GENOMIC IMPRINTING
AND
SILVER-RUSSELL SYNDROME:
CANDIDATE GENES ON CHROMOSOME 7

Emma L. Wakeling

Institute of Child Health,
University of London,
30 Guilford Street,
London WC1N 1EH

Submitted for the degree of
Doctor of Philosophy
to the University of London
June 1999
Silver-Russell syndrome (SRS) is characterised by intrauterine and postnatal growth retardation, triangular facies, fifth finger clinodactyly and lateral asymmetry. Maternal uniparental disomy of chromosome 7 (mUDP7) has been demonstrated in approximately 7% of patients. No consistently isodisomic regions have been found. It is likely, therefore, that one or more imprinted gene(s) on chromosome 7 are involved in SRS. Five candidate genes located on this chromosome have been studied.

The human chromosomal region 7p11.2-13 is homologous to an imprinted region on mouse proximal chromosome 11. Maternal UPD for this region leads to mice with prenatal growth failure. Three growth-related genes, IGFBP1 and 3 (insulin-like growth factor binding proteins 1 and 3) and EGFR (epidermal growth factor receptor), lie within this region and have been proposed as candidates for SRS. The imprinting status of these genes was previously unknown. Their parental allele expression was therefore investigated in normal fetal tissues using transcribed polymorphisms. Biallelic expression of all three genes was observed. Expression of IGFBP3 and EGFR was also seen in cell lines from mUPD7 patients. Since no evidence for imprinting of these three genes was found, their involvement in SRS seems unlikely.

Recently, MEST (mesoderm specific transcript) became the first imprinted gene to be identified on chromosome 7. Its possible role in SRS was investigated by studying allelic methylation patterns using methylation-specific PCR. Normal differential methylation of MEST was found in 46, non-UPD SRS patients. It is therefore unlikely that this gene plays a major role in SRS.

Finally, the growth suppressing gene GRB10 (growth factor receptor binding protein 10) has also recently been proposed as a candidate for SRS. A de novo duplication of 7p11.2-p12, including GRB10, was observed in one SRS proband, providing further support for a role for this gene in SRS. However, Southern blot hybridisation showed normal GRB10 dosage in 36 other, non-UPD patients. Further investigation into the potential role of GRB10 and/or other genes within the region 7p11.2-p12 is needed.
Acknowledgements

First and foremost I would like to thank both Michael Preece and Gudrun Moore for all the work they have put into supervising this project. Their continuous support, encouragement and advice has always been greatly appreciated.

I started work at Queen Charlotte’s expecting to only stay for three months. Now, over three years later, I would like to thank everyone in the laboratory for making my time there so enjoyable. I owe many people for their valuable suggestions and help along the way, including Sayeda Abu-Amero, Phil Stanier, Mrs Ali, Megan Hitchins, Dave Monk, Donna Slater, Vikki Allport, William Dennes, Claire Braybrook, Jane Edelstone, Kit Doudney, Hilary O’Donnell, Cesare Campagnoli and Richard Scrivener.

This study would not have been possible without the cooperation of the Silver-Russell syndrome patients and their families for which I am very grateful. I would particularly like to thank the family of patient 49 and Eric Schmitt for his time spent liaising with them.

Thanks to Steve Sherer for making me so welcome for two weeks at the Hospital for Sick Children in Toronto and for his continuing help with the project. I am also grateful to several others for their contribution to this study: Sue Price for her clinical expertise; Sue Blunt for helping to generate fibroblast cell cultures; Rodney Meredith and Terry Ballard for help with karyotypic analysis; and the medical staff at Queen Charlotte’s Hospital for their efforts collecting maternal blood samples. I would especially like to thank Louis Lefebvre for his technical assistance optimising the methylation-specific PCR assay and, more generally, for many thought-provoking discussions.

Special thanks must go to those closest to me. Firstly to my husband, Phil, for his constant optimism and patience when mine failed. His encouragement and time spent proof-reading has made the writing of this thesis much easier. Particular thanks also goes to my parents for their love and continual support. Finally, I must mention Joe whose baby distractions have kept life in perspective over the last few months.

This work was supported by funding from Action Research and from Children Nationwide and Pharmacia and Upjohn.
Publications


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>Angelman syndrome</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BWS</td>
<td>Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-guanine</td>
</tr>
<tr>
<td>CPM</td>
<td>confined placental mosaicism</td>
</tr>
<tr>
<td>CSHl</td>
<td>chorionic somatomammotrophin hormone 1</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GRB10</td>
<td>growth factor receptor binding protein</td>
</tr>
<tr>
<td>IC</td>
<td>imprinting centre</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IGF1R</td>
<td>insulin-like growth factor-I receptor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IPL</td>
<td>imprinted in placenta and liver</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IUGR</td>
<td>intrauterine growth restriction</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>Meg1</td>
<td>maternally expressed gene 1</td>
</tr>
<tr>
<td>MEST</td>
<td>mesoderm-specific transcript</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinosropanesulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mUPD</td>
<td>maternal UPD</td>
</tr>
<tr>
<td>pUPD</td>
<td>paternal UPD</td>
</tr>
<tr>
<td>PAC</td>
<td>phage PI-derived artificial chromosome</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Peg1</td>
<td>paternally expressed gene 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PWS</td>
<td>Prader-Willi syndrome</td>
</tr>
<tr>
<td>QCCH</td>
<td>Queen Charlotte’s and Chelsea Hospital</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SNRPN</td>
<td>small nuclear ribonucleic protein N</td>
</tr>
<tr>
<td>SRS</td>
<td>Silver-Russell syndrome</td>
</tr>
<tr>
<td>SSCP</td>
<td>single-stranded conformational polymorphism</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor α</td>
</tr>
<tr>
<td>TNDM</td>
<td>transient neonatal diabetes mellitus</td>
</tr>
<tr>
<td>UPD</td>
<td>uniparental disomy</td>
</tr>
<tr>
<td>URL</td>
<td>uniform resource locator</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumour 1</td>
</tr>
<tr>
<td>XIC</td>
<td>X-inactivation centre</td>
</tr>
<tr>
<td>XIST</td>
<td>X-inactive specific transcript</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td></td>
<td>SI units have been used throughout.</td>
</tr>
</tbody>
</table>

**Abbreviations**

- **EBV**: Epstein Barr virus
- **LB**: Luria-Bertani
- **MMLV**: Moloney murine leukaemia virus
- **OLB**: oligonucleotide labelling buffer
- **TEMED**: N,N',N'‐tetramethyl‐1,2‐diaminoethane
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Publications</td>
<td>4</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>5</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>7</td>
</tr>
<tr>
<td>List of Tables</td>
<td>15</td>
</tr>
<tr>
<td>List of Figures</td>
<td>16</td>
</tr>
</tbody>
</table>

## Chapter 1

**Introduction**

1.1 Intrauterine growth restriction (IUGR)                          20

1.2 Silver-Russell syndrome                                          21

1.2.1 The Silver-Russell syndrome phenotype                          21

1.2.1.1 Postnatal growth retardation and pubertal development       24

1.2.1.2 Facial dysmorphism                                           25

1.2.1.3 Lateral asymmetry                                            26

1.2.1.4 Cognitive ability                                            26

1.2.2 Genetic heterogeneity in Silver-Russell syndrome               27

1.2.2.1 Familial inheritance                                         27

1.2.2.2 Chromosomal abnormalities                                   29

1.2.2.3 Maternal uniparental disomy for chromosome 7                 30

1.3 Uniparental disomy (UPD)                                         32

1.3.1 Mechanisms of UPD                                              33

1.3.2 Phenotypic effects of mUPD7                                    35

1.3.2.1 Confined placental mosaicism                               35

1.3.2.2 Reduction to homozygosity                                   36

1.3.2.3 Imprinting effects                                           37
1.4 Imprinting: background evidence

1.4.1 Pronuclear transplantation, hydatidiform moles and triploidy

1.4.2 Mouse imprinting phenotypes

1.4.2.1 Mouse imprinted regions homologous to human chromosome 7

1.5 Imprinting disorders in man

1.5.1 Prader-Willi and Angelman syndromes

1.5.2 Beckwith-Wiedemann syndrome

1.6 Imprinting mechanisms

1.6.1 DNA methylation

1.6.1.1 Developmental regulation of methylation

1.6.1.2 Differential methylation of imprinted genes

1.6.1.3 Evidence for methylation as a genomic imprint

1.6.2 Chromatin structure

1.6.3 Local and regional imprinting control mechanisms

1.6.3.1 Local control mechanisms

1.6.3.2 Regional control mechanisms

1.6.3.3 Implications for other imprinted regions

1.6.4 Imprinting plasticity

1.6.4.1 Developmental, tissue and species-specific imprinting

1.6.4.2 Somatic allele switching

1.6.4.3 Differential usage of parental alleles

1.7 Imprinting: a role in growth regulation

1.7.1 The parent-offspring conflict hypothesis

1.8 Summary

Chapter 2

Experimental aims and general strategy

2.1 Candidate genes

2.1.1 IGFBP1, IGFBP3 and EGFR

2.1.1.1 Investigation of expression in fetal tissues

2.1.1.2 Identification of expressed polymorphisms

2.1.1.3 Investigation of parental allele expression
Chapter 3
Materials
3.1 Silver-Russell Syndrome patients
  3.1.1 SRS cohort
  3.1.2 Patient 49: case report
3.2 Fetal tissues
3.3 Reagents
3.4 Cell culture media
3.5 Enzymes
3.6 Radioisotopes
3.7 Gel electrophoresis
3.8 Photography, blotting and autoradiography
3.9 Transformations
3.10 Fluorescence in situ hybridisation (FISH) equipment
3.11 Oligonucleotides
3.12 Clones

