within one of the cosmids and this sequence was represented elsewhere on 15q, then, analysis of the smaller phage clones would provide more precise information as to the location of such a sequence. Localising this sequence to a smaller region could help identify the apparent deletion in 3731.
Four phage clones were analysed, Lambda 31, Lambda 64 which is LS6-1 positive, Lambda 4 and Lambda 30 (figure 25). The results for each phage clone were compared to an AS deletion control, who had been shown to be deleted by microsatellite analysis for TD3-21, LS6-1 and the 196 tetranucleotide repeat and one or two normal controls. FISH proved possible with these phage clones in contrast to the phage clones used around SNRPN (section 3.7.4 and 4.4.1). The results are shown in table 14.

Figure 25. A diagram indicating the approximate positions of the A229A2 phage, 11HE12 cosmids, LS6-1, TK-4 and 196 microsatellite markers and single copy fragments P1.1-6, P1.1-9 and 42P1.1-5.

3.3.5.1 Lambda 31

The results for Lambda 31 showed that signal of equal intensity was detected on both chromosome 15 homologues in 20/20 metaphases analysed from 3731 (figure 26). Signal of equal intensity was detected on both chromosome 15's in 10/10 metaphases analysed from the normal control and signal was detected on one chromosome 15 homologue only in 10/10 metaphases analysed from the deletion control (figure 26). Therefore, 3731 is not deleted for Lambda 31.
<table>
<thead>
<tr>
<th>PHAGE CLONE</th>
<th>CASE</th>
<th></th>
<th></th>
<th>AS DELETION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3731</td>
<td>NORMAL 1</td>
<td>NORMAL 2</td>
<td>(D15S210-196)</td>
</tr>
<tr>
<td>LAMBDA 31</td>
<td>N 20 / 20</td>
<td>10 / 10</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D -</td>
<td>-</td>
<td>-</td>
<td>10 / 10</td>
</tr>
<tr>
<td></td>
<td>P -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAMBDA 64</td>
<td>N 16 / 20</td>
<td>13 / 20</td>
<td>15 / 20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D 1 / 20</td>
<td>3 / 20</td>
<td>2 / 20</td>
<td>10 / 10</td>
</tr>
<tr>
<td></td>
<td>P 3 / 20</td>
<td>4 / 20</td>
<td>3 / 20</td>
<td>-</td>
</tr>
<tr>
<td>LAMBDA 150</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>LAMBDA 4</td>
<td>N 20 / 20</td>
<td>10 / 10</td>
<td>10 / 10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D -</td>
<td>-</td>
<td>-</td>
<td>10 / 10</td>
</tr>
<tr>
<td></td>
<td>P -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAMBDA 30</td>
<td>N 20 / 20</td>
<td>NT</td>
<td>NT</td>
<td>14 / 20</td>
</tr>
<tr>
<td></td>
<td>P -</td>
<td>-</td>
<td>-</td>
<td>4 / 20</td>
</tr>
<tr>
<td></td>
<td>D -</td>
<td>-</td>
<td>-</td>
<td>2 / 20</td>
</tr>
</tbody>
</table>

**Table 14.** A229A2 phage FISH results, 3731, normal controls and AS deletion controls.

N - Signal on both 15's (equal intensity), P - Signal on both 15's (unequal intensity), D - Signal on one 15 only and NT - Not tested.
3.3.5.2 Lambda 64

Lambda 64 is LS6-1 positive and detected signal of equal intensity in 16/20 (80%) metaphases analysed from 3731 (figure 27). Signal of differing intensity was detected in 3/20 (15%) metaphases and signal on one chromosome 15 homologue only was detected in 1/20 (5%) metaphases analysed. This compares to the two normal controls where signal of equal intensity was detected in 13/20 (65%) metaphases and 15/20 (75%) metaphases analysed. Signal of differing intensity between the two chromosome 15's was detected in 4/20 (20%) and 3/20 (15%) metaphases, while signal was detected on one chromosome 15 homologue only in 3/20 (15%) and 2/20 (10%) metaphases, respectively. Signal was detected on one chromosome 15 homologue only in 10/10 metaphases analysed from the deletion control (figure 27). Since Lambda 64 detected signal on both 15's of equal and unequal intensities and signals on one chromosome 15 homologue only in metaphases from both 3731 and the normal controls, it is likely that 3731 is not deleted for Lambda 64.

3.3.5.3 Lambda 4

Lambda 4 detected signal of equal intensity on both chromosome 15's in 20/20 (100%) metaphases analysed from 3731 (figure 28). For the two normal controls tested, signal of equal intensity was detected in 10/10 metaphases analysed, while signal was detected on one chromosome 15 homologue only in 10/10 metaphases analysed (figure 28).

In summary, 3731 is not deleted for Lambda 31, the LS6-1 positive phage clone Lambda 64 or Lambda 4.

3.3.5.4 Lambda 30

FISH analysis with Lambda 30 did not give reproducible results. Signal was detected in 20/20 metaphases analysed from 3731. However, analysis of the deleted control showed a positive hybridisation signal on one chromosome 15 homologue only in 2/20 (10%) metaphases analysed. In 14/20 (70%) metaphases, signal of equal intensity was detected on both chromosome 15 homologues (figure 29A) and in 4/20 (20%) metaphases, signal was detected on both chromosome 15 homologues but with reduced intensity on one homologue compared to the other.
Figure 26. Comparison of a metaphase spread from (A) 3731 and (B) an AS individual with a known deletion of 15q11-q13, probed with Lambda 31 and cosmid MR-60. Chromosome 15's marked with arrows.
Figure 27. Comparison of a metaphase spread from (A) 3731 and (B) an AS individual with a known deletion of 15q11-q13, probed with Lambda 64 and cosmid MR-60. Chromosome 15's marked with arrows.
Figure 28. Comparison of a metaphase spread from (A) 3731 and (B) an AS individual with a known deletion of 15q11-q13, probed with Lambda 4 and cosmid MR-60. Chromosome 15's marked with arrows.
To test the reproducibility and suitability of Lambda 30 as a FISH probe, five further AS patients with a known deletion of 15q were studied. The results are shown in table 15.

<table>
<thead>
<tr>
<th>DELETION CASE</th>
<th>SIGNAL ON ONE 15 ONLY</th>
<th>SIGNAL ON BOTH 15'S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/10</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10/10</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>14/20</td>
<td>6/20</td>
</tr>
<tr>
<td>4</td>
<td>18/20</td>
<td>2/20</td>
</tr>
<tr>
<td>5</td>
<td>10/10</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 15. Lambda 30 FISH results in AS patients with a known deletion of 15q11-q13.

In a further 2/5 known AS deletion cases, signal was detected on both chromosome 15 homologues in a proportion of the metaphases analysed, indicating that Lambda 30 is unsuitable as a probe for FISH analysis as it does not accurately reflect the genotype and conclusions can not be drawn. Figure 29B shows a metaphase with positive hybridisation signals on both chromosome 15 homologues from AS deletion case 3, probed with Lambda 30 and cosmid MR-60.

These results could be explained by (a) the presence of a related sequence that is represented elsewhere on 15q (b) sequences that are present in Lambda 30 may have been retained during the rearrangement which formed the deletion in these individuals or (c) the map position of Lambda 30 is incorrect.

3.4 Blind analysis of cosmid E42

As the phage clones did not show the same pattern of hybridisation that was seen with the cosmid clones and phage FISH analysis failed to confirm the presence of a deletion in 3731, the original 11HE12 cosmid FISH experiments were repeated. However, this time the experiment was carried out blind.
Figure 29. Metaphases from (A) the AS deletion control and (B) AS deletion case 3, probed with Lambda 30 and cosmid MR-60, showing the presence of signal on both chromosome 15 homologues.
To test for possible operator bias in the analysis of the FISH results an experiment was set-up to study the LS6-1 positive cosmid E42 in 3731 versus four normal controls. Slides were prepared for FISH analysis from 3731 and four normal controls. The normal controls were chosen so that the mitotic index and the chromosome length of the preparations were as similar as possible to those for 3731. Once the slides had been prepared, they were assigned letters by a volunteer and mixed up so that it was not possible to identify the slides. The results for cosmid E42 are shown in table 16.

<table>
<thead>
<tr>
<th>CASE</th>
<th>SIGNAL ON ONE 15 ONLY</th>
<th>SIGNAL ON BOTH 15'S (EQUAL INTENSITY)</th>
<th>SIGNAL ON BOTH 15'S (UNEQUAL INTENSITY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 / 20</td>
<td>14 / 20</td>
<td>5 / 20</td>
</tr>
<tr>
<td>B</td>
<td>2 / 20</td>
<td>13 / 20</td>
<td>5 / 20</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>18 / 20</td>
<td>2 / 20</td>
</tr>
<tr>
<td>D</td>
<td>1 / 20</td>
<td>17 / 20</td>
<td>2 / 20</td>
</tr>
<tr>
<td>E</td>
<td>1 / 20</td>
<td>14 / 20</td>
<td>5 / 20</td>
</tr>
</tbody>
</table>

Table 16. Results of cosmid E42 hybridised to metaphase spreads from 3731 (D), compared to normal controls (A, B, C and E).

E42 detected a similar pattern of metaphases with positive hybridisation signals of equal and unequal intensities on both chromosome 15's and metaphases with positive hybridisation signals on one chromosome 15 homologue only, in 3731 and the normal controls. 5-10% of the total number of metaphases analysed showed an apparent deletion of one chromosome 15 homologue in the majority of cases. 10%-25% of all metaphases analysed showed signal of different intensities on both chromosome 15's, while 65%-90% of the total number of metaphases analysed were showed with signals of equal intensity. It can be concluded that 3731 is not deleted for the LS6-1 positive cosmid E42 and as a degree of bias was also detected in the positive controls themselves, it is unlikely that there was operator bias in the analysis of metaphases showing signals of differing intensities from this individual.

3.5 Assessment of repeats in the 11HE12 and A229A2 phage
The anomalous results could be due to the presence of repetitive sequences in the 11HE12 cosmids and A229A2 phage which are also represented in variable copy number. To obtain a rough estimate of the number of repetitive sequences in the cosmids and phage, DNA was digested with Cfo I, blotted and probed with total human DNA (figure 30). The ethidium bromide stained agarose gel (figure 30) was photographed alongside a ruler, with the wells marking the baseline. The distance that each band had travelled through the agarose gel was then measured and compared to the bands that were present on the autoradiograph, following hybridisation with total human DNA. The bands on the autoradiograph represent DNA fragments that contain repetitive sequences, while those that are present on the ethidium bromide stained agarose gel and not on the autoradiograph, represent single copy fragments. The number of bands on the ethidium bromide stained agarose gel were assessed just beyond the 702 bp Lambda Bst E II fragment. As this was a rough estimate of the repeat sequences in the cosmids and phage and as the vector bands were not immediately apparent, all bands observed were included in the estimation of potential single copy fragments. The number of bands on the ethidium bromide stained agarose gel compared to the number of bands on the autoradiograph for each cosmid and phage is shown in table 17.

<table>
<thead>
<tr>
<th>Cosmid / Phage</th>
<th>Bands on ethidium bromide gel</th>
<th>Bands on autoradiograph</th>
<th>Potential single copy fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>E19</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>E24</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>E42</td>
<td>11</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>E16</td>
<td>12</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Lambda 31</td>
<td>9</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Lambda 64</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Lambda 150</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lambda 4</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Lambda 30</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 17. Estimation of single copy fragments in the 11HE12 cosmids and the A229A2 phage.
Figure 30. A) Cfo I digested cosmids and phage run on a 0.8% agarose gel stained with ethidium bromide.
B) Cfo I digested cosmids and phage probed with total human DNA (0.1XSSC / -70°C overnight).
The results show that in general the cosmids and phage contain few potential single copy sequences although it is unknown how this compares to cosmids and phage derived from other regions of the genome.

A more accurate estimate of the number of repetitive sequences would be obtained by digesting DNA from each cosmid and phage with a different frequent cutting restriction enzymes such as Taq I or Hinf I and probing the filters with (1) total human DNA (2) the whole cosmid or phage as probe and (3) the vector. Each band could then accurately be assigned as repetitive, single copy or vector. However, this does not give any information as to the possibility of a similar repetitive sequence elsewhere on chromosome 15q or the copy number.

3.6 Molecular analysis of 3731

The observation that the cosmid and phage probes contain mainly repetitive elements with little single copy sequence means that either (a) a deletion in 3731 is masked by these repetitive elements or (b) 3731 does not have a deletion of the LS6-1 (D15S113) locus.

3.6.1 Dosage analysis

Three single copy Pst I fragments were subcloned from the LS6-1 positive 11HE12 cosmid E42 (J. Buxton, pers. comm.). These were designated 42P1.1-5 which detects a Hind III polymorphism and had been shown to be heterozygous in 3731 and 7187 (J. Buxton, pers. comm.), P1.1-6 and P1.1-9. The position of these probes is shown in figure 25.

The single copy fragments P1.1-6 and P1.1-9 were used for dosage studies in 3731 and 7187. In both cases, a control probe from chromosome 17, 409R3A, was used to compare the signal intensities. The results obtained with the single copy fragment P1.1-6 are shown in table 18 and the results for P1.1-9 are shown in table 19.

3.6.1.1 P1.1-6 studies

P1.1-6 is a 1.6 kb Pst I fragment which contains an EcoR I site. DNA samples from normal individuals and AS individuals with known deletions were digested with EcoR I. Two bands were therefore visualised for P1.1-6 on the Southern blot (figure 31).
Figure 31. Southern blot used for dosage analysis probed with P1.1-6 and 409R3A (0.1XSSC / -70°C overnight).
Normal: lanes 1, 3-5, 8, 13, 14, 16 and 19. Deleted: lanes 9-12, 15, 18 and 20.
3731: lanes 6 and 17. 7187: lane 7.
<table>
<thead>
<tr>
<th>CASE</th>
<th>P1.1-6 UP (1)</th>
<th>P1.1-6 LP (2)</th>
<th>409R3A</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>472.3</td>
<td>1032</td>
<td>859.1</td>
<td>0.550</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>894.8</td>
<td>1862</td>
<td>1488</td>
<td>0.601</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>804.3</td>
<td>2242</td>
<td>1695</td>
<td>0.475</td>
<td>1.32</td>
</tr>
<tr>
<td>4</td>
<td>251.5</td>
<td>610.2</td>
<td>413.3</td>
<td>0.609</td>
<td>1.48</td>
</tr>
<tr>
<td>5</td>
<td>266.2</td>
<td>658.4</td>
<td>492.8</td>
<td>0.540</td>
<td>1.34</td>
</tr>
<tr>
<td>6</td>
<td>380.1</td>
<td>981.7</td>
<td>777.1</td>
<td>0.489</td>
<td>1.26</td>
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<td>DELETED</td>
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</tr>
<tr>
<td>1</td>
<td>292.3</td>
<td>853.2</td>
<td>971.4</td>
<td>0.301</td>
<td>0.878</td>
</tr>
<tr>
<td>2</td>
<td>259.5</td>
<td>483.5</td>
<td>686.6</td>
<td>0.378</td>
<td>0.704</td>
</tr>
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<td>3</td>
<td>158.2</td>
<td>375.2</td>
<td>505.7</td>
<td>0.313</td>
<td>0.742</td>
</tr>
<tr>
<td>4</td>
<td>344.1</td>
<td>975.9</td>
<td>1205</td>
<td>0.286</td>
<td>0.810</td>
</tr>
<tr>
<td>3731a</td>
<td>269.7</td>
<td>687.6</td>
<td>555.6</td>
<td>0.485</td>
<td>1.24</td>
</tr>
<tr>
<td>3731b</td>
<td>283.5</td>
<td>689.3</td>
<td>576.1</td>
<td>0.492</td>
<td>1.20</td>
</tr>
<tr>
<td>7187</td>
<td>192.9</td>
<td>481.9</td>
<td>361.1</td>
<td>0.534</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Table 18. Results of dosage analysis with P1.1-6 in 3731 and 7187 compared to normal individuals and known AS deletion cases.
**Ratio 1**

Ratio 1 is the intensity of the upper P1.1-6 band compared to the intensity of the 409R3A band.