Chapter 4
Methods
4.1 Solutions
4.2 DNA Protocols
  4.2.1 Extraction of DNA from peripheral blood leucocytes
  4.2.2 Extraction of DNA from fetal tissue
  4.2.3 Small scale preparation of plasmid/cosmid DNA (miniprep)
  4.2.4 Large scale preparation of cosmid/PAC DNA (midiprep)
  4.2.5 Determination of DNA concentration
  4.2.6 Restriction enzyme digestion of DNA
  4.2.7 Agarose gel electrophoresis
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.8</td>
<td>Polyacrylamide gel electrophoresis</td>
<td>85</td>
</tr>
<tr>
<td>4.2.8.1</td>
<td>Denaturing polyacrylamide gel electrophoresis</td>
<td>86</td>
</tr>
<tr>
<td>4.2.8.2</td>
<td>Non-denaturing polyacrylamide gel electrophoresis</td>
<td>87</td>
</tr>
<tr>
<td>4.2.9</td>
<td>Southern blotting</td>
<td>87</td>
</tr>
<tr>
<td>4.2.10</td>
<td>DNA radiolabelling</td>
<td>88</td>
</tr>
<tr>
<td>4.2.10.1</td>
<td>Labelling of double-stranded DNA probes</td>
<td>88</td>
</tr>
<tr>
<td>4.2.10.2</td>
<td>Removal of unincorporated nucleotides from labelled probe</td>
<td>88</td>
</tr>
<tr>
<td>4.2.10.3</td>
<td>Competition of labelled probe</td>
<td>89</td>
</tr>
<tr>
<td>4.2.11</td>
<td>Southern hybridisation</td>
<td>89</td>
</tr>
<tr>
<td>4.2.11.1</td>
<td>Prehybridisation</td>
<td>89</td>
</tr>
<tr>
<td>4.2.11.2</td>
<td>Hybridisation</td>
<td>89</td>
</tr>
<tr>
<td>4.2.11.3</td>
<td>Removal of unbound probe (washing procedure)</td>
<td>90</td>
</tr>
<tr>
<td>4.2.11.4</td>
<td>Removal of probe for re-hybridisation of membranes</td>
<td>90</td>
</tr>
<tr>
<td>4.2.11.5</td>
<td>Quantitative analysis using the PhosphorImager</td>
<td>90</td>
</tr>
<tr>
<td>4.2.12</td>
<td>Polymerase chain reaction (PCR)</td>
<td>90</td>
</tr>
<tr>
<td>4.2.13</td>
<td>Single-stranded conformational polymorphism (SSCP) analysis</td>
<td>91</td>
</tr>
<tr>
<td>4.2.13.1</td>
<td>Vertical gel electrophoresis with radiolabelled PCR products</td>
<td>92</td>
</tr>
<tr>
<td>4.2.13.2</td>
<td>Horizontal gel electrophoresis</td>
<td>92</td>
</tr>
<tr>
<td>4.2.13.3</td>
<td>Silver staining</td>
<td>93</td>
</tr>
<tr>
<td>4.2.14</td>
<td>Subcloning of PCR products</td>
<td>93</td>
</tr>
<tr>
<td>4.2.14.1</td>
<td>Preparation of PCR products</td>
<td>93</td>
</tr>
<tr>
<td>4.2.14.2</td>
<td>Ligation reaction</td>
<td>94</td>
</tr>
<tr>
<td>4.2.14.3</td>
<td>Preparation of competent cells</td>
<td>94</td>
</tr>
<tr>
<td>4.2.14.4</td>
<td>Transformation of ligated products</td>
<td>94</td>
</tr>
<tr>
<td>4.2.15</td>
<td>DNA sequencing</td>
<td>95</td>
</tr>
<tr>
<td>4.2.15.1</td>
<td>Sequencing of PCR-amplified DNA</td>
<td>95</td>
</tr>
<tr>
<td>4.2.15.2</td>
<td>Sequencing of plasmid DNA</td>
<td>96</td>
</tr>
<tr>
<td>4.2.15.3</td>
<td>Automated sequencing</td>
<td>97</td>
</tr>
<tr>
<td>4.2.16</td>
<td>'Clean-up' protocols for PCR products</td>
<td>97</td>
</tr>
<tr>
<td>4.2.16.1</td>
<td>PEG precipitation</td>
<td>98</td>
</tr>
<tr>
<td>4.2.16.2</td>
<td>Gene Clean procedure</td>
<td>98</td>
</tr>
<tr>
<td>4.2.17</td>
<td>Sodium bisulfite treatment of DNA</td>
<td>98</td>
</tr>
</tbody>
</table>
4.3 Preparation of cell lines and cytogenetic analysis
   4.3.1 Transformed lymphoblastoid cell lines
   4.3.2 Fibroblast cell lines
   4.3.3 Preparation of lymphocytes from blood
   4.3.4 Cytogenetic analysis

4.4 RNA protocols
   4.4.1 Preparation of solutions and equipment
   4.4.2 Extraction of total RNA
      4.4.2.1 Fetal tissue
      4.4.2.2 Cell pellets
   4.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)
   4.4.4 Electrophoresis of RNA
   4.4.5 Northern blotting
   4.4.6 Northern hybridisation
      4.4.6.1 Prehybridisation
      4.4.6.2 Hybridisation
      4.4.6.3 Removal of unbound probe (washing procedure)

4.5 Fluorescence in situ hybridisation (FISH)
   4.5.1 Preparation of metaphase spreads
   4.5.2 Nick translation
   4.5.3 FISH

Chapter 5
IGFBP1 and IGFBP3

5.1 Introduction
5.2 Experimental strategy
5.3 Expression of IGFBP1 and IGFBP3 in first and second trimester tissues
   5.3.1 Northern analysis
   5.3.2 RT-PCR
      5.3.2.1 IGFBP1
      5.3.2.2 IGFBP3
   5.4 Demonstration of IGF2 imprinting using SSCP analysis
5.5 Investigation of IGFBP1 imprinting status