95% of the values are expected to lie in the interval:

\[
\text{Mean} \pm 1.96 \times \text{Standard Deviation (sd)}
\]

**Normal Samples:** Range is between 0.475 and 0.609
- Mean = 0.544
- sd = 0.0533

If the data is normally distributed 95% of the values for the normal samples lie in the interval:

\[
0.544 \pm 1.96 \times 0.0533 = 0.440 \text{ and } 0.648
\]

**Deleted Samples:** Range is between 0.286 and 0.378
- Mean = 0.320
- sd = 0.0405

95% of the values for the deleted samples lie in the interval:

\[
0.320 \pm 1.96 \times 0.0405 = 0.241 \text{ and } 0.399
\]

The upper ratio results indicate that both 3731 (0.485 and 0.492) and 7187 (0.534) fall into the normal limits.

**Ratio 2**

Ratio 2 is the intensity of the lower P1.1-6 band compared to the intensity of the 409R3A band.

**Normal Samples:** Range is between 1.2 and 1.48
- Mean = 1.31
- sd = 0.0981
95% of the normal samples lie in the interval:

$$1.31 +/- 1.96 \times 0.0981 = 1.50 \text{ and } 1.12$$

Deleted Samples: Range is between 0.704 and 0.878

Mean = 0.784
sd = 0.0768

95% of the deleted samples lie in the interval:

$$0.784 +/- 1.96 \times 0.0768 = 0.935 \text{ and } 0.633$$

The results for the lower ratio indicate that 3731 (1.24 and 1.20) and 7187 (1.33) fall into the normal range. It can therefore be concluded that the single copy Pst I fragment P1.1-6 is not deleted in 3731 or 7187.

3.6.1.2 P1.1-9 studies

DNA samples from normal individuals and AS individuals with known deletions were digested with EcoR I. Figure 32 shows a Southern blot used for dosage studies probed with 1.1-9 and 409R3A.

**Figure 32.** Southern blot used for dosage analysis probed with P1.1-9 and 409R3A (0.1XSSC, overnight exposure -70°C overnight).

<table>
<thead>
<tr>
<th>CASE</th>
<th>P1.1-9</th>
<th>409R3A</th>
<th>RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td>1 1350</td>
<td>1129</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>2 3001</td>
<td>2265</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>3 2981</td>
<td>2530</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>4 848.2</td>
<td>565.9</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>5 823.5</td>
<td>589.6</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>6 1379</td>
<td>1124</td>
<td>1.23</td>
</tr>
<tr>
<td>DELETED</td>
<td>1 766.5</td>
<td>1189</td>
<td>0.645</td>
</tr>
<tr>
<td></td>
<td>2 433.5</td>
<td>792.6</td>
<td>0.547</td>
</tr>
<tr>
<td></td>
<td>3 429.7</td>
<td>469.1</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>4 1335</td>
<td>1681</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>5 154.2</td>
<td>275.5</td>
<td>0.560</td>
</tr>
<tr>
<td>3731a</td>
<td>1021</td>
<td>855.4</td>
<td>1.19</td>
</tr>
<tr>
<td>3731b</td>
<td>903.4</td>
<td>850.3</td>
<td>1.06</td>
</tr>
<tr>
<td>7187</td>
<td>854.6</td>
<td>581.9</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Table 19. Results of dosage analysis with P1.1-9 in 3731 and 7187, compared to normal individuals and known AS deletion cases.
Normal samples: Range is between 1.18 and 1.50
    Mean = 1.31
    sd = 0.126

95% of the normal samples lie in the interval:
    1.31 +/- 1.96 x 0.126
    = 1.06 and 1.57.

Deleted samples: Range is between 0.547 and 0.916
    Mean = 0.692
    sd = 0.159

95% of the deleted samples lie in the interval:
    0.692 +/- 1.96 x 0.159
    = 0.380 and 1.00.

The results indicate that the ratios for 3731 (1.19 and 1.06) and 7187 (1.47) fall into the normal range, thus P1.1-9 is not deleted in these individuals.
3.6.2 Microsatellite analysis

The dosage studies indicate that 3731 does not have a deletion of the LS6-1 (D15S113) locus. The original LS6-1#1 CA(n) repeat result could be explained by non-amplification of the maternal allele or the cell line metaphases and the peripheral blood metaphases were not derived from the same individual.

3.6.2.1 Confirmation that the samples were derived from 3731

To confirm that the samples were derived from 3731, microsatellite markers from the AS/PWS region were tested to confirm that the DNA prepared from the original blood sample (A) and DNA from the blood sample from which the lymphoblastoid cell line was prepared (B), were identical and that these results were the same as those previously reported (Buxton et al., 1994). The results indicate that the samples used throughout this project were the same (figure 33).

3.6.2.2 Analysis of the LS6-1 (D15S113) locus

The LS6-1 (D15S113) locus was analysed in 3731 and her mother (figure 34) using an alternative primer pair LS6-1#2 (200bp) (section 2.1.3), in which the sequence of the reverse primer differed. The results indicate that 3731 is heterozygous for LS6-1#2 (200bp) and has an allele in common with her mother confirming that the original LS6-1#1 (130-140bp) CA(n) repeat result was due to non-amplification of the maternal allele.

3.6.2.3 Mapping of TK4 and 196

Two further microsatellite markers from the LS6-1 (D15S113) region, TK4 (D.Ledbetter, pers. comm.) and 196 (Trent et al., 1995), a tetranucleotide repeat isolated from the A229A2 YAC and shown to be present in phage clone 92 were mapped.

TK4 was mapped to ascertain its position in relation to the A229A2 and 11HE12 YACs, the 11HE12 cosmids and the A229A2 phage (figure 35), whereas 196 was mapped to ascertain its position in relation to the 11HE12 YAC and corresponding cosmids (figure 36).
Figure 33. Analysis of microsatellite markers IR4-3R, TD3-21, LS6-1 # 1, GABRB3 and MS14 in 3731 blood sample DNA (A), 3731 cell line DNA (B) and 7187 (C).
Figure 34. Comparison of (A) the original LS6-1#1 result with (B) LS6-1#2, in 3731 (a-blood sample DNA, b-cell line DNA) and 7187 (c).
Figure 35. Mapping of TK4 to the YACs, cosmids and phage from the LS6-1 (D15S113) region. (A) 100bp ladder (B) 11HE12 (C) A229A2 (D) E19 (E) E24 (F) E42 (G) E16 (H) Lambda 31 (I) Lambda 30 (J) Lambda 64 (K) Lambda 150 (L) Lambda 4 (M) Genomic DNA and (N) Blank.

Figure 36. Mapping of 196 to YAC 11HE12 and its corresponding cosmids. (A) 100bp ladder (B) A229A2 (C) 11HE12 (D) E19 (E) E24 (F) E42 (G) E16 (H) Genomic DNA (I) Blank.
The results show that TK4 (figure 25) maps to the LS6-1 positive YACs 11HE12 and A229A2, cosmid E42 and Lambda 64, both of which are LS6-1 positive cosmid and phage clones. 196 (figure 25) maps to YAC A229A2 but not to YAC 11HE12 and the corresponding 11HE12 cosmids. This places 196 close but distal of the D15S113 locus.

The 196 tetranucleotide repeat was used to analyse DNA from 3731 and her mother, 7187. 3731 is heterozygous for the 196 tetranucleotide repeat and shares an allele in common with her mother (figure 37). 196 is clearly not deleted in 3731 and thus, 196 provides an alternative to the markers at LS6-1 (D15S113) for the analysis of AS individuals and their families.

![Figure 37. Microsatellite analysis with 196, (A-E) Normal controls, (F) 7187 and (G) 3731.](image)

In summary, the results show that 3731 does not have a deletion of the D15S113 locus.

### 3.7 Characterisation of AS individual 13581

The mother of AS individual 13581 presented for counselling when 7 weeks pregnant. The deletion in 13581 had been shown by molecular probes to be atypical (section 3.1) and it was necessary to determine whether the deletion in 13581 was de novo or if it was the outcome of a balanced translocation in the mother.
3.7.1 FISH analysis

**LS6-1**
The LS6-1 (D15S113) locus was studied in 13581 and 13580 with cosmid E24. Signal was produced on one chromosome 15 homologue only in 13581 in 10/10 metaphases analysed (figure 38), whereas signal was produced on both chromosome 15’s in 10/10 metaphases analysed from 13580 (figure 38). This result shows that the deletion in 13581 is de novo and was not the outcome of a balanced translocation in the mother.

**TD3-21**
An Oncor cosmid probe for the TD3-21 (D15S10) locus, which also contains a chromosome 15q cosmid marker (PML) that lies outside of the AS/PWS region, was analysed. This was used according to the manufacturers instructions. The TD3-21 cosmid produced signal on both chromosome 15 homologues (figure 39) in 10/10 metaphases analysed from the mother (13580). This result shows that the mother (13580) is intact at the TD3-21 (D15S10) locus and that there has not been a chromosomal rearrangement of this locus.

132D4 is a 600 kb TD3-21 positive YAC (figure 6). Labelled YAC 132D4 DNA was hybridised to metaphases from 13581 and an AS deletion control who was deleted for all loci from D15S11-D15S24. YAC 132D4 produced signal on one chromosome 15 homologue only in 10/10 metaphases analysed from both 13581 and the AS deletion control (figure 40). This result indicates that 13581 is also deleted for the TD3-21 (D15S10) locus.

3.7.2 Microsatellite analysis

The microsatellite markers D15S128, D15S210 and 196 were analysed in DNA samples from 13580 and 13581 (figure 41). The father was unavailable. 13580 is heterozygous for the D15S128 microsatellite marker, whereas her daughter (13581) could either be homozygous or hemizygous (figure 41). However, as they both share the same allele this marker is uninformative. The D15S210 result (figure 41) shows that the allele inherited by 13581 is not maternal in origin and she is therefore deleted for D15S210. The 196 result (figure 41) shows that 13580 is heterozygous for 196. The allele inherited by 13581 is not maternal in origin and therefore 13581 is deleted for 196.
Figure 38. Metaphase spreads from (A) 13581 and (B) 13580 probed with cosmids E24 and MR-60. Chromosome 15's marked with arrows.
Figure 39. Oncor cosmid TD3-21 and internal 15q control (PML), hybridised to a metaphase spread from 13580. Chromosome 15's marked with arrows.
Figure 40. Comparison of metaphases probed with YAC 132D4 and cosmid MR-60 from (A) 13581 and (B) an AS individual with a known deletion of 15q11-q13. Chromosome 15’s marked with arrows.
In summary, the results of the microsatellite analysis indicate that 13581 is deleted for the D15S210 and D15S128 loci.

3.7.3 SNRPN exon alpha methylation studies

Exon alpha of SNRPN was studied in 13581 to ascertain her methylation status at this locus. A Southern blot was performed with XbaI/NotI double digestion of genomic DNA from 13581, her mother 13580, a normal individual, a PWS deletion case, an AS deletion case, an AS deletion and a PWS deletion case (figure 42).

The result shows that 13581 does not have the 4.2 kb XbaI paternal band. Paternal DNA methylation imprints (2.0 kb and 0.9 kb bands only) were present in 13581, consistent with the absence of methylation of the NotI sites on the paternal chromosome. Her mother and the normal control exhibit both methylated and unmethylated alleles, consistent with the presence of normal maternal and paternal imprints. The AS deletion case and AS deletion case with paternal DNA methylation present in 13581, the 0.9 kb maternal bands present in the PWS deletion case, and the 2.0 kb maternal band have methylated paternal bands, which have been identified in an imprinting association with paternal SNRPN 13581. This result confirms the clinical diagnosis, and the deletion includes SNRPN (excision) as well as the paternal SNRPN deletion.

Figure 41. Analysis of microsatellite markers from the AS/PWS region within 15q11-q13 (A) D15S128, (B) D15S210 and (C) 196 in 13581 and 13580.

FISH analysis with L48.8 produced signal on both chromosome 15 homologues in 18/20 metaphases (90%) analysed from 13581 (figure 44). In 4/20 (20%) metaphases, signal was produced on one chromosome 15 homologue only. Positive hybridisation signals were observed on both chromosome 15 homologues in 18/20 metaphases (90%) analysed from the normal control, while in 2/20 (10%) metaphases, positive hybridisation signals
In summary the results of the microsatellite analysis indicate that 13581 is deleted for the D15S210 and D15S113 loci.

### 3.7.3 SNRPN exon alpha methylation studies

Exon alpha of SNRPN was studied in 13581 to ascertain her methylation status at this locus. A Southern blot was performed with Xba I / Not I double digestion of genomic DNA from 13581, her mother 13580, a normal individual, a PWS disomy case, an AS disomy case, an AS deletion and a PWS deletion case (figure 42).

The result shows that 13581 does not have the 4.2 kb Xba I maternal band. Paternal DNA methylation imprints (3.0 kb and 0.9 kb bands only) were present in 13581, consistent with the absence of methylation of the three Not I sites on the paternal chromosome. Her mother and the normal control exhibit both methylated and unmethylated alleles consistent with the presence of normal maternal and paternal chromosomes. The AS disomy patient and AS deletion case have paternal DNA methylation imprints (3.0 kb and 0.9 kb paternal bands only) and the PWS disomy patient and PWS deletion case have maternal methylation imprints (4.2 kb maternal band only).

This result could be explained either by (a) the deletion in 13581 includes SNRPN (exon alpha) or (b) the proximal deletion breakpoint in 13581 lies close to this exon and this deletion exerts a positional effect on exon alpha.

### 3.7.4 FISH analysis with phage from the SNRPN and PW71 loci

A 160 kb phage contig was established which encompassed the PW71 and SNRPN loci (Buiting et al., 1995). Two phage clones from this contig, L48.8 and L48.25 (figure 43), were analysed by FISH to localise the proximal breakpoint in 13581. The results are shown in table 20.

**L48.8**

FISH analysis with L48.8 produced signal on both chromosome 15 homologues in 16/20 metaphases (80%) analysed from 13581 (figure 44). In 4/20 (20%) metaphases, signal was produced on one chromosome 15 homologue only. Positive hybridisation signals were observed on both chromosome 15 homologues in 18/20 metaphases (90%) analysed from the normal control, while in 2/20 (10%) metaphases, positive hybridisation signals
Figure 42. Detection of differential methylation at the SNRPN CpG island in AS individual 13581(A), 13580 (B), normal individual (C), PWS patient with maternal UPD (D), AS patient with paternal UPD (E) known AS individual (F) and known PWS individual (G).