5.5.1 Screening for IGFBP1 polymorphism using SSCP analysis

5.5.2 Demonstration of IGFBP1 polymorphism by direct sequencing

5.5.3 Investigation of IGFBP1 imprinting status in fetal liver

5.6 Investigation of IGFBP3 imprinting status

5.6.1 Screening for IGFBP3 polymorphism using SSCP analysis

5.6.2 Demonstration of IGFBP3 polymorphism by direct sequencing

5.6.3 Investigation of IGFBP3 imprinting status in fetal tissues

5.7 Expression of IGFBP1 and IGFBP3 in mUPD7 patients

5.7.1 IGFBP1

5.7.2 IGFBP3

5.8 Summary

Chapter 6

EGFR

6.1 Introduction

6.2 Experimental strategy

6.3 Expression of EGFR in first and second trimester fetal tissues

6.4 Identification of informative fetal samples using an expressed EGFR polymorphism

6.5 Investigation of EGFR imprinting status in fetal tissues

6.5.1 Demonstration of biallelic expression of EGFR

6.5.2 Search for contaminating maternal DNA in fetal samples

6.6 Expression of EGFR in SRS patients with mUPD7

6.7 Summary

Chapter 7

MEST

7.1 Introduction

7.2 Experimental strategy

7.2.1 Analysis of MEST allelic methylation in SRS patients

7.2.2 Analysis of MEST expression in SRS cell lines

12
7.3 Methylation analysis in SRS patients

7.3.1 Southern blot analysis 150

7.3.2 Methylation-specific PCR 153

7.3.2.1 Design of parent-specific primers 153

7.3.2.2 Optimisation of PCR conditions 156

7.3.2.3 Investigation of parental allele methylation in a cohort of SRS patients 157

7.3.2.4 Exclusion of suspected mUPD7 using methylation-specific PCR 157

7.4 Analysis of MEST imprinting status in cell lines 162

7.4.1 MEST imprinting status in normal cell lines 162

7.4.2 Expression of MEST in SRS patients 165

7.4.3 Comparison of MEST expression in cell lines and fetal tissues 167

7.5 Summary 168

Chapter 8

GRB10 170

8.1 Introduction 170

8.2 Experimental strategy 172

8.3 De novo 7p11.2-p12 duplication in a SRS patient 174

8.3.1 Cytogenetic analysis 174

8.3.2 Fluorescence in situ hybridisation (FISH) studies 175

8.4 Investigation of GRB10 dosage by Southern blot analysis 177

8.4.1 Optimisation of Southern blot hybridisation 177

8.4.1.1 GRB10 probe 177

8.4.1.2 Restriction enzymes 178

8.4.1.3 Control probe, A6121-1 179

8.4.2 Quantitative analysis of GRB10 dosage in SRS patients 181

8.4.2.1 Calculation of gene dosage 181

8.4.2.2 Gene dosage in SRS cohort 182

8.5 Summary 186
Chapter 9

Discussion 187

9.1 *IGFBP1, IGFBP3* and *EGFR* 187

9.2 *MEST* 193

9.2.1 Allelic methylation in SRS patients 193

9.2.2 *MEST* expression in cell lines 196

9.3 *GRB10* 201

9.4 Conclusion 207

9.5 Future Work 209

Summary 211

References 213

Appendices 237
List of Tables

Chapter 1. Introduction
Table 1.1 Frequency of symptoms and signs in Silver-Russell syndrome 24
Table 1.2 Case reports of mUPD7 in association with growth failure 32
Table 1.3 Phenotypes associated with mouse imprinted regions 41
Table 1.4 Molecular mechanisms in Prader-Willi and Angelman syndromes 45
Table 1.5 Genetic groups in Beckwith-Wiedemann syndrome 48

Chapter 3. Materials
Table 3.1 Primer sequences 76
Table 3.2 cDNA clones 78
Table 3.3 Genomic clones 78

Chapter 5. IGFBP1 and IGFBP3
Table 5.1 Potential polymorphisms within the 3' UTR of IGFBP3 125

Chapter 6. EGFR
Table 6.1 Biallelic expression of EGFR in tissues from five heterozygous fetuses 144

Chapter 7. MEST
Table 7.1 Analysis of allele inheritance in patient K using tetranucleotide markers from chromosome 7q 161

Chapter 8. GRB10
Table 8.1 Schematic diagram of the domain structure of human GRB10 171

Chapter 9. Discussion
Table 9.1 Clinical features of patients with partial trisomy 7p involving the region 7p11.2-p12 206
# List of Figures

**Chapter 1. Introduction**
- Figure 1.1 Clinical features of Silver-Russell syndrome 23
- Figure 1.2 Mechanisms by which uniparental disomy may arise 34
- Figure 1.3 Autoradiograph of Southern blot showing parental allele inheritance in three SRS patients 35
- Figure 1.4 Distribution of hetero- and isodisomy on chromosome 7 for five SRS probands with mUPD7 38
- Figure 1.5 Mouse imprinting maps of chromosomes 6 and 11 43
- Figure 1.6 Inheritance of imprinting centre (IC) mutations 46
- Figure 1.7 Temporal changes in DNA methylation during development 50

**Chapter 2. Experimental aims and general strategy**
- Figure 2.1 Ideogram of human chromosome 7 illustrating regions homologous to mouse imprinted regions 66

**Chapter 3. Materials**
- Figure 3.1 Patient 49 and her mother 72

**Chapter 5. IGFBP1 and IGFBP3**
- Figure 5.1 Northern analysis of IGFBP1 and IGFBP3 in mid-trimester fetal liver 111
- Figure 5.2 Gel electrophoresis of RT-PCR products showing GAPDH expression in fetal tissues 113
- Figure 5.3 Gel electrophoresis of RT-PCR products showing IGFBP1 expression in fetal tissues 113
- Figure 5.4 Gel electrophoresis of RT-PCR products showing IGFBP3 expression in fetal tissues 115
- Figure 5.5 Demonstration of IGF2 imprinting using ApaI polymorphism 117
- Figure 5.6 Demonstration of IGF2 imprinting by SSCP analysis 118
- Figure 5.7 Sequence of IGFBP1 surrounding polymorphic site in exon 4 119
Figure 5.8  SSCP analysis of 167 bp region predicted to contain IGFBP1 Polymorphism

Figure 5.9  Demonstration of polymorphism within exon 4 of IGFBP1

Figure 5.10  Biallelic expression of IGFBP1 in fetal liver, demonstrated using primers IGFBP1-BF and –ER

Figure 5.11  Biallelic expression of IGFBP1 in fetal liver, demonstrated using primers IGFBP1-EF and –ER

Figure 5.12  Sequence of IGFBP3 exon 5

Figure 5.13  Schematic diagram of IGFBP3 showing position of primers and restriction sites used to screen exon 5 by SSCP analysis

Figure 5.14  Demonstration of an expressed polymorphism within the 3’ UTR of IGFBP3

Figure 5.15  Biallelic expression of IGFBP3 in brain from one 10 week heterozygous fetus

Figure 5.16  Gel electrophoresis of RT-PCR products showing IGFBP3 expression in fibroblasts from UPD and non-UPD SRS patients

Chapter 6. EGFR

Figure 6.1  Gel electrophoresis of RT-PCR products showing EGFR expression in fetal tissues

Figure 6.2  Schematic map of EGFR cDNA

Figure 6.3  Polymorphism for BstNI restriction site within EGFR demonstrated using primer pair B1/B2

Figure 6.4  Polymorphism for BstNI restriction site within EGFR demonstrated using primer pair A1/A2

Figure 6.5  Biallelic expression of EGFR in placenta from heterozygous fetus, F17

Figure 6.6  Evidence for contaminating maternal cDNA in one of two tissues from fetus F15

Figure 6.7  Gel electrophoresis of RT-PCR products showing EGFR expression in fibroblasts from UPD and non-UPD SRS patients
Chapter 7. MEST

Figure 7.1 Restriction map of the 4.3 kb HindIII probe for MEST 151
Figure 7.2 Methylation analysis of the 5' end of MEST by Southern blot hybridisation 152
Figure 7.3 Schematic representation of bisulfite treatment and amplification of methylated and unmethylated DNA 155
Figure 7.4 Methylation-specific PCR analysis of patients and normal controls 157
Figure 7.5 Exclusion of mUPD7 in patient K using methylation-specific PCR analysis 159
Figure 7.6 Investigation of allele inheritance in patient K using tetranucleotide markers located on chromosome 7q 160
Figure 7.7 Schematic diagram of MEST and position of AflIII polymorphism 162
Figure 7.8 Ethidium-bromide stained gel showing MEST imprinting status in cell lines from heterozygous control (G) 164
Figure 7.9 Autoradiograph showing MEST imprinting status in cell lines from heterozygous control (M) 165
Figure 7.10 MEST and GAPDH expression in fibroblasts from UPD and non-UPD SRS patients 166
Figure 7.11 Northern analysis of MEST expression in cell lines and fetal tissues 168

Chapter 8. GRB10

Figure 8.1 Schematic diagram of the domain structure of human GRB10 171
Figure 8.2 Partial karyotype with ideogram showing duplication of chromosome 7p11.2-12 in patient 49 174
Figure 8.3 FISH analysis showing duplication of GRB10 in patient 49 176
Figure 8.4 Southern blot showing hybridisation of GRB10 probe 179
Figure 8.5 Identification of XbaI fragment containing non-repetitive sequence for use as control probe in GRB10 dosage study 180
Figure 8.6 Gene dosage analysis using Southern blot hybridisation 182
Figure 8.7 Histograms showing the range of GRB10 to control signal ratios determined by quantitative analysis of Southern blot hybridisation 183
Figure 8.8  Gene dosage analysis in patient 49 and her family using Southern blot hybridisation

Figure 8.9  Southern blot demonstrating paternally inherited XbaI RFLP in patient 1
Chapter 1. Introduction

Silver-Russell syndrome (SRS) is characterised by intrauterine growth restriction* (IUGR), postnatal short stature, a triangular face and, in some cases, lateral asymmetry. It is recognised as a relatively common cause of growth failure in children presenting to paediatric growth clinics. The incidence may be as high as that of Turner syndrome (1 in 7000) since both present at similar rates. The aetiology of fetal growth is complex, involving both environmental and genetic factors. SRS is of particular interest to those studying the origins of prenatal growth failure for two reasons. Firstly, the characteristics that make up this syndrome have been well defined. Secondly, there is strong evidence for an underlying genetic cause. Recent studies indicate that it is one of a growing number of recognised disorders of genomic imprinting. For these reasons SRS is an excellent model for the investigation of the molecular mechanisms underlying fetal growth restriction.

1.1 Intrauterine growth restriction (IUGR)

IUGR has been defined as the failure of an infant to achieve his/her genetic growth potential in utero (Goldenberg and Cliver, 1997). In reality, however, it is impossible to determine growth potential. IUGR is generally diagnosed in infants with birth weights below the 10th centile who fall into the category known as ‘small for gestational age’ (SGA). However, not all SGA babies have IUGR since some merely represent the lower end of the normal range for birth weight.

It is now well recognised that poor fetal growth is not only a major cause of perinatal morbidity and mortality (Danielian et al., 1994) but is also associated with long-term sequelae such as hypertension, diabetes and coronary heart disease (Barker et al., 1989; Hales et al., 1991). Fetal growth is a complex, dynamic process controlled by a wide range of factors of maternal, placental and fetal origin (Bernstein and Divon, 1997). As a result, the aetiology of IUGR is also multifactorial. Maternal factors such as infection, nutrition, smoking, alcohol and medical conditions including hypertension, diabetes and

* The term growth retardation is increasingly being replaced by growth restriction. In this thesis the two are used interchangeably.
collagen vascular disease have all been implicated. Placental pathology such as abnormal implantation or abruption can also give rise to IUGR. Fetal risk factors for poor growth include multiple pregnancy, chromosomal abnormality and other genetic disorders. The cause of IUGR in most infants, however, remains undetermined.

The interplay between genetic and environmental factors in the regulation of fetal growth is currently poorly understood. In one study, chromosomal abnormalities were identified in 7% of IUGR fetuses where the abdominal circumference was below the 10th percentile (Heydanus et al., 1994). Common karyotypic abnormalities include trisomy 21, 18 and 13, Turner syndrome, deletions, translocations and supernumerary marker chromosomes. Single gene disorders (for example, many skeletal dysplasias) can also lead to IUGR. There are numerous additional syndromes associated with prenatal onset of growth failure in which the underlying molecular mechanism is currently undetermined (Jones, 1997; Gross, 1997). In many of these, however, the pattern of inheritance suggests a genetic basis. Studies of these disorders, including SRS, provide a key to understanding the role of genetic factors in prenatal growth control.

1.2 Silver-Russell syndrome

1.2.1 The Silver-Russell syndrome phenotype

The characteristic features of this syndrome were originally described independently by Silver et al. (1953) and Russell (1954). Silver et al. reported two unrelated patients with congenital hemihypertrophy, low birth weight, short stature and raised urinary gonadotrophins. The following year Russell described five unrelated children with extreme IUGR and characteristic facial features. In two cases there was also asymmetry. Although different findings were emphasised by the two authors, the common features were subsequently recognised as a single syndrome, now known as the Silver-Russell syndrome (Black, 1961).

The range of phenotypic variance in SRS is difficult to assess since diagnostic criteria have not been established and the features associated with the syndrome are rather non-specific. Patients with mild clinical features may go unrecognised (Tanner, 1975)
while others with severe SRS may be misdiagnosed (Donnai et al., 1989). It has been suggested that SRS children may merely represent the severe end of the spectrum of low birth weight infants. However, it is now generally accepted that SRS does constitute a separate clinical entity. Severe IUGR, postnatal short stature, typical facial features with a small triangular face, down-turned corners of the mouth and a relatively large forehead and asymmetry of trunk, head and/or limbs are characteristic. Other abnormalities are less constant but include short fifth fingers with clinodactyly, syndactyly, male genital abnormalities, ear anomalies, café-au-lait patches and blue sclerae (Figure 1.1). Generalised camptodactyly has recently been noted in 22% of patients, many of whom also had distal arthrogryposis (Price et al., in press). Severe feeding difficulties and excessive sweating, sometimes associated with hypoglycaemia, are commonly seen in the neonatal period (Marks and Bergeson, 1977). Overall, there is a wide range of phenotypic variability in SRS. This is likely to reflect, at least in part, the heterogeneous aetiology of the disorder.
Figure 1.1. Clinical features of Silver-Russell syndrome. (A) Triangular facies with relative macrocephaly. (B) Lateral asymmetry affecting the face. (C) Fifth finger clinodactyly. (D) Syndactyly of second and third toes.
Chapter 1. Introduction

A comprehensive review of clinical findings and growth patterns in 386 Silver-Russell syndrome patients has been published by Wollmann et al. (1995). One hundred and twenty patients were seen between 1970 and 1993 and additional, well defined cases obtained from the literature. Table 1, below, shows the frequency with which characteristic features of the syndrome were noted in this study.

Table 1.1. Frequency (%) of symptoms and signs in children with SRS (n=143), adapted from Wollmann et al. (1995).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short stature</td>
<td>99</td>
</tr>
<tr>
<td>Birth weight &lt; 3rd percentile</td>
<td>94</td>
</tr>
<tr>
<td>Triangular face</td>
<td>79</td>
</tr>
<tr>
<td>Fifth finger clinodactyly</td>
<td>68</td>
</tr>
<tr>
<td>Relative macrocephaly</td>
<td>64</td>
</tr>
<tr>
<td>Ear anomalies</td>
<td>53</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>51</td>
</tr>
<tr>
<td>Fifth finger brachydactyly</td>
<td>48</td>
</tr>
<tr>
<td>Down-slanting corners of the mouth</td>
<td>46</td>
</tr>
<tr>
<td>Psychomotor retardation</td>
<td>37</td>
</tr>
<tr>
<td>Syndactylies</td>
<td>28</td>
</tr>
<tr>
<td>Café-au-lait spots</td>
<td>19</td>
</tr>
<tr>
<td>Early/precocious puberty</td>
<td>13</td>
</tr>
</tbody>
</table>

1.2.1.1 Postnatal growth retardation and pubertal development

The postnatal growth pattern in SRS is characteristic (Wollmann et al., 1995). Growth velocity is adequate throughout childhood. However, 'catch-up' growth, as is usually seen in children with IUGR caused by progressive placental insufficiency, does not occur. Short stature is therefore persistent. Most of the children described by Wollmann et al. had severe feeding problems in infancy, often leading to prolonged periods of hospitalisation and tube feeding. A tendency for early or prompt puberty in these children
lead to further compromise of their final height. In this study the mean final height (± SD) was 151.2 ± 7.8 cm in males and 139.9 ± 9.0 cm in females.

The report by Wollmann et al. (1995) included many of the 39 SRS patients described in a previous longitudinal study of growth by Tanner et al. (1975). Although the criteria for diagnosis adopted by Tanner et al. were less stringent, the results of their study correspond well with those of Wollmann et al.. Average height at referral was 3.6 SD below the mean. Follow up of 18 of these patients through adolescence showed that the pattern of puberty and adolescent growth was essentially normal (Davies et al., 1988). Again, the timing of puberty was found to be marginally earlier than usual, though in this small series this difference did not reach statistical significance. Nineteen of the original cohort of 39 patients were treated with growth hormone without lasting effect.

The characteristic pattern of growth seen in SRS, with IUGR but normal postnatal growth velocity, suggests that the molecular mechanisms involved are predominantly prenatal. Investigation of growth failure in this disorder has therefore focused on events in utero.

1.2.1.2 Facial dysmorphism

Some of the difficulty in identifying patients with SRS arises from the wide phenotypic variability in facial appearance. Many affected children have only mild facial dysmorphism while others have the classical features described by Russell (1954). These include a triangular shaped face with a broad forehead tapering to a small, pointed chin, a prominent nasal bridge and a wide, thin, 'shark-like' mouth with down-turned corners. The relatively 'soft' dysmorphic features associated with SRS may lead to inappropriate diagnosis which may, in turn, account for some of the genetic heterogeneity associated with the syndrome.
Chapter 1. Introduction

1.2.1.3 Lateral asymmetry

For some time attempts were made to draw a distinction between patients with and without asymmetry (Tanner and Ham, 1969). In their study of growth in SRS, Tanner et al. (1975) measured the degree of limb asymmetry. The distribution overlapped with the range of the normal subjects and there was no evidence of bimodality. It was therefore not possible to identify two distinct groups of patients and the authors speculated that some mechanism controlling bilateral symmetry is disturbed in SRS.

In some disorders affecting lateral symmetry, asymmetry results from mosaicism. For example, mosaicism for segmental uniparental disomy is thought to be the cause of the hemihypertrophy seen in Beckwith-Wiedemann syndrome (BWS) (Henry et al., 1993; Slatter et al., 1994). However, the observation that some asymmetric SRS patients also suffer from bilateral camptodactyly (Price et al., in press) argues against mosaicism as a cause in this disorder. Alternative explanations for the loss of symmetry in SRS may result from better definition of the underlying molecular basis of this disorder.