0.1XSSC, overnight exposure -70°C.
Figure 43. A map of the 15q11-q13 region between PW71 (D15S63) and SNRPN (Buiting et al., 1995) indicating the positions of phage clones Lambda 48.8 and Lambda 48.25.

were observed on one chromosome 15 homologue only. L48.8 detected signal on one chromosome 15 homologue only in 19/20 (95%) metaphases analysed from the AS deletion control (figure 44) and in 1/20 (5%) metaphases signal was detected on both chromosome 15 homologues. As L48.8 contained repetitive sequences that were not sufficiently suppressed, a second FISH experiment was performed in which the amount of Cot-1 DNA was increased to 30μg (L48.8a). However, the results of the second experiment were not significantly different.

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<td>18 / 20</td>
</tr>
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<tr>
<td></td>
<td></td>
<td>NORMAL FAILED</td>
</tr>
</tbody>
</table>

Table 20. L48.8 and L48.25 FISH results in 13581, an AS deletion control deleted for loci encompassing D15S11-D15S24 and a normal control.
Figure 44. Comparison of metaphases probed with L48.8b and MR-60 from (A) 13581 and (B) an AS individual with a known deletion of 15q11-q13. Chromosome 15’s marked with arrows.
L48.25 contains exon alpha of SNRPN and was used as a probe for FISH analysis to determine whether exon alpha is deleted in 13581. The results (table 19) show that L48.25 produced signal on both chromosome 15 homologues in 10/20 (50%) metaphases analysed from 13581 (figure 45). In 10/20 (50%) metaphases, signal was produced on one chromosome 15 homologue only (figure 45). In comparison, signal was produced on one chromosome 15 homologue only in 10/10 metaphases analysed from the AS deletion control. The normal control failed.

The possible interpretations of these results are (a) L48.8 and L48.25 are not deleted in 13581 and the metaphases with signal on one chromosome 15 homologue only, could be due to poor hybridisation of the two probes or (b) the proximal deletion breakpoint in 13581 could fall within either L48.8 or L48.25. The proximal deletion breakpoint can not fall within both L48.8 and L48.25. The L48.8 results showed a similar number of metaphases with signal on one chromosome 15 homologue only, in both 13581 and the normal control and this probably represents poor hybridisation of the probe, indicating that L48.8 is unlikely to be deleted in 13581. The normal control for L48.25 failed and therefore, it is not known whether the L48.25 result is due to poor hybridisation of the probe or whether the proximal breakpoint falls within this phage clone.

3.7.5 L48.25 whole phage hybridisation

To study whether the proximal deletion breakpoint in 13581 falls within L48.25 which contains SNRPN (exon alpha), the whole L48.25 phage was hybridised to Taq I and EcoR V digested DNA from 13581 and her mother to identify a junction fragment. Unfortunately, the probe was sufficiently repetitive (K. Buiting, pers. comm.) that no clear differences in the bands between 13581 and 13580 were observed (figure 46).

In conclusion, AS individual 13581 has a de novo deletion of her maternal chromosome 15 encompassing the D15S10/D15S210 and D15S113 loci and a paternal DNA methylation imprint only at SNRPN (exon alpha). The distal breakpoint lies between the tetranucleotide repeat 196 and GABR83 and the proximal breakpoint lies proximal to YAC132D4 which is completely deleted, within the resolution of FISH.
Figure 45. Comparison of metaphases from 13581 probed with L48.25 and MR-60 showing (A) a deletion of one of the chromosome 15's with (B) the presence of signal on both chromosome 15's.
3.8 AS (4;15) translocation family

A family was reported (Reis et al., 1999) in which the mother of an AS individual carried a balanced reciprocal translocation 46, XX, t(4;15); (q36.2;q13). An ideogram of the translocation is shown in figure 46. The son of this individual inherited an unbalanced version of this translocation and has AS, although some of the characteristic EEG findings were absent. The AS individual has inherited the derivative 4 but not the derivative 15 and is therefore monosomic for this region. The karyotype of this individual is 46, XY, -4, -15, +der(4)(14;15) (q36.2;q13).

3.8.1 FISH Analysis of the Translocation

Further studies were done with probes 425, 231 and E10 from the LSE-1 region to ascertain the translocation more accurately. Slides from the metaphases were kindly provided by Dr. André Reis. Unfortunately, the slides were too dark to be analysable, the results of the analysis of the metaphases which were not analysable, the results of the hybridisation at the long arm of the der(4) are presented in figure 47. The hybridisation of Lambda 48.25 to genomic DNA from 13581 (I) and 13580 (II) digested with Taq I (A) and EcoR V (B). 0.1XSSC, overnight exposure -70°C.

Figure 46. Hybridisation of Lambda 48.25 to genomic DNA from 13581 (I) and 13580 (II) digested with Taq I (A) and EcoR V (B). 0.1XSSC, overnight exposure -70°C.

Figure 47. Cosmid E10 hybridised to the long arm of the der(4).
3.8 AS t(4;15) translocation family

A family was reported (Reis et al., 1993) in which the mother of an AS individual carried a balanced reciprocal translocation 46, XX, t (4;15) (q35.2;q13). An ideogram of the translocation is shown in figure 48. The son of this individual inherited an unbalanced version of this translocation and has AS, although some of the characteristic EEG findings were absent. This AS individual has inherited the derivative 4 but not the derivative 15 and is therefore monosomic for this region. The karyotype of this individual is 45, XY, -4, -15, +der(4) t (4;15) (q35.2;q13).

3.8.1 FISH Analysis of the Translocation

Further studies were undertaken with cosmids 420, 231 and E19 from the LS6-1 region to ascertain the translocation breakpoint more accurately. Slides from the mother were kindly provided by Dr. André Reis. Unfortunately, the slide quality was poor and it was only possible to analyse 2-3 metaphases per slide. However, in the metaphases which were analysable, the results showed that all three cosmids were present on the long arm of the derived 4. Figure 47 shows the hybridisation of cosmid E19 to the long arm of the derived 4.

Figure 47. Cosmid E19 hybridised to the long arm of the derived 4.
Figure 48. Ideogram of the balanced reciprocal translocation 46, XX, t (4; 15) (q35.2; q13).
The translocation breakpoint therefore appears to lie proximal to cosmid E19 between D15S10 and E19. The son is therefore monosomic for 15pter to the region proximal of E19, the maternal chromosome being deleted.

3.9 Maternal Deletion of 15q without AS

A mother and son were reported with a deletion of 15q that includes the D15S113 (LS6-1) locus but neither individual had the characteristics of AS (Michaelis et al., 1995).

3.9.1 FISH analysis

FISH analysis was carried out with four YAC clones, 132D4, 230H12, 142A2 and 11HE12 (figure 6) which had previously been shown to be non chimeric (Mutirangura et al., 1993b). The FISH results are shown in table 21.

<table>
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</tr>
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<td>-</td>
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<tr>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 21. Results of FISH analysis with YAC clones from the AS/PWS region in BS and DH.

D = Deleted, ND = Non-deleted and P = Partial deletion.

The results show that the LS6-1 positive YAC 11HE12 and YAC A142A2 produced signal on one chromosome 15 homologue only in 10/10 metaphases analysed from BS and DH (figures 49 and 50). This confirms that BS and DH are deleted for the LS6-1 (D15S113) locus.

YAC 132D4 which contains the D15S10 locus, produced signal on both chromosome 15 homologues in 10/10 metaphases analysed from BS and DH (figure 51). The D15S10 locus is therefore not deleted in BS and DH.
Figure 49. A metaphase from BS probed with YAC 11HE12 and cosmid MR-60. Chromosome 15's marked with arrows.
Figure 50. A metaphase from BS probed with YAC 142A2 and cosmid MR-60. Chromosome 15's marked with arrows.

Figure 51. A metaphase from BS probed with YAC 132D4 and cosmid MR-60. Chromosome 15's marked with arrows.
YAC 230H12 which overlaps with YAC 132D4 and YAC 142A2 (figure 6), produced positive hybridisation signals on both chromosome 15’s, with the signal intensity consistently much greater on one chromosome 15 homologue compared to the other. This was observed in 15/20 (75%) metaphases analysed from BS (figure 52A) and in 13/20 (65%) metaphases analysed from DH. Signals were produced on one chromosome 15 homologue only in 5/20 (25%) metaphases analysed from BS (figure 52B) and in 7/20 (35%) metaphases analysed from DH. These results indicate that the proximal deletion breakpoint in BS and DH probably lies within YAC 230H12, distal of the D15S10 locus.

3.10 Analysis of Markers and Probes in AS Individuals with no known molecular defect

As 3731 was shown not to be deleted for the D15S113 locus and the deletion in 13581 encompassed both the D15S10/D15S210 and D15S113 loci, further AS individuals in whom no molecular defect had been identified were studied with the microsatellite markers, D15S210 and 196.

3.10.1 Microsatellite Analysis

Previous studies of some AS individuals had been incomplete and as further probes became available, these individuals were studied further. AS individuals EA and KC were heterozygous for the microsatellite markers IR4-3R (D15S11), GABRB3 and MS14 (D15S97) and AS individual HR was heterozygous for IR4-3R (D15S11) only. All other markers tested were uninformative (J. Chan, pers. comm.). The results show that EA, KC and HR are heterozygous for D15S210 (figure 53) and 196 (figure 54), inheriting both a maternal and paternal allele. These individuals are therefore not deleted for either of these two markers.
Figure 52. Comparison of metaphase spreads from BS probed with YAC 230H12 and cosmid MR-60, showing the presence of (A) signals of differing intensities on both 15’s and (B) a deletion of one of the chromosome 15 homologues.
Figure 53. Analysis of D15S210 in (A) KC (B) EA and (C) HR

Figure 54. Analysis of 196 in (A) KC (B) EA and (C) HR
4.0 Fine mapping of the methylation control locus
4.1 Introduction

A sub-set of AS individuals exists in which there is biparental inheritance of markers from 15q11-q13 and no detectable deletion. This sub-set of individuals has been divided into those who have normal DNA methylation imprints and those who have aberrant DNA methylation imprints (section 1.2.6). The experiments in this section were designed to look for deletions or recombination events to separate the two loci.

4.2 Family R

Family R were referred for genetic counselling when the mother of two siblings with AS was 17 weeks pregnant. Microsatellite analysis in the two affected siblings indicated biparental inheritance of 15q11-q13 markers. Parental DNA methylation imprints (6.6 kb maternal and 4.7 kb paternal bands) were normal, whereas only paternal PW71B Hind III / Hpa II fragments (figures 4 and 55), SNRPN (intron 5) Cfo I / Bgl II fragments and SNRPN (exon alpha) Xba I / Not I fragments were present in both siblings (J. Buxton, pers. comm.). The presence of a paternal DNA methylation imprint on both chromosome 15's of these individuals is suggestive of an imprinter mutation. DNA prepared from a 21 week fetal blood sample showed normal DNA methylation imprints at PW71B (D15S63) (figures 4 and 55). Re-probing the same Hind III / Hpa II filter with 42P1.1-5 (section 3.6.1), showed that the 21 week fetus and both AS siblings shared a 42P1.1-5 allele (J. Buxton, pers. comm.).

![Figure 55. Pedigree of family R showing the results of the PW71B analysis.](image)

The results obtained for family R are apparently contradictory. The PW71B methylation result would predict that the fetus is normal, whereas the 42P1.1-5 result would predict the fetus to be affected. The possibilities are that
42P1.1-5 does not truly reflect the AS critical region or that the structural gene is close to 42P1.1-5, but the locus regulating imprinting is elsewhere. A haplotype was therefore constructed to test these possibilities.

4.2.1 Haplotype analysis

Microsatellite markers were chosen from either side of the 42P1.1-5 marker. The location of these markers in the 15q11-q13 region is shown in figure 7 and the results of the analysis with the 15q11-q13 microsatellite markers, including the 42P1.1-5 result (J. Buxton, pers. comm.) are shown in table 22. The haplotypes inferred by these markers are demonstrated in figure 56.

<table>
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Table 22. Segregation analysis of microsatellite markers from 15q11-q13 in family R.

The mother of family R was heterozygous for the markers at D15S18-1 (IR39-1), D15S128 (AFM273yf9) (figures 57 and 58) and D15S113 (42P1.1-5). She was homozygous and therefore uninformative for the markers at D15S11 (IR4-3R), D15S210 (AFM320vd9) and D15S97 (MS14) (figures 57 and 58). Two probes were fully informative, 42P1.1-5 (near D15S113) and D15S128 (near SNRPN). At D15S128, the fetus has inherited the same maternal allele as the two affected siblings, while at 42P1.1-5, the fetus has inherited the same maternal allele as at least one of the affected siblings. A minimum cross-over haplotype can be drawn, consistent with a whole haplotype. This indicates that the fetus most likely has the whole haplotype in common with the two affected siblings.
Figure 56. Segregation analysis of chromosome 15 markers from the AS/PWS region on 15q11-q13.
Figure 57. Analysis of 15q11-q13 microsatellite markers in family R, D15S18-1, D15S11 and D15S128.
The possible interpretation of these results are either that there has been (a) a double recombination between D15S128 and D15S191; (b) the methylation locus lies proximal of D15S16-1; (c) the methylation control locus lies distal of D15S113; (d) a methylation control locus that is not on chromosome 15q11-q13 (e) genomic mosaicism (f) non-paternity or (g) the fetal sample had been switched for sibling 2 when the PCR DNA dilutions were prepared.

4.2.1 Exclusion of a sample switch in family R

To check that the DNA samples from the fetus and sibling 2 had not been switched, an additional microsatellite marker from another chromosome was tested. The marker D7S183 from chromosome 7, which has 12 alleles and a heterozygosity of 0.63 was chosen. The result showed that sibling 2 and the fetus were both heterozygous, having one allele in common of their other allele DNA. (Figure 58, table 23). The fetal DNA sample which had not therefore have been switched with the DNA sample of sibling 2. The result also showed that sibling 2 had apparently inherited both 15q11-q13 markers from their maternal chromosome 15. The absence of paternal alleles at the markers had been an essential test, also suggesting the disomy or trisomy of chromosome 15. A deletion or expansion of one of the maternal alleles.

To ensure that DNA samples were not mixed up, fresh DNA dilutions were made from both fetal and maternal DNA and these were compared against dilutions of DNA samples, which had been prepared from the blood samples. The results were done (Figure 58). The results showed that the samples had not been mixed up and that the call line DNA and these were consistent with the DNA samples, which had been prepared from the blood samples. The call lines were done for sibling 2 with D15S97 and D15S210. This result implies that sibling 2 had its maternal chromosome 15q11-q13. The result implies that sibling 2 had be a new mutation of one of the maternal alleles.

4.2.2 UPD analysis of chromosome 7, sibling 2

Further microsatellite markers from chromosome 7 were tested to ascertain whether sibling 2 had inherited both paternal chromosome 7's (UPD 7 pat) or whether there had been a new mutation of one of the maternal alleles at meiosis. The position of these markers on chromosome 7 is shown in figure 60 and the results are shown in table 23.