1.2.1.4 Cognitive ability

Early on in the literature there were suggestions that the incidence of learning disability in SRS might be raised (Silver, 1964) but this was not confirmed in subsequent cohorts investigated for growth (Tanner et al., 1975). In his review of SRS in 1988, Patton wrote that ‘normal IQ is the rule’. However, a more recent study of 25 affected children found that approximately half had significant impairment of their cognitive abilities (Lai et al., 1994). The mean IQ (± SD) was 86 (± 24) with 32% scoring below 70, within the learning disability range. Nearly half the group required remedial education and a similar proportion were receiving speech therapy.

The underlying explanation for these findings may be complex. Mental development scores for children with IUGR starting early in pregnancy tend to be lower than those with later onset growth retardation (Chiswick, 1985). The timing of the onset of growth failure in SRS is not well documented. Prenatal growth failure has been detected as early as 12 weeks gestation but is more commonly recognised around the start of the third trimester (S. Price, personal communication). Those SRS children affected earlier in
pregnancy may be at greater risk of cognitive deficit as a result. Some of the factors known to influence the cognitive development of children with IUGR may not have been relevant to the group studied by Lai et al. (1994). In particular, complications during pregnancy and at birth were infrequent and most patients came from relatively advantaged social backgrounds. However, many of the patients did have persistent neonatal hypoglycaemia which may well have compromised subsequent neurological development. The authors suggest that although the findings show that the cognitive abilities of children with SRS are at the lower end of the range for low birth weight children, they may be similar to those of children whose IUGR is of early onset. Comparison is difficult since low birth weight is an extremely heterogeneous condition and studies do not always distinguish between growth retardation in early and late pregnancy. Uncertainty therefore remains as to whether there is an underlying disorder of cognitive development which is specific to SRS.

1.2.2 Genetic heterogeneity in Silver-Russell syndrome

The majority of cases of SRS are sporadic. However, a number of patients have been reported with either familial inheritance or various chromosomal abnormalities. These cases not only provide evidence for a underlying genetic basis to the syndrome but also suggests genetic heterogeneity exists between patients.

1.2.2.1 Familial inheritance

Pedigrees consistent with autosomal dominant, autosomal recessive and X-linked inheritance have all been described (Duncan et al., 1990). In a minority of patients, direct transmission of the disease from one parent to one or more offspring has been observed, supporting a dominant mode of inheritance (Escobar et al., 1978; Duncan et al., 1990). Duncan et al. reported seven affected cases within two three-generation families, six of whom had lateral asymmetry. In addition, they reviewed 190 SRS cases from the literature and found 21 families where relatives of the proband had complete or 'partial' expression of the syndrome. In 17 of these families there were multiple maternal relatives with some features of SRS. However, the majority of these relatives simply had short stature without other characteristic signs of SRS; only four of the mothers had classical features of SRS.
Chapter 1. Introduction

Therefore, although the facial features of SRS tend to become less obvious during adulthood, the true incidence of dominant transmission maybe significantly less than is suggested by these figures.

Reports of paternal transmission are exceptionally rare. There are several explanations for this observation. Firstly, more detailed data is generally available on maternal height and appearance since mothers more frequently accompany children to clinics. Secondly, it is possible that hypogonadism associated with SRS may reduce male transmission. Finally, a genomic imprinting effect may be at work. The relationship between imprinting and SRS is discussed more fully in Section 1.3.2.

Several families have been described where both parents are of normal stature but SRS has been diagnosed in siblings (Duncan et al., 1990). In these families it seems likely that there is autosomal recessive transmission. Teebi’s (1992) description of six SRS siblings in one consanguinous Arab family strongly supports autosomal recessive inheritance. Seven children with SRS phenotype in three, unrelated, consanguinous Bedouin families have also been reported by Gouda et al. (1996). The overall incidence of such cases, however, is small.

Concordance for SRS in monozygotic twins and discordance in dizygotic twins has been observed, supporting the theory that SRS has a genetic basis. However, monozygotic twins discordant for SRS have also been reported by Samn et al. (1990). Two possible interpretations were given by the authors for this observation: 1) that expression of SRS requires the interaction of both genetic and environmental factors or 2) that SRS is genetically heterogeneous with some cases being of non-genetic origin. It is also possible that mosaicism for an underlying genetic abnormality in one twin could give rise to this phenomenon.

SRS is usually an isolated diagnosis in an otherwise normal family. Given the weight of evidence that SRS has a genetic cause, sporadic cases are probably the result of new mutations at an autosomal locus. Some conditions where this is seen are associated with increased parental age but studies have failed to demonstrate such an effect in SRS (Escobar et al., 1978).
1.2.2.2 Chromosomal abnormalities

Reports of several, different chromosomal abnormalities found in association with the SRS phenotype provide further evidence for genetic heterogeneity in SRS. Two patients have been described with typical features of SRS, both with balanced translocations involving chromosome 17q25. The first had inherited a balanced translocation t(17;20)(q25;q13) from her unaffected father (Ramirez-Dueñas et al., 1992). The origin of her father's translocation was not investigated but this observation suggests that if one or more gene(s) at the breakpoint are involved in the phenotype, the parent of origin may be important. The second patient had a balanced \textit{de novo} translocation t(1;17)(q31;q25) (Midro et al., 1993). Using a positional cloning approach an attempt is being made to map the breakpoint at 17q25 in this individual (Doerr et al., 1999). Since the human growth hormone gene cluster is localised close to the breakpoint 17q25 (17q22-q24), Eggermann et al. (1998) screened 54 SRS families for deletions within this cluster. A deletion of the chorionic somatomammotrophin hormone 1 (CSH1) gene was identified in one patient. His father, who also carries the deletion, has short stature (-2.2 SD), though he is not described as having the full SRS phenotype. The significance of these findings is at present unclear since deletions of \textit{CSH1} have been reported previously without any phenotypic consequences (Wurzel et al., 1982).

Several patients have been described with ring chromosome 15 (r(15)) who also have features characteristic of SRS (Tamura et al., 1993). Features described in association with r(15) include growth retardation and a triangular face but also, less typical of SRS, microcephaly, hypertelorism and variable mental retardation. Severe growth failure is commonly seen in association with ring chromosomes possibly due to instability of ring chromosomes (Kosztolányi, 1987) and resultant mosaicism. However, in patients with r(15), resultant hemizygosity for distal sub-bands of 15q may also be involved in the phenotype. At least 18 other patients with distal chromosome 15q deletions and features of SRS have also been reported (Roback et al., 1991; Tamura et al., 1993). The phenotype of 15q- patients is rather variable, probably in part due to the extent of the associated deletion. However, in many, IUGR, short stature, a triangular face and 5th finger clinodactyly are described. As with r(15), several other features not typical of SRS have been reported in
15q- patients, including microcephaly, micrognathia, renal anomalies and mental retardation.

Together these cases suggest that there may be a locus involved in SRS within the distal segment of 15q. The insulin-like growth factor-I gene, *IGFIR*, is located at 15q26.3 and has been proposed as a candidate (Roback et al., 1991). However, two studies have failed to find any evidence for *IGFIR* hemizygosity in SRS patients without chromosome 15 abnormalities (Rogan et al., 1996; Abu-Amero et al., 1997). Thus the SRS phenotype in patients with 15q abnormalities may be the result of reduced expression of *IGFIR* but this does not appear to be a universal explanation for the disorder.

Other chromosomal abnormalities have been described in patients with features less characteristic of SRS. These include a girl with an interstitial deletion of proximal 8q and a boy with deletion of the entire short arm of chromosome 18 (Schinzel et al., 1994). In the first case, features of SRS were found in association with other less typical findings such as microcephaly. In the second, the patient had findings consistent with the 18p- syndrome in addition to pre- and postnatal growth retardation, lower leg asymmetry and clinodactyly. In isolated cases such as these it seems likely that the phenotypic resemblance to SRS is purely coincidental. However, the possibility remains that a mutation in one or more genes in these regions may be responsible for the phenotype in at least a proportion of patients. Trisomy 18 mosaicism (Chauvel et al., 1975) and diploid/triploid mosaicism (Graham et al., 1981) have been reported in association with two patients with SRS-like phenotypes. In these, growth failure and asymmetry may be the result of asymmetric somatic mosaicism.

### 1.2.2.3 Maternal uniparental disomy for chromosome 7

Uniparental disomy (UPD) is the abnormal inheritance of two copies of a specific chromosome from only one parent (Engel, 1980). UPD was first recognised as a cause for human disease with the identification of two patients with cystic fibrosis and short stature who had both inherited two copies of one chromosome 7 homologue from their mothers (Spence et al., 1988; Voss et al., 1989). Two further cases of maternal UPD for chromosome 7 (mUPD7) associated with low birth weight and poor postnatal growth were described subsequently (Spotila et al., 1992; Langlois et al., 1995).
Interestingly, paternal UPD for chromosome 7 (pUPD7) appears to have no phenotypic effect on growth (Höglund et al., 1994). A 23 year old female was investigated following diagnosis of congenital chloride diarrhoea and found to have paternal isodisomy for chromosome 7. She was of normal stature and had no other dysmorphic features.

A case of maternal UPD7q and paternal UPD7p has also been reported in a child with normal birth weight but postnatal growth retardation (Eggerding et al. 1994). Some minor dysmorphic features compatible with SRS were also noted. These included a triangular-shaped facies, prominent occiput, slight limb asymmetry and very mild, bilateral clinodactyly. Since pUPD7 is associated with a normal phenotype, postnatal growth retardation in this patient must be the result of maternal UPD7q. The authors suggest that intrauterine growth may require normal expression of genes on chromosome 7p whereas genes localised to 7q may be involved in the regulation of postnatal growth.

The clinical details of all four patients with mUPD7 described above are detailed in Table 2 (below). Some of these cases had additional features reminiscent of SRS. Thus not only is mUPD7 associated with growth failure but, in several cases, with other features of SRS. This observation led investigators to search for evidence of mUPD7 in SRS patients. Kotzot et al. (1995) investigated 35 patients with either SRS or primordial growth retardation to look for mUPD7. Using multiple microsatellite PCR markers they found four patients with mUPD7. Similar observations have been made by other groups, including ours (Preece et al. 1997; Eggermann et al. 1997). Fifty three cases of SRS have now been investigated at Queen Charlotte’s and Chelsea Hospital (QCCH) and five patients with mUPD7 identified (Preece et al., 1999). In total 11 cases of mUPD7 have been detected out of 115 SRS patients investigated in these three studies. However there have been other studies where no cases of mUPD7 were identified or the rate of mUPD7 detection was much lower (Ayala-Madrigal et al., 1996; Penaherrera et al., 1996; Shuman et al., 1996; Joyce et al., 1997; Cogliati et al., 1998). Taking the numbers from these studies into account, the true prevalence of mUPD7 in SRS is probably around 7%.

31
Chapter 1. Introduction

Table 1.2. Case reports of mUPD7 in association with growth failure.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diagnoses</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spence et al., 1988</td>
<td>Cystic fibrosis; mUPD7 (isodisomy)</td>
<td>16 year old girl with postnatal short stature (&gt; -2 SD) and lateral asymmetry. Normal IQ and facial appearance. Birth weight not reported.</td>
</tr>
<tr>
<td>Voss et al., 1989</td>
<td>Cystic fibrosis; mUPD7 (isodisomy)</td>
<td>4 year old boy with low birth weight (&gt; -2 SD), and postnatal short stature (&gt; -2 SD). No dysmorphic features reported.</td>
</tr>
<tr>
<td>Spotila et al., 1992</td>
<td>Homozygosity for COL1A2 mutation; mUPD7 (mixed hetero- and isodisomy)</td>
<td>30 year old man with low birth weight (&gt; -2 SD); postnatal short stature (&gt; -2 SD) and slightly pointed facies. Normal IQ but required speech therapy at 11 years.</td>
</tr>
<tr>
<td>Langlois et al., 1995</td>
<td>Confined placental mosaicism for trisomy 7; mUPD7 (heterodisomy)</td>
<td>3 year old girl with low birth weight (-3 SD) and height (-3 SD) but relative sparing of head size (-1 SD); postnatal short stature (-2 SD). No dysmorphic features and normal IQ.</td>
</tr>
</tbody>
</table>

1.3 Uniparental disomy (UPD)

UPD was first proposed as a mechanism for disease in man by Engel in 1980. Experimental UPD in mice was subsequently shown to result in both normal and pathological phenotypes depending on the chromosome or chromosomal region involved (Cattanach and Kirk, 1985) (Section 1.4.2). In the last fifteen years, numerous highly informative polymorphic markers have been developed, allowing the parental origin of a particular chromosome to be determined (Strachan and Read, 1996). These include both minisatellites (variable number of tandem repeats (VNTRs)) and microsatellites (di-, tri- and tetranucleotide repeats). Microsatellites have proved particularly useful since they can
be rapidly typed using polymerase chain reaction (PCR) technology. Molecular analysis using these markers has provided mounting evidence for UPD in man.

As in mice, the phenotype resulting from UPD in man is chromosome specific. UPD for some human chromosomes is associated with a normal phenotype. However for others, UPD has been described in association with specific anomalies. For example, mUPD15 is found in some cases of Prader-Willi syndrome (Nicholls et al., 1989) and pUPD15 in a small percentage of patients with Angelman syndrome (Malcolm et al., 1991). Paternal UPD11 has been described in association with Beckwith-Wiedemann syndrome (Henry et al., 1991). In addition, various phenotypic effects of UPD have been described for at least five other chromosomes (Morison and Reeve, 1998). SRS can now been added to the growing list of disorders associated with UPD in man.

1.3.1 Mechanisms of UPD

Several mechanisms have been postulated to explain the origins of UPD. These include (i) gamete complementation; (ii) monosomy duplication; (iii) trisomic rescue; and (iv) somatic recombination (Engel and DeLozier-Blanchet, 1991) (Figure 1.2). Contrary to Engel's original prediction (1980), only a small proportion of UPD cases seem to arise from gamete complementation. Instead the majority of cases result from trisomic rescue where loss of the extra chromosome leaves two chromosomes (Hahnemann and Vejerslav, 1997). Loss of the additional chromosome has a 1 in 3 chance of resulting in UPD. Most cases of UPD are maternal in origin since the incidence of aneuploidy is significantly greater in oocytes compared to spermatozoa (Abruzzo and Hassold, 1995).

In UPD the two chromosomes (or chromosomal regions) can either be two identical copies (isodisomy) or one copy of each (heterodisomy) of the given parent's chromosomes (Figure 1.3). The presence and extent of isodisomy depends on two factors: (i) whether the error leading to UPD occurred at meiosis I, meiosis II or mitosis; and (ii) whether chromosomes have been modified by crossing-over. With meiosis I nondisjunction, chromosomes are heterodisomic unless meiotic recombination has occurred. Conversely, with meiosis II nondisjunction chromosomes are isodisomic unless modified by crossing-over. In order to distinguish between UPD arising from meiosis I and meiosis II errors,
Chapter 1. Introduction

centromeric markers are necessary. Recombination is unlikely to result in complete isodisomy following meiotic nondisjunction. Instead, when all markers on the chromosome pair are isodisomic it is assumed that the extra chromosome has resulted from a postzygotic error in mitosis.

Figure 1.2. Mechanisms by which uniparental disomy (UPD) may arise, adapted from Lindgren (1997). Only the chromosome pair involved is shown. Opposite parental alleles are depicted by shaded/non-shaded areas. In gamete complementation, a nullisomic gamete is fertilised by a disomic gamete, resulting in UPD. Chromosomal duplication in a monosomic gamete also leads to UPD. In trisomic rescue, loss of the extra chromosome leaves a normal chromosome complement but has a 1 in 3 chance of resulting in UPD. Gamete complementation, monosomy duplication and trisomic rescue all result in UPD for an entire chromosome. Somatic recombination produces a mosaic individual with both normal and partially isodisomic cell lines.
Figure 1.3. Autoradiograph of Southern blot showing inheritance of parental alleles in three SRS patients. DNA was restricted with *Hinfl* and hybridised with VNTR markers D7S21 or D7S22. (A) Normal biparental inheritance in patient 46. (B) and (C) Maternal uniparental disomy in patients 19 and 4, respectively. In both of these families, the proband (P) has inherited two alleles from the mother (M) and none from the father (F). Note that patient 19 has one copy of each maternal allele (heterodisomy) whereas patient 4 has two identical maternal alleles (isodisomy). (Clinical details of all three cases are given in Appendix 1).

1.3.2 Phenotypic effects of mUPD7

An abnormal phenotype associated with UPD may result from one or more of the following mechanisms: (i) the effect of associated trisomy on the placenta or fetus; (ii) autosomal disease due to reduction to homozygosity; and (iii) the effect due to imprinted gene(s) on that chromosome. In order to understand the molecular basis of SRS it is important to analyse the relative contribution of these mechanisms to the mUPD7 phenotype.