Figure 58. Analysis of 15q11-q13 microsatellite markers D15S210 and D15S97 in family R.
The possible interpretation of these results are either that there has been (a) a double recombination between D15S128 and D15S18-1 (b) the methylation locus lies proximal of D15S18-1 (c) the methylation control locus lies distal of D15S113 (d) a methylation control locus that is not on chromosome 15q11-q13 (e) germline mosaicism (f) non-paternity or (g) the fetal sample had been switched for sibling 2 when the PCR DNA dilutions were prepared.

4.2.1 Exclusion of a sample switch in family R

To check that the DNA samples from the fetus and sibling 2 had not been switched, an additional microsatellite marker from another chromosome was tested. The marker D7S513 from chromosome 7, which has 13 alleles and a heterozygosity of 0.82 was chosen.

The result showed that sibling 2 and the fetus are both heterozygous, having one allele in common and their other allele different (figure 59, table 23). The fetal DNA sample could not therefore have been switched with the DNA sample from sibling 2. The result also showed that sibling 2 had apparently inherited the same alleles as her father with no apparent inheritance of a maternal allele (figure 60). This indicates that either (a) the samples had been mixed up (b) that sibling 2 also has paternal uniparental disomy of chromosome 7 (UPD 7 pat) or (c) there has been a new mutation of one of the maternal alleles at meiosis.

To confirm that the DNA samples used for PCR had not been mixed up, fresh DNA dilutions were prepared from recently prepared cell line DNA and these were compared to the original DNA samples, which had been prepared from the blood from which the cell lines were derived (figure 59). The results confirm that the samples had not been mixed up and that the cell line DNA was identical to the DNA prepared from the original blood samples. This result implies that either (a) sibling 2 has UPD 7 pat or (b) there has been a new mutation of one of the maternal alleles.

4.2.3 UPD analysis of chromosome 7, sibling 2

Further microsatellite markers from chromosome 7 were tested to ascertain whether sibling 2 had inherited both paternal chromosome 7's (UPD 7 pat) or whether there had been a new mutation of one of the maternal alleles at meiosis. The position of these markers on chromosome 7 is shown in figure 60 and the results are shown in table 23.
**Figure 59.** Analysis of the marker D7S513 in family R.

(A) Old DNA dilutions (B) New DNA dilutions.
Figure 60. Long range map of chromosome 7 microsatellite markers.
Table 23. Segregation analysis of chromosome 7 markers in family R.

Sibling 2 was informative for the CFTR dinucleotide repeat marker in intron 17b of the CFTR gene on chromosome 7q (Zielenski et al., 1991) and for the marker at D7S488 (figure 61). Sibling 2 was uninformative for the markers at D7S493, D7S517 and D7S507 (figures 61 and 62). The results indicate biparental inheritance of maternal and paternal alleles at D7S488 and for the TA repeat in intron 17b of the CFTR gene. The results also indicate that there has probably been a cross-over between D7S488 and D7S493 on the maternal chromosome in sibling 2. Thus, disomy of chromosome 7 was ruled out distal to D7S488 but without testing further markers, partial disomy of proximal 7p cannot be ruled out.
Figure 61. Analysis of microsatellite markers from chromosome 7 in family R, CFTR(17b), D7S493 and D7S488 showing biparental inheritance of D7S488 in sibling 2.
4.3 Family W

The two affected AS siblings in family W were shown to have paternal DNA methylation imprints at PWW718 (D15S93). The mother of these children was shown to have the 6.6 kb maternal band only at PWW718 (D15S93) and the same abnormal pattern was seen in her father and his identical twin brother. This pattern is normally seen in individuals with PWS; however, these individuals are phenotypically normal. This mother's half-brother and the normal siblings of the two affected children had normal DNA methylation imprints (J. Buxton, pers. comm.) (figure 63).

Figure 62. Analysis of chromosome 7 microsatellite markers in family R, D7S507 and D7S517.

Figure 63. Pedigree of Family W showing the results of the DNA analysis.
4.3 Family W

The two affected AS siblings in family W were shown to have paternal DNA methylation imprints at PW71B (D15S63). The mother of these children was shown to have the 6.6 kb maternal band only at PW71B (D15S63) and the same abnormal pattern was seen in her father and his identical twin brother. This pattern is normally seen in individuals with PWS, however, these individuals are phenotypically normal. The mothers half-brother and the normal siblings of the two affected children had normal DNA methylation imprints (J. Buxton, pers. comm.) (figure 63).

![Family W Pedigree](image)

**Figure 63.** Pedigree of family W showing the results of the PW71B analysis.

Family W were studied further to observe whether any recombinants in this family could further define the AS methylation locus.

4.3.1 Microsatellite analysis

DNA samples from nine individuals in family W were studied to ascertain whether the disease locus in this family co-segregates with the microsatellite markers from the AS/PWS region on chromosome 15q11-q13. The father of the affected children and the mother of (II2)'s half-brother were unavailable for study. The results are shown in table 24 and the haplotypes suggested by these markers in family W are depicted in figure 64.
Table 24. Segregation analysis of chromosome 15q11-q13 markers in family W.
Figure 64. Segregation analysis using markers from the AS/PWS region on chromosome 15q11-q13.
Family W were uninformative for the markers at D15S11 (figure 65) and D15S97 (figure 67). D15S128 was partially informative. The grandfather and mother of the four siblings were informative. The mother (II2) inherited allele 3 from her father and allele 2 from her mother. The two affected siblings (III1 and III2) could have inherited either the grandmaternal allele 2 or the grandpaternal allele 3 from their mother and are therefore uninformative for this marker, whereas the two normal siblings (III3 and III4) could only have inherited the grandmaternal allele 2 (figure 65). While the two affected siblings are uninformative at D15S128, it is consistent that they have also inherited the grandpaternal allele 3 at this locus, whereas the two unaffected siblings have clearly inherited the grandmaternal allele 2. D15S210 and 196 were fully informative. The two affected siblings have inherited different maternal alleles at these loci compared to their normal siblings and these alleles are grandpaternal in origin (figure 66). A minimum cross-over haplotype can be drawn, consistent with a whole haplotype. This shows that no recombinants were found between any of the tested markers from 15q11-q13 and the AS locus in this family.

An approximate LOD score for a phase known family can be ascertained using the following equation (Morton 1955, 1956):

\[ Z (\theta) = \log_{10} 2^a (1-\theta)^b \theta^c \]

\[ = \log_{10} 2^5 (1-0)^5 0^0 \]

\[ = 1.5 \]

where 
- \( a \) = total number of offspring scored
- \( b \) = number of non-recombinant offspring
- \( c \) = number of recombinant offspring.

This result suggests linkage between chromosome 15 markers and the disease locus in this family. As no recombinants were observed, it does not narrow the region down or separate the methylation control locus from the structural gene locus.
Figure 65. Analysis of 15q11-q13 microsatellite markers D15S11 and D15S128 in family W.
Figure 66. Analysis of 15q11-q13 microsatellite markers D15S210 and the tetranucleotide repeat 196 in family W.
Figure 67. Analysis of the 15q11-q13 microsatellite marker D15S97 in family W.

4.4 Individual 6468

AS individual 6468 was an isolated AS case, shown to have biparental inheritance of 15q11-q13 markers (S. Cottrell, pers. comm.) and a paternal DNA methylation imprint at PW71B (D15S63) (J. Buxton, pers. comm.).

4.4.1 FISH analysis

In collaboration with Karin Buiting, phage clones covering the region found to be deleted in other imprinter mutations (Buiting et al., 1995), were used as FISH probes in this patient. These microdeletions, had a breakpoint that lay within one of two phage clones, Lambda 48.8 or Lambda 48.3 (figure 43). FISH analysis was undertaken with these phage clones to determine if 6468 also had a deletion of this region. The experiments with the AS deletion control and the normal control for L48.8 showed that this probe was suitable for the FISH detection of deletions (table 25).
Table 25. L48.8 FISH analysis from 6468, an AS deletion control deleted for loci encompassing D15S11-D15S24 and a normal control.

In 6468, L48.8 produced signal on both chromosome 15 homologues in 15/20 (75%) metaphases analysed. In 5/20 (25%) metaphases signal was produced on one chromosome 15 homologue only. Due to the presence of repetitive sequences which were not sufficiently suppressed, the experiment was repeated and the amount of Cot-1 DNA increased to 30μg (L48.8a). However, the results were not significantly different. Positive hybridisation signals were observed on both chromosome 15 homologues in 18/20 (90%) of metaphases analysed from the normal control, whereas positive hybridisation signals were observed on one chromosome 15 homologue only in 19/20 (95%) of metaphases analysed from the AS deletion control.

These results indicate that either (a) there is poor hybridisation of the probe or (b) a deletion breakpoint lies within L48.8. Since signal was observed on one chromosome 15 homologue only in both 6468 and the normal control, it is unlikely that 6468 is deleted for L48.8, but further experiments would be required to confirm this finding. Figure 68 shows a metaphase from 6468 with positive hybridisation signals on both chromosome 15 homologues compared to a metaphase from a known AS deletion case with signal on one chromosome 15 homologue only.

The experiments with Lambda 48.3 failed to give sufficient analysable signal in 6468 and the controls studied, suggesting that the FISH detection of deletions in this region may be difficult. This may be due to the presence of known repetitive sequences in the phage (K. Buiting, pers. comm.).
Figure 68. Comparison of (A) a metaphase spread from 6468, probed with Lambda 48.8 and MR-60 with (B) a metaphase spread from an AS individual with a known deletion of 15q11-q13. Chromosome 15's marked with arrows.
5.0 Discussion
5.1 The AS and PWS critical regions

The identification of patients with visible chromosomal rearrangements has proved extremely useful in mapping disease loci and has allowed the rapid isolation of disease genes by positional cloning strategies (section 1.7). Once a candidate region for a disease gene has been assigned, all the transcripts contained within that region are potential candidates and have to be screened for mutations.

Most individuals with AS and PWS have a large de novo deletion extending from D15S9 to D15S12 (figure 6), encompassing approximately 4 Mb. It has been estimated that on average there is one gene per 40-50 kb in the human genome (Fields et al., 1994) and it is therefore possible that the deleted segment in AS and PWS could contain approximately 100 genes. Several candidate genes for PWS have been isolated including SNRPN, ZNF127, PAR-5, PAR-1 and IPW (Özçelik et al., 1992; Glenn et al., 1993b; Sutcliffe et al., 1994; Wevrick et al., 1994). These genes have all shown allele-specific expression from the paternal chromosome only (section 1.3.3) and the expression of ZNF127, PAR-5, PAR-1 and IPW have all been shown to be controlled by SNRPN (Sutcliffe et al., 1994; Saitoh et al., 1996).

The presence of a familial, non-deletion, non-UPD, non-imprintor class in AS is consistent with a single maternally expressed gene and a region that regulates its exclusive maternal expression. Since virtually no cases of AS with small deletions exist, AS is most probably caused by a single gene rather than a cluster of genes. In contrast, the absence of a non-deletion, non-UPD, non-imprintor class in PWS, suggests that multiple or co-ordinately regulated genes may have a role to play in the aetiology of PWS. However, it is possible that, for example, point mutations of a single PWS gene (which would give rise to familial cases) could result in affected individuals presenting with a different phenotype and therefore, these cases may not have been diagnosed.

The patients are the most valuable resource available in the search for the gene involved in the aetiology of AS and the genetic mechanisms that underlie the disorder. The isolation, identification and screening of genes is a labour intensive process and the identification of a critical region for a given genetic disorder reduces the interval that needs to be screened for candidate genes. The identification of patients with atypical deletions or translocations and the observation of recombination events within families segregating for the disease phenotype would narrow the region within chromosome 15q11-
q13 that needs to be screened for an AS gene and this was the approach adopted in this thesis.

At the start of this project, the AS critical region was defined by an inherited deletion of approximately 1.5 Mb (Section 1.2.5). The deletion in this family was too large to be polymorphic and inheritance of the deletion through the male germline did not cause PWS, whereas inheritance through the female germline resulted in AS. This clearly indicated genomic imprinting and the existence of a separate locus for PWS.

SNRPN was the first gene to be mapped to the smallest region of deletion overlap for PWS on chromosome 15q11-q13. A region of less than 100 kb around and upstream of the first exon of SNRPN has been shown to be affected in AS and PWS patients with imprinter mutations (Sutcliffe et al., 1994; Buiting et al., 1995; Saitoh et al., 1996). Microdeletions which included the first exon of SNRPN were observed in PWS imprinter mutation families and these were telomeric to those observed in AS imprinter mutation families. Novel transcripts which represent alternative transcripts of the SNRPN gene have recently been identified from this region and mutations in this transcription unit have been described in AS and PWS imprinter mutation families (Dittrich et al., 1996). ZNF127, SNRPN, PAR-5, IPW and PAR-1 which are normally expressed from the paternal allele, were not expressed in lymphoblasts from these PWS patients (Saitoh et al., 1996). In contrast, AS patients with imprinter mutations showed biparental expression of SNRPN and IPW. These findings suggest a role for alternative SNRPN transcripts in imprint switching. SNRPN has also been implicated as a major determinant of PWS in a PWS patient with a de novo balanced reciprocal translocation which disrupts the SNRPN locus (Sun et al., 1996). In contrast to the PWS patients with imprinter mutations, this translocation does not appear to affect the expression of ZNF127, IPW, PAR-5 or PAR-1 in fibroblasts, although whether these genes are expressed in the brain cells and other tissues of this patient is unknown. To date, mutations in the coding region of the SNRPN gene have not been identified in the majority of PWS or PWS-like patients and it is unlikely that SNRPN alone is responsible for the PWS phenotype in these individuals. This is strongly supported by evidence from a PWS patient with a de novo balanced reciprocal translocation (Schulze et al., 1996). The paternal SNRPN allele is unaffected by the translocation in this individual, implying that SNRPN is not involved in the majority of clinical features observed in this patient.
5.2 Criteria for defining a candidate AS gene

To date, an inherited maternally expressed gene which would be a candidate for AS, has not been isolated. As the AS locus is imprinted, not only the AS gene itself but sequences which control its exclusive maternal expression could also be disrupted.

AS individuals who have a mutation in the imprinting process have recently been recognised (Reis et al., 1994; Buiting et al., 1995; Saitoh et al., 1996). Microdeletions were identified in some of these individuals, defining a genetic element termed the imprinting centre (IC) (Buiting et al., 1995; Saitoh et al., 1996). It has been hypothesised that the IC product functions in resetting the 15q11-q13 imprint and mutations of the IC block this resetting. A deletion of the IC which arises on an ancestral paternal chromosome is predicted to fix a paternal epigenotype on that chromosome. Maternal transmission leads to effective homozygosity for a paternal epigenotype and as a consequence of this, the maternally expressed AS gene is predicted to be silenced (figure 69). Approaches for the identification of an AS gene would either be to use a technique which specifically isolates an imprinted gene or to identify a 'critical region' and isolate any gene from that region. The latter approach was mainly adopted in this thesis.

5.3 Identification of imprinted genes

Direct cDNA selection using YACs which formed a contig encompassing the PWS deletion interval on chromosome 15 was successful in identifying a paternally expressed gene, IPW (Wevrick et al., 1994). The YACs used to isolate IPW were suitable for the PWS region but not for the AS region, since they only extended by approximately 25 kb into this region. No candidate AS gene was reported. YACs which form a contig across the AS critical region could also be used for direct selection. The technique of direct selection is dependent on the expression of the relevant gene in the tissue from which the cDNA is prepared. As AS and PWS are neurogenetic disorders, it might be expected that the AS and PWS genes would be found in the same tissue and a similar approach could therefore be adopted in the search for an AS gene. Screening fetal brain cDNA libraries with genomic DNA fragments isolated from the 15q11-q13 region, that had been preselected for hybridisation to radiolabelled cDNA probes (Nakao et al., 1994; Sutcliffe et al., 1994), was
Figure 69. Schematic representation of the effects of AS and PWS imprintor mutations.
successful in isolating several ESTs and a known gene, UBE3A (Huibregtse et al., 1991, 1993). UBE3A maps to the AS critical region and is expressed in cultured fibroblasts and lymphoblasts from AS and PWS patients and normal controls. It has been shown to function as a ubiquitin protein ligase in the ubiquitination of p53 (Scheffner et al., 1993), which does not suggest any obvious correlation with the AS phenotype. The mouse homologue of UBE3A has recently been isolated (Sutcliffe et al., 1996) and paternal expression was observed in different tissues of mice with paternal UPD for the syntenic region. Thus, UBE3A is unlikely to represent the AS gene, although it can not be eliminated as a candidate for AS, since imprinting may occur in tissues relevant to the phenotype, although this is unlikely.

PAR-1, PAR-2, PAR-4, PAR-5, PAR-6 and PAR-7 were identified from the AS/PWS region. Imprinting analysis in cultured fibroblasts and lymphoblasts from AS and PWS patients and normals showed that PAR-2 was not imprinted in these tissues. PAR-1 and PAR-5 which map to the region critical for PWS, were shown to be expressed exclusively from the paternal allele and since there is loss of expression of these transcripts in PWS patients with imprintor mutations (Sutcliffe et al., 1994; Saitoh et al., 1996), they could have a role to play in the aetiology of PWS. The PAR-2 transcript which maps to the region critical for AS is not a strong candidate for AS, as it is not imprinted, but it can not be eliminated as a candidate since imprinting may occur in other tissues relevant to the AS phenotype. PAR-4 and PAR-7 were insufficiently expressed to allow imprinting analysis and PAR-6 was not characterised. Since it is unknown whether these three transcripts are imprinted, they can not be eliminated as candidates for either AS or PWS.

The molecular mechanisms of genomic imprinting are unknown, although allele specific DNA methylation appears to be a characteristic of imprinted genes (Brandeis et al., 1993). DNA methylation is known to maintain the inactive X chromosome in its inactive state (Singer-Sam and Riggs, 1993) and differences in DNA methylation could identify genes which are exclusively expressed from the maternal or paternal alleles. DNA methylation was the basis of a strategy that was implemented in the search for candidate imprinted genes involved in the aetiology of AS and PWS by Driscoll et al., (1992). The highly evolutionary conserved cDNA, DN34, identified distinct differences in DNA methylation of the parental alleles at the D15S9 locus and this led to the isolation of the paternally expressed gene ZNF127, which encodes a novel zinc finger protein (Glenn et al., 1993b). It has been shown that PWS patients with imprintor mutations do not express ZNF127 (Saitoh et
al., 1996) and this gene is therefore a candidate to play a role in the pathogenesis of PWS.

Screening techniques which scan the whole genome for imprinted genes have been successful in identifying imprinted genes in the mouse on chromosomes 6 and 11. The technique of restriction landmark genome scanning (RLGS) is based on the assumption that the alleles of imprinted genes will be differentially methylated. The paternally expressed gene U2afbp-rs, which is also known as SP2 was isolated by RLGS (Hatada et al., 1993; Hayashizaki et al., 1994). A second screening technique was based on cDNA subtraction hybridisation. The paternally expressed genes Peg 1 / Mest and Peg 3 were identified using subtraction hybridisation between cDNAs from normal and parthenogenetic embryos (Kaneko-Ishino et al., 1995). The technique of RLGS relied on back-crosses in mice and the subtractive hybridisation technique relied on parthenogenetic embryos and are therefore only applicable to mice, but as conservation of imprinted genes between humans and mice has been observed (table 4), it would be possible to identify an imprinted gene in the mouse and then search for the human homologue.

Tissue specific patterns of expression of genes involved in the aetiology of AS can not be predicted, although as AS is a neurogenetic disorder, it is probable that transcripts could be expressed in brain tissue. It is also conceivable that these genes may not show allele specific expression in all tissues and that there could be a switching between monoalleleic and biallelic expression during development. This has been observed with IGF2, which is only expressed from the paternal allele in fetal liver (Ohlsson et al., 1993; Giannoukakis et al., 1993), but in adult liver, the choroid plexus and leptomeninges, biallelic expression is observed (Ohlsson et al., 1994). Biallelic expression of the maternally expressed p57KIP2 gene has also been observed in some tissues. In fetal brain and some embryonal tumours, the levels of expression of the paternal allele are comparable to those of the maternal allele, while low levels of expression of the paternal allele have been observed in most tissues (Matsuoka et al., 1996).

5.4 Contribution of this project to the AS critical region

AS individual 3731 had normal DNA methylation patterns at ZNF127 (D15S9), PW71 (D15S63) and SNRPN (intron 5), but had failed to inherit a maternal allele for the CA(n) repeat at LS6-1 (D15S113). The LS6-1 result in 3731
indicated the possibility of a deletion of this locus and as the D15S113 locus lies within the 1.5 Mb AS critical region, confirmation of a deletion in this individual could potentially narrow the region for searching for a structural AS gene. The apparent deletion of the LS6-1 marker could also be the result of non-amplification of the maternal allele. Allele non-amplification has previously been described with microsatellite polymorphisms (Weber et al., 1991; Koorey et al., 1993) and can potentially cause confusion in the detection of deletions.

Detailed molecular characterisation of the AS and PWS critical regions had been difficult due to the relatively low polymorphic content of DNA markers from the region (Nicholls et al., 1989). The isolation of YACs for previously mapped chromosome 15q11-q13 DNA probes, enabled FISH deletion analysis in AS and PWS patients, to define the critical region for each disorder (Kuwano et al., 1992).

FISH analysis was the approach adopted in this thesis to analyse AS individual 3731 and provided a method for assessing the possibility of allele non-amplification of the LS6-1 marker. The results of FISH analysis with the LS6-1 positive 11HE12 cosmids indicated that the D15S113 locus was deleted in 3731 (section 3.3.2). However, the results with the series 200 cosmids did not indicate that any single cosmid was completely deleted in 3731 (section 3.3.3) and this conflicted with the original 11HE12 cosmid results. To clarify the discrepancies in 3731, FISH analysis with phage clones derived from the A229A2 YAC clone was performed. The results indicated that 3731 did not have a deletion of the LS6-1 (D15S113) locus.

The primers for LS6-1#1 have been shown to detect a poorly amplified allele, which has a frequency of 3/56 normal chromosomes studied (S. Rickard, pers. comm.). It was subsequently shown that while the LS6-1#1 primers did not detect a maternal allele in 3731, when the alternative primers LS6-1#2 were used, 3731 was shown to be heterozygous. The original LS6-1#1 result was therefore shown to be due to non amplification of the maternal allele. The presence of the poorly amplified LS6-1 allele is probably due to a polymorphism in the reverse primer sequence, since LS6-1#1 and LS6-1#2 share the same forward primer sequence. Further evidence from a new polymorphic marker (196) located within 60 kb of LS6-1 and two single copy fragments, indicated that 3731 is not deleted for the LS6-1 locus or surrounding loci (section 3.6).

The most likely explanation for the conflicting FISH results with the 11HE12 and series 200 cosmids was that the sensitivity and specificity of the FISH
method used to analyse the series 200 cosmids (method 2), was greater than for the method used to analyse the 11HE12 cosmids (method 1). Changes in method 2 which might increase the specificity include the temperature increases of the 50%/2XSSC formamide washes from 42°C to 45°C and the 0.1XSSC washes from 42°C to 60°C and the increase in the stringency of the SSC washes from 1XSSC to 0.1XSSC. This would increase the dissociation of the non-specific and mismatched hybrids from the metaphase chromosomes and hence, reduce non-specific amplification. The sensitivity may have been increased by increasing the concentration of the Cot-1 DNA which would reduce the non-specific hybridisation of the probe during hybridisation. Therefore, it is possible that by increasing the sensitivity and specificity of the method, a weaker signal which was initially not detected, could now be visualised. This appeared to be confirmed when the 11HE12 cosmid FISH results were re-analysed using method 2 (section 3.4.4). The number of metaphases where signal was detected on one chromosome 15 only was significantly reduced from 90% of all the metaphases analysed from 3731 to 15% and 5% respectively for the two LS6-1 positive cosmids E24 and E42.

FISH analysis of normal individuals with the 11HE12 LS6-1 positive cosmids frequently detected positive hybridisation signals on both chromosome 15's with a significantly reduced signal on one homologue compared to the other in a small number of the analysed metaphases. This was also observed in other studies with these cosmids (L. Willett, pers. comm.). It is difficult to assess whether a probe is 'partially' deleted by FISH as the amount of signal on each homologue is difficult to quantify, due to several variables. The computer programme provided with the digital imaging equipment enables the intensity of the signal on each homologue to be measured, however, the amount of signal detected is dependent on the amount of labelled probe that hybridises to each homologue and the number of amplification steps. The intensity of signals observed on both homologues could also be dependent on the position in which the two homologues are lying within a metaphase. Similarly, a comparison of the amount of signal on each homologue due to the test probe with the amount of signal on each homologue due to the control probe, would also be affected by the same variables. Thus, both analysis by eye or using the computer programme, will result in a degree of inaccuracy in assessing the intensity of the signal on each homologue and a degree of caution should therefore be used in the interpretation of 'partial' deletions by FISH.
It is possible that there could be a sequence elsewhere on chromosome 15q that is related to a sequence that is contained in the cosmids. This sequence may be present in different copy numbers on the two chromosome 15's. Low copy repeats have been described with multiple loci on chromosomes 15 and 16 (Buiting et al., 1992; Nagaoka et al., 1994; Tomlinson et al., 1994) and it has been shown that the presence of low copy repeats in cosmids isolated from the DiGeorge and velo-cardio-facial syndrome critical region within 22q11 made these cosmids difficult to use in FISH experiments (Halford et al., 1993).

Alternatively, the cosmids may contain so many repeat sequences that the competition is variable. The presence of numerous repeat sequences in proximal 15q was first demonstrated using 15q11-q13 specific markers, isolated from flow sorted inv dup (15) chromosomes (Donlon et al., 1986), where DNA segments were difficult to clone in this region when propagated in recombination proficient hosts. It was shown that these clones contained inverted repeats and the presence of high copy repeat sequences such as these could affect FISH analysis. Repetitive DNA is distributed throughout the whole genome, with the most abundant repetitive DNA being the Alu-repeat family. These interspersed repetitive sequences (IRS) need to be competed out to prevent their hybridisation to the whole chromosome complement (Landegent et al., 1987; Lichter et al., 1988a; Pinkel et al., 1988). Competing these repeat sequences with an excess of unlabelled competitor DNA, results in the suppression of these repeats. However, the degree of suppression of these repeats will vary between experiments and this has also been observed in Southern blot analysis with highly repetitive probes.

The results in 3731 remove evidence for the LS6-1 (D15S113) locus being the critical region for an AS gene and no further evidence for the involvement of the D15S113 locus was found in the studies of AS individuals with no known molecular defect (section 3.10).

Evidence which apparently excluded D15S113 from the AS critical region was provided by the t (4; 15) translocation patient (Reis et al., 1993), who was shown to be intact for the D15S113 locus. The translocation breakpoint in this individual could define the distal AS boundary although it is now known that imprinting control elements are deleted in this individual (Buiting et al., 1995). Loss of these imprinting control elements could be the cause of AS in this individual. These elements are predicted to act as a switch, but their continued presence may also be required for the maternal expression of the
AS gene. Alternatively, the AS gene itself has also been deleted. Further evidence excluding the D15S113 locus was provided by a child with a large maternally inherited deletion of proximal 15q, which included the D15S113 locus (Michaelis et al., 1995). This patient did not have the characteristic features of AS and was studied further (section 3.9) in order to identify the centromeric breakpoint.

FISH analysis of the deletion in the proband (DH) and his mother (BS) indicated that the centromeric deletion breakpoint could be contained within the 520 kb YAC 230H12, which was either present on both chromosome 15 homologues in DH and BS but with a consistently reduced signal intensity on one homologue compared to the other or was present on one chromosome 15 homologue only. The 600 kb D15S10 positive YAC 132D4 which maps centromeric to 230H12, was present on both chromosome 15 homologues in DH and BS, whereas the 520 kb telomeric YAC 142A2 was present on one chromosome 15 homologue only in DH and BS. The most likely interpretation of the results is a 'partial' deletion of YAC 230H12. YAC clones are several hundred kb larger than cosmid clones and a difference in the intensity of the signals between the two homologues is much easier to visualise accurately. Since the difference in signal intensity between the two homologues was so striking in this case (figure 52A) and as the AS deletion control was completely deleted for this YAC, the results in DH and BS are likely to reflect a genuine 'partial' deletion of the YAC clone. However, as the interpretation of 'partial' deletions is not always reliable, further molecular studies are required to confirm this finding. It has recently been shown that D15S10 also maps within YAC 230H12 (J.Buxton, pers. comm.), placing this YAC further centromeric (figure 71). The result in patient DH indicates that the telomeric boundary of the AS critical region may lie approximately 150 kb distal of the D15S10 locus.

The apparently mild phenotype that is observed in both DH and BS is surprising. The mother has a borderline intellectual defect and slightly downslanting palpebral fissures, while her son has mild to moderate mental retardation and mild facial anomalies. Interstitial deletions of proximal 15q without a typical AS or PWS phenotype have been reported (Tonk et al., 1995) and the majority of these cases are associated with malformations that are more severe than those reported in patient DH. Further cytogenetic and molecular analysis of these individuals is required to ascertain the extent of the deletions, the degree of overlap between the deletions, which genes are deleted, any additional cytogenetic abnormalities such as a subtle insertion
and the clinical similarities. Without this information, a degree of caution should be employed in interpreting the data from DH. The telomeric boundary for the AS critical region as defined by patient DH could however be contradicted by an argument based on a translocation patient (Burke et al., 1996) (section 5.5).