1.3.2.1 Confined placental mosaicism

Mosaicism is the presence of two or more karyotypically different cell lines within an individual. It originates early in embryogenesis and may be either generalised or confined to particular tissues. Confined placental mosaicism (CPM) is seen in 1-2% of chorionic villus samples taken for prenatal diagnosis from viable pregnancies at 9-12 weeks gestation (Wang *et al.*, 1993). The rates and consequences of CPM vary for each individual
chromosome. Overall there is a high incidence of IUGR in pregnancies where CPM is present at term (Kalousek et al., 1991). Trisomy 16 is commonly associated with miscarriages and is also the most common form of CPM seen in IUGR (Kalousek, 1994). Poor prenatal growth is seen both with and without associated UPD16 (Wolstenholme, 1995).

Trisomy 7 is a lethal aneuploidy which accounts for 0.9% of spontaneous abortions. In comparison, trisomy 16 accounts for 7.5% (Hassold, 1994). However CPM for trisomies 7 and 16 occur at similar frequencies (Kalousek et al., 1993). This suggests that most cases of CPM7 result from somatic duplication, not trisomic rescue. If this is the case then the risk of fetal UPD in association with CPM would be expected to be low. Kalousek et al. (1996) reported a study of 14 pregnancies with CPM for trisomy 7. DNA analysis in nine showed that fetal UPD7 was present in just one case; in the other eight cases biparental inheritance was seen. As predicted, DNA analysis showed that in the majority (six of seven informative cases) the trisomy was somatic in origin. One trisomy resulted from meiosis nondisjunction and was associated with fetal UPD. The infant with UPD had severe IUGR. The other eight infants with biparental inheritance all had normal birthweights. In contrast to trisomy 16, there was no evidence that intrauterine growth was affected by the presence of a trisomy 7 cell line in the placenta. It therefore seems unlikely that CPM is responsible for the significant growth retardation seen in patients with mUPD7.

1.3.2.2 Reduction to homozygosity

In regions of isodisomy, inheritance of two mutant copies of a recessive gene can lead to an abnormal phenotype. For example, cystic fibrosis, homozygosity for a COLIA2 mutation and congenital chloride diarrhoea have all been reported in association with UPD7 (Spence et al., 1988; Voss et al., 1989; Spotila et al., 1992; Höglund et al., 1994). It is therefore possible that the SRS phenotype could result from exposure of another recessive gene in a region of isodisomy common to all mUPD7 patients.

The five patients identified at QCCH as having mUPD7 were investigated using 40 polymorphic markers distributed along the length of chromosome 7 (Preece et al., 1999). Mixed hetero- and isodisomy was seen (Figure 1.4). In all five, centromeric markers were
heterodisomic suggesting that the UPD had arisen as the result of maternal meiosis I nondisjunction and subsequent trisomic rescue. Significantly, no regions on chromosome 7 were found to be consistently isodisomic in all patients. This observation makes exposure of a recessive gene an unlikely explanation for the SRS phenotype.

1.3.2.3 Imprinting effects

Another consequence of UPD is the production of an abnormal phenotype via an imprinting effect. Imprinting is the differential expression of a gene dependent on its parent of origin. Maternal and paternal copies of chromosomes or chromosomal regions containing such genes are not functionally equivalent. This phenomenon explains the pathological phenotype seen in mice with experimental UPD of particular chromosomes or chromosomal regions (Cattanach and Kirk, 1985). Similarly, either absence of expression of a paternally expressed gene(s) or over-expression of a maternally expressed gene(s) could be responsible for the SRS phenotype seen in patients with mUPD7. Since no evidence can be found to support a role for either CPM or reduction to homozygosity, it seems that imprinting is the most likely explanation for the SRS phenotype.
Table 1.4. Distribution of hetero- and isodisomy on chromosome 7 for five SRS probands with mUPD7 (from Preece et al., 1999). Heterodisomic markers are represented by , isodisomy by and non-informative markers by . Markers not studied in a particular individual have no symbol. The distances of each marker from pter are given in cM. The shaded area marks the approximate location of the centromere.
1.4 Imprinting: background evidence

Genes inherited in a traditional Mendelian fashion are expressed equally from both maternally and paternally inherited alleles. Until relatively recently it was assumed that this pattern of inheritance would apply to all genes. However, several studies have now demonstrated that male and female contributions to an offspring’s genome are not functionally equivalent. This has led to the concept of imprinting: the differential expression of certain genes dependent on their parental origin.

1.4.1 Pronuclear transplantation, hydatidiform moles and triploidy

The first evidence of an imprinting effect came from the elegant pronuclear transplantation experiments performed by McGrath and Solter (1984). Gynogenetic (two maternal pronuclei) and androgenetic (two paternal pronuclei) zygotes were constructed and transferred into pseudopregnant mice. Neither were viable, despite having normal, diploid chromosome numbers. Gynogenetic zygotes formed embryos with relatively normal growth but small placentas. In contrast, androgenetic zygotes had relatively normal placental growth but very poor embryonic development.

Human analogies to these experiments exist in the form of the naturally occurring hydatidiform mole and ovarian teratoma. The complete hydatidiform mole is an abnormal conceptus composed of hydropic trophoblastic tissue alone with no embryonic development. Its genotype is entirely paternal (Lawler et al. 1982). By contrast, ovarian teratomas are embryonic tumours with tissues from three embryonic germ layers but no placental tissue. They have an entirely maternal genotype (Linder et al. 1975). Human triploid embryos have two haploid sets of chromosomes from one parent and a third from another parent. Triploid with one maternal and two paternal sets typically presents with a large, cystic placenta and fetal IUGR. Triploid conceptuses with one paternal and two maternal sets are far less common. They abort early in gestation and have small, underdeveloped placentas (MacFadden et al., 1991).
Chapter 1. Introduction

These observations all support the hypothesis that paternally expressed genes are largely responsible for development of the extraembryonic tissues whereas maternally expressed genes are more important to embryonic development.

1.4.2 Mouse imprinting phenotypes

Studies on mice with UPD have provided further evidence for differential function of the two parental genomes (Cattanach and Kirk, 1985). Robertsonian or reciprocal translocations have been used to generate animals with UPD for whole or selected chromosome regions, respectively. UPD for most chromosomal regions is associated with a normal phenotype. However, UPD of certain regions results in 'imprinting' phenotypes with effects on growth, behaviour and survival (Beechey and Cattanach, 1998). There are specific 'imprinted' regions in the genome within which both parental alleles are required for normal development. Ten such regions located on six chromosomes (2, 6, 7, 11, 12 and 17) are currently recognised in the mouse (Table 1.3).

Although this approach to studying imprinting effects has proved extremely powerful, it has its limitations. Firstly, the maps generated are not exhaustive as some imprinting effects may be subtle. Additional regions (on chromosomes 9, 14 and 19) are known to contain imprinted genes but have no associated phenotype. Secondly, most of the imprinting regions are relatively large, covering up to 30% of individual chromosomes, and are currently too extensive for detailed mapping. The maps are refined and regions narrowed as suitable translocations become available. Finally, it is only possible to determine whether a phenotype results from absence of one parental copy or the presence of two by creating chromosomal imbalance of the region. This has been possible in some instances. For example, mice with two maternal copies and one paternal copy of the central region of chromosome 7 do not show the imprinting phenotype associated with mUPD of the region (Cattanach et al., 1995). This suggests that absence of the paternal copy is responsible for the postnatal lethality.
Table 1.3. Phenotypes associated with mouse imprinted regions, adapted from Beechey and Cattanach (1998). These phenotypes occur in mice with uniparental duplications of either maternal or paternal origin. Imprinted genes identified within these regions are listed.