Individual 13581 (section 3.7) has an atypical deletion of 15q11-q13 and is interesting from several points of view. The telomeric breakpoint lies between the 196 and GABRB3 loci. Since 13581 is heterozygous for the microsatellite marker at GABRB3, this may exclude the GABRB3 locus from the AS critical region. This could differ from the telomeric breakpoint described in the Japanese family (Saitoh et al., 1992; Greger et al., 1993), which lies within intron 3 of the GABRB3 gene. YAC 132D4 which maps distal to SNRPN, was shown to be deleted in 13581, placing the centromeric deletion breakpoint proximal to this YAC, close to SNRPN. The DNA methylation pattern at SNRPN (exon alpha) was altered in this individual indicating that the deletion may include the SNRPN locus. As FISH analysis with a phage clone containing exon alpha was unreliable, it was not established whether exon alpha was deleted in this individual or whether the altered DNA methylation pattern could be due to a position effect exerted by the deletion itself. The DNA methylation pattern at PW71B (D15S63) was not altered. Since the deletion in 13581 does not affect the methylation pattern at PW71B (D15S63), the methylation at this locus is probably not affected and therefore, it is unlikely that the deletion affects the imprinting pattern. FISH analysis with L48.8 indicated that 13581 is unlikely to be deleted for this phage clone. L48.6I is a 6.8 kb genomic EcoR I fragment which maps to L48.8. This fragment is deleted in 5/6 AS imprintor mutation families (Buiting et al., 1995; Saitoh et al., 1996) and contains exon BD3 which is part of an alternative SNRPN transcript (Dittrich et al., 1996). These results imply that the deletion may not affect the imprinting control elements which appear to require exons upstream of SNRPN for their functioning in AS.

The region that is deleted in AS imprintor families is also deleted in all but one PWS imprintor mutation patients (Buiting et al., 1995; Saitoh et al., 1996), suggesting that either additional elements are required for the IC to function or that the PWS IC is different but adjacent to that involved in AS. It will be of great interest to determine whether these imprinting control elements, as well as the AS structural gene, are deleted in 13581 or if the deletion itself exerts a position effect on the IC. As transcripts from the IC are further characterised (Buiting et al., 1995; Dittrich et al., 1996), mutation analysis in this individual
could determine whether the deletion involves the IC. The diagnostic implications of this case are discussed in section 5.6.

Two AS imprinted mutation families (families R and W) and one isolated case (6468) were identified (sections 4.2, 4.3 and 4.4). The results of the haplotype analysis in family W were consistent with the localisation of the mutation in this family to chromosome 15q11-q13. In individual 6468, FISH studies were unable to detect a deletion of the IC and to date, further studies including SSCP analysis of IC exons, have not identified a mutation of the IC in either this individual or family W (K. Buiting, pers. comm.). It is probable that the mutations in family W and individual 6468 are smaller than the microdeletions that have been observed in those patients with imprinted mutations reported to date (Sutcliffe et al., 1994; Buiting et al., 1995; Saitoh et al., 1996). It is also not inconceivable, that the imprinted mutation in individual 6468 involves a trans-acting factor which is not on chromosome 15. The haplotypes observed in family W imply that the possibility of a trans-acting factor in this family is unlikely.

In family R, both affected siblings had paternal DNA methylation imprints only at PW71 (D15S63). Prenatal diagnosis was attempted in this family and both normal maternal and paternal DNA methylation imprints were observed in the fetus. Haplotype analysis showed that the fetus and the two affected siblings had inherited the same maternal alleles at D15S113 and D15S128, a distance of 2.9 cM, based on the sex-specific map derived from the work of Robinson and Lalande (1995). The results indicate that the fetus most likely has the whole haplotype in common with the two affected siblings.

D15S128 lies in the vicinity of SNRPN and the proposed methylation control locus. One explanation of the findings in family R would be a double recombination between D15S128 (assuming that it lies distal of the methylation control locus) and a marker which lies proximal to these control elements. However, double recombination events within 3 cM are rare and alternative explanations which would also explain these findings are germline mosaicism or the methylation status in the fetal blood sample may not reflect the true methylation status of the fetus. Hypomethylation has been observed at the D15S63 locus in extraembryonic tissues (section 5.6), although there is no evidence for hypomethylation at this locus in somatic tissues. It is likely that the 'imprint' is established early in development. Differential methylation of Xist, H19 and Igf2r has been observed in the gametes (Stöger et al., 1993; Tremblay et al., 1995) and this differential methylation survives preimplantation erasure. Since imprinting occurs when differences in
epigenetic modifications of the gametes survive erasure in early development, the 'imprint' is likely to be passed from the gametes into the soma. It has also been shown that normal maternal and paternal DNA methylation imprints were present at PW71B (D15S63) in fetal blood samples from individuals without either AS or PWS (J. Buxton, pers. comm.) and it is probable that the methylation status of the fetal blood sample does reflect the methylation status of the fetus.

A deletion of the imprinting control locus was subsequently identified in family R (Saitoh et al., 1996). The deletion was estimated to span approximately 10 kb. Two probes (L48.3I and L48.6I) detected an 11.0 kb EcoR I junction fragment in the two affected siblings and their mother. This junction fragment was not detected in the fetus (J. Buxton, pers. comm.). In two further imprinter mutation families (Saitoh et al., 1996), the mothers showed a reduction in intensity of the breakpoint fragments compared to their affected children. This indicates that they are mosaic for the deletion. As germine mosaicism would also explain the findings in family R, it is possible that this could be a relatively frequent observation in imprinter mutation cases. The observation of germine mosaicism indicates an early somatic origin for the deletions. The deletion would therefore apparently 'fix' the grandpaternal epigenotype into the chromosome, prior to resetting the new imprint in the germine of the mother (figure 69).

The deletions of the IC do not cause the disease phenotype themselves as the deletions are also present in phenotypically normal parents and ancestors (Sutcliffe et al., 1994; Buiting et al., 1995; Saitoh et al., 1996). The IC encodes alternative transcripts (BD transcripts) of the SNRPN gene (Dittrich et al., 1996). Deletions of the BD exons in several AS families were shown to be associated with a block in the paternal to maternal imprint switch, whereas deletions of the first SNRPN exon in several PWS families blocks the maternal to paternal imprint switch. These findings indicate that the IC may have a bipartite structure. The authors proposed a model in which the IC consists of an imprinter, which encodes the BD transcript and is transcribed from the paternal chromosome only. The imprinter acts in cis on the switch initiation site which could be the first exon of SNRPN, the SNRPN promoter or a site nearby. In the female germine, the maternally inherited chromosome retains the maternal imprint, whereas the acquisition of a maternal imprint on the paternally inherited chromosome, requires a trans-acting factor which acts on the switch initiation site. If the BD-imprinter on the paternal allele is mutated, then the trans-acting factor would not have access to the paternal
chromosome, resulting in failure of the paternal to maternal switch. In the male germline, in the absence of a maternal trans-acting factor, the paternal imprint is retained on the paternally inherited chromosome, whereas the maternally inherited chromosome loses the maternal imprint starting from the switch initiation site, either by default or via other factors. If the first exon of SNRPN is mutated on the maternal allele, then this would result in failure of the maternal to paternal switch. However, it has been argued that failure of imprint erasure may be occurring in PWS (Ferguson-Smith, 1996) and that the first exon of SNRPN could be involved in this process. This would make a single switch step less likely than one that involves erasure of the imprint and resetting in the germline.

5.5 Current status of the critical region

Various patients throw light on the possible boundaries of the AS critical region (figure 70). The centromeric boundary of the AS critical region was initially defined by D15S174, the proximal breakpoint of the deletion in the Japanese family described by Saitoh et al., (1992). A familial case of AS in which the two affected siblings inherited different maternal alleles between D15S63 and D15S122 but inherited the same maternal allele at D15S113 (Wagstaff et al., 1993; Greger et al., 1994) reduces the centromeric boundary to D15S122. However, DNA methylation imprints were not reported in this family and while the inheritance of different maternal alleles at D15S63 would make an imprinter mutation unlikely in this family it is not impossible that one of the siblings could have an imprinter mutation, whereas the other could have a mutation within the AS structural gene itself, although this is highly unlikely.

The results presented in this thesis indicate that the telomeric boundary of the AS region could lie in a 150 kb region, distal of the D15S10 locus, as defined by the proximal breakpoint of the inherited maternal deletion without AS in patient DH (section 5.4). Previously, the telomeric boundary of the AS region was defined by the distal breakpoint of the deletion described in the Japanese family (Saitoh et al., 1992). The distal deletion breakpoint in this family lies within intron 3 of the GABRB3 gene (Greger et al., 1993), approximately 1.4 Mb from the D15S10 locus. The result in patient DH would place the telomeric boundary of the AS region approximately 1.25 Mb centromeric, to that originally defined by the Japanese family. However, while the deletion in patient DH encompasses loci from D15S113 to D15S12, only a mild
Figure 70. A map of chromosome 15q11-q13 depicting the putative AS critical region based on molecular analysis in atypical patients and a review of the current literature (see text).
phenotype is produced and a degree of caution should be used in interpreting the data from patient DH.

It has been suggested that the telomeric boundary for the AS critical region could be further defined by the breakpoint of a familial cryptic translocation between chromosomes 14 and 15 (Burke et al., 1996). The mother and the proband's sister carry an apparently balanced form of the translocation, whereas the proband inherited an unbalanced form of the translocation, 46, XX, -15, +der 14, t (14; 15) (q11.2; q11.2) mat. The mother and unaffected daughter had normal DNA methylation patterns at ZNF127, D15S63 and SNRPN, whereas the proband had a paternal methylation pattern only. FISH analysis placed the translocation breakpoint between SNRPN and D15S10. Recently, the breakpoint of this translocation has been localised between two overlapping cosmids, cosmids 34 and 23 (Boyar et al., 1996). These cosmids are part of a contig across the region and map to the telomeric end of YAC 132D4, distal of the PAR-2 locus (Sutcliffe et al., 1994). Cosmids 34 and 23 presumably lie proximal of the D15S10 locus, although in the YAC and PAC map which is currently being constructed by J. Buxton and S. Rickard at the Institute of Child Health (figure 71), PAR-2 was mapped distal of D15S10, indicating discrepancies in the marker order in this region. The authors state that the smallest region of overlap is defined as the region between D15S122 and the translocation breakpoint. Since D15S122 has been shown to map within YACs 132D4, B230E3 and 230H12, between D15S10 and D15S113 (Malcolm and Donlon, 1994; J. Buxton, pers. comm.), this would place the AS gene in the region which spans D15S10 and D15S122. If this were the case, then the translocation breakpoint would disrupt the AS gene and the normal sister, who inherited the balanced form of the translocation from her mother would also have AS. The authors argue that loss of the IC is unlikely to be the cause of AS in this individual. They state that if the translocation separated the IC and the AS structural gene, then the proband's sister who is clinically normal, would have AS, as the IC can no longer act in cis. However, an alternative explanation is that the IC and any sequences that it codes for are able to act in trans as well as in cis and the balanced form of the translocation would not result in an AS phenotype. The chromosome 15 IC is known to act in cis (Sutcliffe et al., 1994; Buiting et al., 1995; Saitoh et al., 1996) and in the model proposed by Dittrich et al., (1996), the existance of a trans-acting factor in the maternal germline has been hypothesised. Both cis and trans models have been proposed to explain the mechanisms of action of imprinted genes (Bartolomei et al., 1993; Yoo-Warren et al., 1988; Stöger et
al., 1993; Barlow, 1994; Forejt and Gregorová, 1992) and conclusions can not be drawn from the t (14; 15) case. If the t (14; 15) translocation breakpoint is discounted, the smallest region of overlap for the AS gene can be defined as the region between D15S122 and the breakpoint in DH, which encompasses a distance of approximately 100 kb.

**Figure 71.** PAC and YAC map of the Angelman syndrome critical region (J. Buxton and S. Rickard, pers. comm.).

### 5.6 Implications for the diagnosis of AS and PWS

The rapid and efficient detection of patients with AS and PWS and the type of mutation is of great clinical importance. These two syndromes are especially difficult to diagnose in newborns and young infants by clinical examination alone and early diagnosis may avoid some of the fatal consequences such as obesity in PWS (Stadler et al., 1988). The majority of AS and PWS cases are sporadic with no recurrence risk in families. However, a proportion of patients with both AS and PWS are familial cases where the recurrence risk is as high as 50%.

High resolution cytogenetic detection of interstitial deletions of 15q11-q13 provided the first laboratory based diagnostic test for these two syndromes.
and considerable reliance was placed on the results of these investigations. In the routine cytogenetic laboratory setting, samples from patients with only a vague clinical diagnosis are often sent for cytogenetic investigations and therefore routine high resolution cytogenetic investigations serves two purposes. It might identify AS and PWS patients with typical deletions, but many studies have shown that it is unreliable and submicroscopic deletions not detected by high resolution cytogenetics have been reported (Chan et al., 1993; Zackowski et al., 1993; Delach et al., 1994; Saitoh et al., 1994; Butler, 1995; Bettio et al., 1995; Smith et al., 1995; Webb et al., 1995). It will also identify patients with other structural rearrangements involving chromosome 15 (section 1.2.2), which can also give rise to either syndrome and it will further identify patients in whom the phenotype is the result of some other chromosomal rearrangement not involving the 15q11-q13 region, which results in non-specific mental retardation.

False positive results could be due to common polymorphisms of proximal 15q which include duplications and deletions (Hood et al., 1986; Brookwell and Veleba, 1987; Hoo et al., 1990; Zackowski et al., 1993) and replication asynchrony which can result in one chromosome 15 homologue appearing shorter than the other (Izumikawa et al., 1991), whereas false negative results are due in part to the higher resolution of DNA analysis compared to high resolution cytogenetics. At the 1000 band level of resolution, one cytogenetic band could contain up to five megabases of DNA.

FISH analysis overcomes the problems of detecting deletions by high resolution cytogenetics (Delach et al., 1994; Bettio et al., 1995; Smith et al., 1995; White and Knoll, 1995; Erdel et al., 1996; Teshima et al., 1996). FISH provides a greater degree of sensitivity compared to standard cytogenetic techniques and the availability of probes specific for the AS and PWS regions, has enabled FISH detection of deletions in interphase nuclei and metaphase chromosomes. To confirm a deletion in the AS critical region, analysis of a D15S10 probe would be the most efficient, whereas confirmation of a deletion within the PWS critical region would require analysis of the SNRPN locus. These two probes would identify all the currently reported AS and PWS patients within the deletion class.

Few atypical deletions of the 15q11-q13 region exist in AS and PWS patients. The Japanese family with a small inherited deletion of 15q11-q13 (Hamabe et al., 1991; Saitoh et al., 1992) and AS individual 13581 reported in this thesis, represent two of the smallest known deletions that have to date been reported in the AS critical region and therefore, it is unlikely that any AS deletion
patient would be missed by studying the D15S10 locus only. Similarly in PWS, atypical deletions are rare and analysis of the SNRPN locus should identify all PWS patients with deletions, including some patients with deletions of the imprinting centre (Teshima et al., 1996). Analysis of the proximal marker, D15S11, in both AS and PWS patients will enable patients with typical deletions and therefore a low familial recurrence risk to be distinguished from patients with atypical deletions and possibly a high familial recurrence risk. FISH analysis proved extremely useful in the detection of a de novo deletion of 15q11-q13 in AS individual 13581. This was an important result as the mother of 13581 was 7 weeks pregnant and the result at PW71B (D15S63) had suggested that 13581 was non-deleted, placing her mother in the high recurrence risk class. Thus, FISH analysis in this family indicated that the risk to the pregnancy was low.

The commercially available cosmid probes (Oncor and Vysis) incorporate an internal control marker (PML) on the long arm of chromosome 15 at q22. The directly labelled SNRPN and D15S10 probes supplied by Vysis also contain the centromeric marker D15Z1, which recognises short repeats related to AATGG in 'classical' satellite DNA located in pericentromeric heterochromatin. The use of a chromosome 15 centromeric probe such as D15Z (Oncor) which is specific for highly repeated centromeric alphoid DNA or D15Z1 (Oncor), is recommended as this will discriminate between those cases that are due to an interstitial deletion and those cases in which a structural rearrangement caused the deletion. It is also essential to analyse the parents of AS and PWS patients to determine whether the deletion is de novo and the recurrence risk low or whether it is inherited or arose as part of a cryptic rearrangement in a parent, which would then impose a higher recurrence risk to the family (Kennerknecht et al., 1992).

Non-commercial cosmid and phage probes from the 15q11-q13 region are available from some research centres and YACs from the same region can be obtained from the Baylor Institute (section 2.1.2). The results presented in this thesis indicate that both a normal control and a known AS deletion control should be used in all FISH experiments, particularly when non-commercial probes are used. This enables the hybridisation efficiency of the probe to be assessed and should prevent the possibility of mis-diagnosis with probes that are either highly repetitive or that contain repeat sequences which are represented elsewhere on 15q.

The results of the FISH studies using phage probes from the region surrounding the imprinting centre presented in this thesis suggest that these
probes are on the limits of detection. Since the sizes of microdeletions found in this region are variable (Buiting et al., 1995; Saitoh et al., 1996), FISH detection of deletions of the imprinting centre is not the most suitable diagnostic test to employ for identifying imprinter mutations, as the majority of findings will be negative or ambiguous. Therefore, FISH analysis with the SNRPN cosmid probe should be used for deletion detection only and other methods should be employed for detecting imprinter mutations.

FISH analysis will detect AS and PWS patients in the deletion class, including some patients with other structural rearrangements of chromosome 15. Recently, asynchronous replication studies in interphase nuclei using probes from the D15S9 and SNRPN loci have been used to distinguish AS and PWS patients with UPD from those patients with biparental inheritance of both chromosome 15's (White et al., 1996). The frequency of interphase cells demonstrating asynchronous replication was significantly lower in patients with UPD compared to those patients with biparental inheritance of chromosome 15 and normal controls. One obvious advantage of this technique is that it does not require parental samples and by combining metaphase and interphase analysis, a diagnosis can be provided in greater than 95% of PWS patients and 75-80% of AS patients. However, this technique requires the analysis of at least 100 interphase nuclei in both the patients and in normal controls, it requires probes with greater than 90% hybridisation efficiency and therefore, each new batch of labelled probe requires testing. It will also not identify AS or PWS patients with imprinter mutations or distinguish those AS patients with imprinter mutations from those who do not have a deletion, UPD or an imprinter mutation.

In PWS, all cases to date can be detected by studying the DNA methylation patterns at loci within the 15q11-q13 region (section 1.2.6). It is a relatively quick and easy test for screening large numbers of patients in whom the clinical diagnosis is uncertain. Studying DNA methylation patterns will not distinguish between those patients with deletions and maternal UPD (low recurrence risk) or those patients with imprinter mutations (high recurrence risk). To distinguish PWS patients with maternal UPD from those with imprinter mutations, it is essential that microsatellite analysis is carried out, since despite being extremely rare, there is a high recurrence risk to the families of PWS patients with imprinter mutations. In AS, analysis of DNA methylation patterns at loci within 15q11-q13 (section 1.2.6) will detect patients with either deletions or paternal UPD (both with low recurrence risk) and AS patients with imprinter mutations (high recurrence risk), although it will
not distinguish between them. Microsatellite analysis is therefore essential to enable AS patients with paternal UPD to be distinguished from those patients with imprinter mutations. It is critical that AS and PWS imprinter mutation cases are distinguished from deletion and UPD cases, as there is a recurrence risk of up to 50% in these families (Reis et al., 1994; Sutcliffe et al., 1994; Buiting et al., 1995; Saitoh et al., 1996). The recurrence risk could however be less than the 50% expected, since germline mosaicism has been reported in some of these cases (Saitoh et al., 1996). One important consideration in these cases is the suggestion that AS patients with imprinter mutations may have a milder phenotype (Burger et al., 1995; M. Pembrey, pers. comm.).

No currently available molecular technique will detect the 20-25% of AS patients in whom biparental inheritance of chromosome 15q11-q13 is observed but no deletion, paternal UPD or imprinter mutation detected. The families of these patients have a high recurrence risk of AS and it is therefore crucial that the clinical diagnosis is correct in these patients. The only test available to these families is to perform prenatal diagnosis and to observe the co-inheritance of maternal 15q11-q13 markers in the fetus.

The majority of diagnostic laboratories have to date used the PW71B probe (Dittrich et al., 1992, 1993) for the analysis of DNA methylation imprints and it is recommended that to obtain consistent results, complete digestion should be tested for (Dittrich et al., 1996). However, DNA methylation patterns at the D15S63 locus were inconsistent in lymphoblastoid cell lines and in chorionic villus samples compared to amniotic fluid cells (Dittrich et al., 1993; van den Ouweland et al., 1995). This was shown to be the result of hypomethylation at the D15S63 locus in extraembryonic and lymphoblastoid tissues (Dittrich et al., 1996) and therefore, PW71B should not be used for methylation studies in these tissues. This inconsistency has also been observed in prenatal diagnosis of the fragile X syndrome, where the EcoR I / Eag I methylation based assay cannot be performed on DNA isolated from chorionic villi, in contrast to DNA isolated from amniotic fluid cells and other fetal tissues (Sutherland et al., 1991; Sutcliffe et al., 1992). SNRPN (exon alpha) will also detect parent of origin differences in DNA methylation (section 1.2.6). The differential methylation associated with SNRPN (exon alpha) is complete using genomic DNA from leukocytes, lymphoblasts, skin fibroblasts and fetal tissues (Glenn et al., 1996) and SNRPN (exon alpha) may therefore prove reliable in the analysis of DNA methylation patterns in chorionic villus samples, although studies will be required to determine the reliability of SNRPN (exon alpha) in extraembryonic tissues. DNA methylation analysis in
AS individual 13581 showed normal parental DNA methylation imprints at
PW71B, but only a paternal methylation imprint was observed at SNRPN
(exon alpha). DNA methylation analysis in two AS imprinter families also
gave inconsistent results with PW71B (J. Buxton and S. Cottrell, pers.
comm.). These findings imply that the SNRPN (exon alpha) probe is the
more accurate probe for DNA methylation studies in AS and PWS patients.

During the course of this work, several new microsatellite markers have been
identified (Malcolm and Donlon, 1994; S. Christian, pers. comm.). D15S210
lies within the AS critical region and D15S128 lies in the vicinity of SNRPN
and may detect microdeletions of the imprinting centre in informative families.
The highly polymorphic tetranucleotide repeat, 196 (R. Trent, pers. comm.), is
a suitable and preferable alternative to the primers at LS6-1 (D15S113) for
which null alleles have been observed (section 5.4). 196 is highly informative
and easy to read as it is based on a tetranucleotide repeat.

Familial AS and PWS patients with imprinter mutations (Glenn et al., 1993b;
Buiting et al., 1994; Reis et al., 1994; Sutcliffe et al., 1994; Buiting et al.,
1995; Saitoh et al., 1996) represent the first AS and PWS patients in whom a
specific defect has been observed. This observation has enabled more
accurate prenatal diagnosis of imprinter mutation cases (Saitoh et al., 1996).

Once the extent of a deletion of the IC has been identified in an AS or PWS
imprinter family, it is possible to look for the junction fragments in DNA from
chorionic villus samples to exclude a microdeletion.

The observation of probable germline mosaicism in family R and in two further
cases (Saitoh et al., 1996) shows that this must be an important consideration
in imprinter mutation families. However, it is interesting to note that in family
W and patient 6468 a deletion of the IC has not yet been detected. While the
microsatellite analysis in family W indicates that the mutation in this family is
most probably on chromosome 15, it is not inconceivable that some imprinter
mutations involve a trans-acting factor which is not on chromosome 15.

In all cases of trisomy 15 encountered in CVS or amniocentesis, UPD testing
should be offered as theoretically one-third of these cases will result in UPD
15 (Cassidy et al., 1992; Purvis-Smith et al., 1992; Morichon-Devallez et al.,
1993; Christian et al., 1996). UPD testing should also be offered in cases
where marker chromosome 15’s, balanced reciprocal translocations or
Robertsonian translocations involving chromosome 15 or isochromosome
15’s are detected.

Finally, this leaves approximately 20-25% of AS patients in whom no deletion,
disomy or imprinter mutation can be detected. In patients where the clinical
diagnosis is consistent with AS and no cytogenetic or molecular aberration is observed, then counselling should be offered to the family as the recurrence risk is up to 50%. These families await the identification of the AS gene. A strategy for the diagnosis of all classes of AS and PWS patients based on observations in both this thesis and a review of the current literature is illustrated in figure 72. This strategy is in agreement with Cassidy et al., (1996).

5.7 Future work

The next advance in understanding the mechanisms involved in the aetiology of AS will be the identification of the AS structural gene. Identification of the gene will enable a definitive diagnosis to be made in the remaining 20-25% of AS patients in whom no molecular aberration has yet been detected. It will also be possible to offer the families of these patients (who have a high recurrence risk for the disorder), accurate prenatal diagnosis.

To isolate the AS gene itself, further mapping and cloning studies are required. At present, the AS critical region is thought to span a 100 kb region between D15S122 and the breakpoint in patient DH (section 5.5). Since two of the YACs which extend across this region may be internally deleted (Nakao et al., 1994; J. Buxton, pers. comm.), it will be necessary to isolate further YACs, PACs and BAC clones to try and fill this gap and to accurately map the markers within this region. As new clones are isolated from the AS critical region, these can be screened by cDNA selection or exon trapping experiments for potential genes. However, since neither of these techniques have proved successful in identifying an AS gene to date and the identification of patients with atypical deletions has also been of limited success, it may be more appropriate to adopt some of the screening techniques such as RLGS and modified cDNA subtraction hybridisation, although these techniques are only possible in mice and therefore rely on conservation between the human and mouse genes (section 5.2). Alternatively, a technique such as differential display (Liang and Pardee, 1992) could be used to compare mRNA isolated from AS patients with mRNA isolated from normal individuals, to look for differences in mRNA expression patterns. However, this technique would be limited to the analysis of mRNA from either peripheral blood lymphocytes or skin fibroblasts and is therefore reliant on the AS gene being expressed in these tissues.
Figure 72. A strategy for the diagnosis of AS and PWS.
Further studies of patients with imprintor mutations and their families will lead
to a greater understanding of genomic imprinting mechanisms on
chromosome 15q11-q13. As the IC is further characterised, this will increase
our understanding of its function and how it acts on imprinted genes in the
15q11-q13 region. It will be of great interest to determine how and when
imprint erasure and re-setting occurs, how SNRPN is involved in this process
and whether trans as well as cis factors are involved in the functioning of the
IC. Mouse chromosome 7 shows homology to the human 15q11-q13 region
(section 1.4.5.1) and as the human germline is not accessible, a murine
model will need to be developed to study these processes.

Further work of interest would be to accurately define the centromeric
breakpoints in patient DH and AS individual 13581. The centromeric
breakpoint in DH could mark the telomeric boundary of the AS critical region
and is poorly defined at present. As the PAC contig across the region is
constructed, analysis of restriction enzyme fragments from the PAC clones
could be used to identify a junction fragment in DH, to accurately determine
the telomeric boundary of the AS critical region as defined by this patient.
The centromeric breakpoint in 13581 could mark the telomeric boundary for
the IC in AS, since the methylation pattern at PW71 (D15S63) was normal in
this individual and deletions distal to this may not alter the DNA methylation
patterns at loci throughout 15q11-q13. It will therefore be important to
determine whether the deletion in this individual affects the IC or whether only
the AS gene itself is affected. This may help to identify the exact region of
15q11-q13 that is critical for the regulation of the exclusive maternal
expression of the AS gene and that which is critical for the AS structural gene.
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Angelman syndrome (AS) is a neurogenetic disorder arising from a lack of genetic contribution from the maternal chromosome 15q11–13. To date, the AS critical region has been defined by an inherited deletion of approximately 1.5 Mb, spanning the 3-21 (D15S10), LS6-1 (D15S113) and GABRB3 loci. We have identified an individual with the typical features of AS who has a deletion of the maternal chromosome which encompasses LS6-1, but does not extend to either flanking marker. This deletion, initially detected by (CA), repeat analysis, was further characterised by fluorescence in situ hybridisation (FISH) using cosmids derived from a 260 kb LS6-1 yeast artificial chromosome (YAC). Neither end cosmids from this YAC clone falls within the deletion, suggesting that the minimal AS region is less than 200 kb. We also studied three loci within 15q11–13 which detect parent-of-origin specific DNA methylation imprints, and found that both normal maternal and paternal patterns were present in this patient.

INTRODUCTION

Angelman syndrome (AS) is characterised by mental retardation, ataxia, absent speech, seizures, inappropriate laughter and hypopigmentation (1). Its incidence is estimated to be 1 in 20,000 (2). AS is associated with a lack of genetic contribution from maternal chromosome 15q11–13, usually arising from a deletion of the maternally derived chromosome (3–7), but occasionally through paternal uniparental disomy (8). Approximately 20% of AS patients, including almost all familial cases, have no detectable chromosomal abnormality (9–12). The AS region is close to, but distinct from that for Prader–Willi syndrome (PWS) (13), a condition associated with a lack of paternal contribution from 15q11–13 (3,14,15). The features of PWS, mental retardation, hypogonadism, infantile hypotonia and obesity (16), are very different from those of AS. This suggests that the disorders are caused by deficiencies of closely linked, oppositely imprinted genes.

The PWS region, which lies centromeric to the AS region (17–19), includes the gene encoding the small nuclear ribonucleoprotein associated polypeptide N (SNRPN) (20). It has been shown that SNRPN, which is involved in RNA splicing in the brain (21), is only expressed from the paternal chromosome in both mouse (22,23) and human (24–26). SNRPN is thus a good candidate gene for PWS, although a lack of SNRPN expression alone is probably not sufficient to cause all the symptoms of PWS. Unlike AS, in which some patients have no detectable chromosomal lesion, all typical PWS patients have either a detectable paternal deletion of 15q11–13, or maternal uniparental disomy of this region (14,17,28). This difference suggests that although AS may possibly arise from a mutation in one putative gene, a mutation solely of SNRPN does not result in typical PWS.

In addition to its functional imprint, SNRPN has an epigenetic, in the form of a parent-of-origin specific DNA methylation pattern (24). Similar methylation imprints have been described for two loci which lie centromeric to SNRPN; the ZNF127 gene (D15S9, DN34) (29) and the anonymous DNA marker PW71 (D15S63) (30,31). The precise role of methylation in genomic imprinting has yet to be elucidated, though methylation of the paternal SNRPN allele is associated with a lack of its expression (24).

The AS region is approximately 1.5 Mb, as defined by an affected family carrying a small inherited deletion (13,32), and another patient with an unbalanced translocation (33). The latter excludes the B3 and A5 subunits of the γ-aminobutyric acid receptor (GABR) as candidate loci responsible for AS. Three further expressed sequences have been isolated from within this critical AS region, though no evidence for their differential parental expression has yet been found (26). The entire PWS/AS region has now been isolated in a 3.5 Mb YAC contig (34), which will assist in the identification of novel coding sequences.

In an attempt to further delineate the AS region, we used several (CA), repeat markers (the locations of which are shown in Fig. 3) to study patients with no detectable chromosomal abnormalities. We report the identification of an AS patient with a microdeletion which includes the LS6-1 (D15S113) (28) locus, but does not extend to either 3-21 (D15S10) (35) or GABRB3 (36). FISH analysis, using cosmids derived from an LS6-1 positive YAC, was subsequently carried out to confirm and estimate the extent of this deletion. We also studied the DNA methylation imprints at the ZNF127, PW71B and SNRPN loci in the patient, to investigate the possibility that this microdeletion
might result in the loss of sequences involved in the epigenetic modification of the AS region, rather than an AS structural gene.

RESULTS

(CA)n repeat and DNA methylation analysis
The AS patient 3731, and her unaffected mother were found to be heterozygous for the IR43R (37), TD3-21 (35), MS14 (38) and GABRB3 (36) (CA)n repeats. However, 3731 has not inherited a maternal allele for LS6-1 (28) (Fig. 1). The patient’s mother is homozygous for the LS6-1 marker, so it could not be determined whether or not she also carries the deletion. DNA methylation analysis of ZNF127, PW71B and SNRPN in the patient’s DNA revealed that both the paternal and maternal imprints were present at all three loci (not shown).

FISH analysis
The apparent deletion of the LS6-1 marker in patient 3731 may have been the result of non-amplification of the maternal allele, perhaps caused by a polymorphism within one of the primer binding sites. FISH was thus used to confirm and further characterise the deletion, initially using a 260 kb LS6-1-positive YAC, 11HE12. This clone gave a positive hybridisation signal on both chromosome 15s (not shown), suggesting that at least one of the deletion breakpoints lay within it. YAC 11HE12 was subcloned, and cosmids for the LS6-1 locus and both ends of the YAC were isolated. Fig. 3 shows the positions of these cosmids, within a restriction map of 11HE12. As shown in Fig. 2, the deletion of LS6-1 was confirmed by FISH, since the cosmid containing this marker (E24) is deleted in patient 3731, whilst two copies are present in the normal control. However, cosmids derived from the left and right ends of 11HE12, E19 and E16 respectively, are not deleted, suggesting that the microdeletion lies entirely within this YAC clone. E19 and E16 were also hybridised to chromosomes from one of the patients whose ~1.5 Mb deletion previously defined the AS region (13) (Fig. 2). As expected, both cosmids were deleted in this control, minimising the concern that E19 and/or E16 contain repeats specific to 15q11–13, which would give misleading results. The FISH results indicate that the deletion associated with AS in patient 3731 is no larger than 200 kb (Fig. 3).

DISCUSSION
We have determined that the maternal deletion within 15q11–13 associated with the typical features of AS in patient 3731 is less than 200 kb. The region may be further delineated by the AS patient carrying an unbalanced translocation t(4;15) (33), since it has been shown that this patient is intact for the LS6-1 locus (44). However, such deletion overlap regions need to be interpreted with caution, since for reasons outlined below, more than one AS ‘critical region’ may exist. Furthermore, the microdeletion in patient 3731 may be part of a more complex rearrangement involving more than one locus within 15q11–13. The possibility that a particular chromosomal lesion merely coincides with the occurrence of AS in an individual also remains. The relevance of such lesions will only be established by analysis of further patients.

Since the AS locus is imprinted, it is possible that the deletion in patient 3731 defines the critical region not for the AS gene

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**Figure 1.** Detection of a microdeletion in patient 3731 using 5 (CA)n repeat markers from the AS/PWS region within 15q11–13. The patient is heterozygous at the IR43R, TD3-21, MS14, and GABRB3 loci, but not LS6-1, where no maternal allele is present. The maternal allele for all the other markers is marked with an arrowhead, except TD3-21, at which both patient and mother are heterozygous for the same two alleles.

**Figure 2.** Detection of the microdeletion in chromosomes from patient 3731 using FISH. The cosmid from the AS region is marked by a white arrow in each case; the other signal present is a control cosmid from 15q26.1. (a) E24 hybridised to 3731, (b) E24 hybridised to a normal control. (c) E19 hybridised to 3731, (d) E19 hybridised to an AS deletion control. (e) E16 hybridised to 3731, (f) E16 hybridised to an AS deletion control.
itself, but for sequences controlling its exclusively maternal expression. It has recently been suggested that disruptions of such ‘imprinting control elements’ (ICEs) may constitute the molecular defect in some AS and atypical PWS patients (39). In these rare cases, aberrant methylation patterns, which are thought to reflect a failure of the imprinting signal, are found at several loci within 15q11–13 (40, 41). In AS individuals of this type, both the maternal and paternal chromosomes carry a paternal methylation imprint, and it is proposed that this results in the inactivation of an otherwise intact AS gene. To investigate this possibility, we looked at DNA methylation at the ZNF127, PW71B and SNRPN loci in lymphocyte DNA from patient 3731, and found that both paternal and maternal patterns were present at all three loci. This implies that chromosomes carrying the normal maternal and paternal imprints are present, and suggests that the < 200 kb deletion may disrupt an unidentified AS gene, rather than imprinting sequences. In contrast, the inherited 1.5 Mb microdeletion described by Saitoh et al. is accompanied by aberrant methylation at the ZNF127 locus (40). One possible explanation of these results is that the larger deletion includes both a structural gene(s) and an element involved in imprinting of the AS region, whereas the smaller, internal deletion described here disrupts only the AS gene. This suggests that the AS gene and a putative imprinting locus both lie within 1.5 Mb of 15q11–13. However, since the mechanisms involved in the control and maintenance of imprinting have yet to be elucidated, it is not possible to draw any definite conclusions. In addition to DNA methylation, replication timing is also thought to play a central role in imprinting (42, 43).

Although no expressed sequences have yet been identified which map within the microdeletion reported here, the cosmids spanning the region will provide a resource for the isolation of candidate AS genes. Furthermore, the LS6-1 cosmid (E24) will be useful for detecting unusual deletions in AS patients, in both diagnostic and research situations.

**MATERIALS AND METHODS**

**Patient details**
Patient 3731 is the third child of healthy parents; her two siblings are unaffected. The neonatal period was uneventful, but she was not sitting at ten months of age, and by two years global developmental delay was apparent. She has marked truncal hypotonia and scoliosis, and has never been able to walk or sit unaided. Her voluntary movements are jerky and she has never developed any speech. She has blonde hair, blue eyes with pale fundi, microbrachycephaly, a wide, smiling mouth and a prominent chin. An EEG performed at 5 years of age showed the slow wave changes characteristic of AS. High resolution chromosome analysis was normal.

**CA**<sub>n</sub> repeats
DNA from the patient and her mother was analysed with six (CA)<sub>n</sub> repeats; IR43R (D15S11) (37), 3-21 (D15S10) (35), LS6-1 (D15S113) (28), GABRB3

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**Figure 3.** A map of the AS/PWS region within 15q11–13 (34), showing the positions of the (CA)<sub>n</sub> repeat markers used in this study (indicated with an asterisk), and the 11HE12 YAC clone. Also indicated are the 1.5 Mb deletion which has defined the AS region to date (13), and the minimum region of deletion overlap for PWS (13, 17). Above the map of the entire region is a restriction map of YAC 11HE12 (orientation unknown), showing the locations of the E19 (right-hand end), E24 (LS6-1) and E16 (left-hand end) cosmids which were used for FISH analysis. All the MluI and BssHII sites present within the YAC clone are shown, but many SacII sites were present. Consequently, only the SacII fragments detected by the left and right YAC vector probes are shown on this map.
Methylation studies

The possible presence of abnormal methylation patterns within 15q11-13 in patient 3731 was investigated using the probes ZNF127 (D15S9) (29), PWT1B (D15S65) (31) and SNRPN (24). 5 μg of lymphocyte DNA was digested with the relevant enzymes, and transferred to Hybond N+ (Amersham) membranes by capillary blotting. After alkali fixation in 0.4 M sodium hydroxide for 15 minutes, the filters were hybridised, washed and exposed as described in the published methods.

YAC characterisation

YAC clone 11HE12 was isolated using the LS6-1 primers, from the ICI total human library via PCR analysis of successive pools (45). High molecular weight DNA in agarose blocks was prepared according to the method of Anand et al. (45). Sizing and restriction mapping were carried out according to standard methods, using pulsed-field gel electrophoresis. In addition, some digests were separated by field inversion gel electrophoresis, using the Biorad FIEG Mapper™ system, and an 8–48 kb size marker (Biorad).

Cosmid library construction and characterisation

The 11HE12 YAC clone was subcloned into the Superco (Stratagene) cosmid vector. Total YAC DNA in agarose blocks was partially digested with Mbol to give fragments >50 kb. The digested DNA in molten agarose was then dephosphorylated, and treated with 0.05× volume of 1 M NTA (nitritolii-acetic acid, Sigma) to incubate the alkaline phosphate. Following two phenol extractions, one chloroform extraction and ethanol precipitation, 0.5 μg of the recovered DNA was cloned into the BamHI site of Superco, and packaged using the Gigapack II Gold packaging extract (Stratagene). 1–1013 recombinants were obtained, and 42 cosmids positive with radiolabelled total human DNA were isolated for further study. Since the average insert size was 40 kb, this represented a 0.5-fold coverage of the YAC. From this subset, a clone positive for LS6-1 (E24) and the right-hand end (E19) of the YAC were isolated, but no cosmids containing the left-hand end were present in the library. However, by hybridising whole cosmids to digested YAC DNA, a clone was identified which localised to the 60 kb SacII fragment detected by the left-hand YAC vector probe (E16). A complete cosmid contig representing the entire YAC could not be constructed, as approximately 80 kb of 11HE12 was not represented in the library. This may have been caused by an uneven distribution of Mbol sites in the region, leaving some regions uncloned. Alternatively, some human-positive cosmids may not have contained much repetitive DNA, and so would not have been detected by the human DNA screen, even though both strongly and weakly hybridising colonies were isolated.

FISH experiments

Metaphase spreads were prepared from lymphoblastoid cell lines and PHA-stimulated lymphocytes according to standard protocols. Cosmid DNA was labelled with biotin by nick translation, using a commercially available kit (Gibco BRL). These images were captured and analysed on a Macintosh Quadra, using the IPLab extension software (Digital Scientific). To ascertain whether or not a cosmid was present in metaphase spreads were denatured in 70% formamide, 2×SSC for 2 minutes at 70°C, and then dehydrated in an ethanol series. The labelled cosmid probes were hybridised to the chromosomes overnight with biotin by nick translation, using a commercially available kit (Gibco BRL).

ACKNOWLEDGEMENTS

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Prepared by S.Rickard 12/95-3/96

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ANGELMAN SYNDROME DATABASE

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METHYLATION
RFLP
(CA)n
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| Case No. | Disorder Type | 6758 | 10404 | 6177 | 10356 | 7188 | 16766 | 5187 | 5188 | 8013 | 10407 | 5852 | 14528 | 14829 | 4517/5793 | 11507 | 4884/4249 | 11836 | 10899 | 11837 | 10407 | 11353 | 12497 | 9496 | 5373 | 9574 | 5960 | 4750 | 9950 | 5710 | 5449 | 5209 | 6592 | 9985 | 14928 | 6613 | 6865 | 6157 | 5832 | 5883 | 4744 | 5058 | 12497 | 9574 | 11993 | 6030 | 7135 | 9727 | 14927/14913 | 3792 | 9496 | 10448 | 11857 | 11856 | 8849 | 8331 | 10999 | 10442 | 105222 | 13822 |
|---------|--------------|-----|-------|------|-------|------|-------|------|-------|-----|-------|------|-------|------|-------|--------|--------|-------|------|-------|------|-------|------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------- |
| 13628 | Y | ND | ND | ND | ND | ND | HET | ND | ND | HET | MET | AS | ND | OD DEL | ND | ND | N | FAMILIAL | IMPRINTOR |
| 7020  | N | ND | ND | ND | ND | ND | ND | NR | HET | HET | HOM | ND | HET | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | NO DEL OR DISOMY |
| 61811 | N | HOM | DEL | ND | ND | HOM | ND | HOM | HR | ND | HOM | ND | HOM | ND | AS | ND | ND | DEL | ND | ND | ND | ND | N | OUT OF DNA | DELETION |
| 8568  | N | HET | HOM | ND | ND | HOM | HOM | HR | HET | HET | HET | HR | HOM | ND | HET | ND | ND | ND | ND | ND | ND | N | NO DEL OR DISOMY |
| 6509  | Y | HET | ND | ND | ND | HOM | DEL | ND | DEL | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 6282  | Y | HET | DEL | ND | ND | HOM | ND | ND | DEL | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 4711  | N | HET | HOM | ND | ND | ND | DEL | HOM | HET | HET | ND | HET | HET | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 7067  | N | HET | ND | ND | HOM | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 7425  | Y | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | OUT OF DNA | DELETION |
| 6241  | Y | HOM | DEL | ND | ND | HOM | ND | ND | HOM | ND | ND | HET | HET | HET | ND | AS | ND | ND | ND | ND | N | DELETION |
| 6180  | N | NR | NR | ND | ND | ND | HET | ND | NR | HET | HET | ND | HET | ND | ND | ND | ND | ND | ND | ND | N | Y |
| 6707  | N | HOM | HOM | ND | ND | ND | DEL | HOM | DEL | ND | DEL | DEL | DEL | DEL | ND | ND | ND | ND | ND | ND | N | DELETION |
| 6720  | N | HET | HOM | ND | ND | HOM | DEL | HET | HET | ND | ND | HOM | HOM | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 5218  | Y | HET | HET | ND | ND | HOM | HET | HET | ND | HET | HET | ND | HET | HET | ND | ND | ND | ND | ND | ND | N | DELETION |
| 5217  | Y | HET | HOM | HOM | ND | ND | HET | HET | HOM | HET | HET | ND | HET | HET | ND | ND | ND | ND | ND | ND | N | IMPRINTOR |
| 5352  | N | HET | HOM | ND | ND | HOM | ND | ND | HET | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 12454 | Y | HET | HOM | ND | ND | ND | ND | ND | HET | HET | HET | HET | HET | HET | HET | ND | ND | ND | ND | ND | N | DELETION |
| 6218  | N | HET | DEL | ND | ND | HOM | ND | ND | DEL | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N | DELETION |
| 3791  | Y | HET | HOM | ND | ND | HOM | DEL | HOM | HOM | ND | HET | HET | HET | HET | ND | ND | ND | ND | ND | ND | N | DELETION |

**ANGELMAN SYNDROME DATABASE**

*Prepared by S. Rickard 12/95-3/96 ANGELMAN SYNDROME DATABASE*