<table>
<thead>
<tr>
<th>Region</th>
<th>Origin</th>
<th>Imprinted loci</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal 2</td>
<td>Maternal</td>
<td></td>
<td>Variable fetal viability with growth retardation.</td>
</tr>
<tr>
<td>Distal 2</td>
<td>Maternal</td>
<td><em>Gnas, Nnat</em></td>
<td>Abnormal neonatal behaviour (hypokinetic) and lethality.</td>
</tr>
<tr>
<td>Distal 2</td>
<td>Paternal</td>
<td><em>Gnas, Nnat</em></td>
<td>Abnormal neonatal behaviour (hyperkinetic) and lethality.</td>
</tr>
<tr>
<td>Proximal 6</td>
<td>Maternal</td>
<td><em>Peg1/Mest</em></td>
<td>Early embryonic lethality.</td>
</tr>
<tr>
<td>Proximal 7</td>
<td>Maternal</td>
<td><em>Peg3/Pwl</em></td>
<td>Neonatal lethality.</td>
</tr>
<tr>
<td>Proximal 7</td>
<td>Paternal</td>
<td><em>Snrpn, Ndn, Dn34, Znf127, Ipw, Ube3a</em></td>
<td>Postnatal growth failure. Reduced viability.</td>
</tr>
<tr>
<td>Central 7</td>
<td>Maternal</td>
<td><em>Snrpn, Ndn, Dn34, Znf127, Ipw, Ube3a</em></td>
<td>Postnatal lethality.</td>
</tr>
<tr>
<td>Distal 7</td>
<td>Maternal</td>
<td><em>Ipl/Tssc3, Impt1, Orc12, p57KIP2, (Cdkn1c), Kvlqt1 (Kcnq1), Tap1, (Cd81), Dn34, Ins2, Igf4, H19</em></td>
<td>Late fetal lethality.</td>
</tr>
<tr>
<td>Distal 7</td>
<td>Paternal</td>
<td><em>Ipl/Tssc3, Impt1, Orc12, p57KIP2, (Cdkn1c), Kvlqt1 (Kcnq1), Tap1, (Cd81), Dn34, Ins2, Igf4, H19</em></td>
<td>Early embryonic lethality.</td>
</tr>
<tr>
<td>Proximal 11</td>
<td>Maternal</td>
<td><em>Megl/Grb10, U2af1-rs1</em></td>
<td>Prenatal growth retardation.</td>
</tr>
<tr>
<td>Proximal 11</td>
<td>Paternal</td>
<td><em>Megl/Grb10, U2af1-rs1</em></td>
<td>Prenatal overgrowth.</td>
</tr>
<tr>
<td>Distal 12</td>
<td>Maternal</td>
<td></td>
<td>Late embryonic/neonatal lethality and growth retardation.</td>
</tr>
<tr>
<td>Distal 12</td>
<td>Paternal</td>
<td></td>
<td>Late embryonic/neonatal lethality and growth retardation.</td>
</tr>
<tr>
<td>Proximal 17</td>
<td>Paternal</td>
<td><em>Ifgr2r, Igf2ras</em></td>
<td>Neonatal lethality.</td>
</tr>
</tbody>
</table>
1.4.2.1 Mouse imprinted regions homologous to human chromosome 7

Regions of human chromosome 7 which share syntenic homology with imprinted regions in the mouse are of particular interest when looking for potential candidates for SRS (Beechey and Cattanach, 1998). An imprinted region on mouse proximal chromosome 11 is homologous to human chromosome 7p11.2-p13 (Figure 1.5A). Maternal UPD for this region leads to prenatal growth retardation. Conversely, paternal UPD results in prenatal overgrowth (Cattanach and Kirk, 1985). Recent studies have demonstrated that in both cases postnatal growth velocity is normal and size differences are merely maintained after birth (Cattanach et al., 1996). This pattern of growth retardation is remarkably similar to that described in SRS (Wollmann et al., 1995). The two disomy growth rates are mirror-images of each other, suggesting that expressed gene dosage is the underlying cause. Analysis of fetal growth was limited to 12.5-17.5 days post coitum. However, by extrapolating the data, both maternal and paternal size differences were calculated to start as early as seven days post coitum. The imprinting effects associated with UPD of this region therefore occur very early in development. Differential fetal growth was observed prior to changes in placental growth suggesting that it is expression of the responsible gene(s) in the fetus which results in growth failure.

The imprinted domain on proximal chromosome 11 has recently been redefined using two new translocations (Cattanach et al., 1998) (Figure 1.5A). The region is located close to the centromere and encompasses two known imprinted genes: U2af1-rs1 and Megl/Grb10. Megl/Grb10 is a particularly good candidate for the chromosome 11 imprinting effects since it encodes a growth factor receptor binding protein. Megl/Grb10 and U2af1-rs1 are located about 15 cM apart and are separated by conserved regions showing homology with two different human chromosomes. The human homologues of Megl/Grb10 and U2af1-rs1 also lie on different chromosomes (7 and 5, respectively). For these reasons it seems likely that the two genes lie within two distinct imprinting domains within the small proximal region of mouse chromosome 11.

Two other regions of human chromosome 7 share syntenic homology with an imprinted region on mouse proximal chromosome 6 (Figure 1.5B). Maternal UPD for this segment in mice results in early embryonic lethality. To date, only one imprinted gene (Peg1/Mest) has been mapped to the region.
Figure 1.5. Mouse imprinting maps of (A) chromosome 11 and (B) chromosome 6 (Beechey and Cattanach, 1998). The positions of reciprocal (T) and Robertsonian (Rb) translocations used to define the regions with imprinting phenotype are shown.
Chapter 1. Introduction

1.5 Imprinting disorders in man

As mentioned in Section 1.3 above, UPD for some human chromosomes has been seen in association with specific disease phenotypes which arise as a result of imprinting effects. Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Wiedemann syndrome (BWS) are the best understood examples of human disease caused by abnormalities of imprinted genes. Multiple mechanisms leading to these disorders have now been described. Study of these molecular mechanisms has been extensive and has contributed much to our understanding of the imprinting process.

1.5.1 Prader-Willi and Angelman syndromes

PWS and AS are both caused by imprinted gene defects within a region of chromosome 15q11-q13 (Table 1.4). PWS is characterised by neonatal hypotonia, mild to moderate mental retardation, hyperphagia and severe obesity, hypogonadism, short stature and small hands and feet. AS patients have severe mental retardation with lack of speech, ataxia, seizures, sleep disorder, hyperactivity, and a happy disposition with frequent paroxysms of laughter (Cassidy, 1997).

The most common molecular defect in both PWS and AS is a large deletion, usually 4 Mb in size. In PWS the deletions are all paternal in origin whereas in AS all deletions are maternal (Magenis et al., 1990). Both syndromes can also arise from UPD (Nicholls et al., 1989; Malcolm et al., 1991). Maternal UPD is common in PWS, being seen in approximately 25% patients. Paternal UPD occurs in AS, though only in about 2% (Nicholls et al., 1998). These observations indicate that PWS arises from the absence/disruption of an imprinted gene(s) expressed from the paternal allele alone. Conversely, AS must result from the absence/disruption of gene(s) expressed exclusively from the maternal allele.
Chapter 1. Introduction

Table 1.4. Molecular mechanisms and their frequency in Prader-Willi and Angelman syndromes, adapted from Nicholls et al. (1998).

<table>
<thead>
<tr>
<th>Molecular mechanism</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prader-Willi syndrome</strong></td>
<td></td>
</tr>
<tr>
<td>Paternal deletion 15q11-q13</td>
<td>70%</td>
</tr>
<tr>
<td>mUPD15</td>
<td>25%</td>
</tr>
<tr>
<td>Imprinting mutation</td>
<td>≤5%</td>
</tr>
<tr>
<td>Balanced translocation</td>
<td>0.1%</td>
</tr>
<tr>
<td><strong>Angelman syndrome</strong></td>
<td></td>
</tr>
<tr>
<td>Maternal deletion 15q11-q13</td>
<td>70%</td>
</tr>
<tr>
<td>pUPD15</td>
<td>2%</td>
</tr>
<tr>
<td>Gene mutation (UBE3A)</td>
<td>20%</td>
</tr>
<tr>
<td>Imprinting mutation</td>
<td>≤5%</td>
</tr>
<tr>
<td>Balanced translocation</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

About 5% of PWS and AS patients have biparental inheritance of chromosome 15 but abnormal DNA methylation and gene expression throughout the imprinted region (Reis et al., 1994; Sutcliffe et al., 1994). This suggests that some patients have cis-acting mutations affecting the imprinting process itself. In some cases it has been shown that this imprinting defect results from small deletions of the region around the gene for small nuclear ribonucleoprotein N (SNRPN) (Sutcliffe et al., 1994; Buiting et al., 1995). These 'imprinting centre' (IC) deletions prevent the switch of the grandparental imprint in the parental germline. They 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. In PWS, IC mutation in the father blocks the maternal -> paternal imprint switch resulting in transmission of a maternal imprint (from the grandmother) to half his gametes. Consequently, 50% of his offspring will inherit maternal imprints from both parents and develop PWS (Figure 1.6A). Conversely, in AS, IC mutation in the mother blocks the paternal -> maternal imprint switch resulting in transmission of a paternal imprint (from the grandfather) to half her gametes. Thus, 50% of her offspring will inherit paternal imprints from both parents and develop AS (Figure 1.6B).
Figure 1.6. Inheritance of imprinting centre (IC) mutations (from Nicholls et al., 1998). The pedigree shows the inherited chromosome 15 imprint in somatic cells. (A) PWS: IC mutations block resetting of the maternal -> paternal imprint. Thus mutation in the male germline results in transmission of a maternal imprint [P(M)] to half his offspring. These children develop PWS. Note that since mutation in the female germline still results in a maternal imprint [M(M)], all her offspring will have a normal phenotype. (B) AS: IC mutations block resetting of the paternal -> maternal imprint. Mutation in the female germline results in transmission of a paternal imprint [M(P)] to half her offspring. These children develop AS. Since mutation in the male germline still results in a paternal imprint [P(P)], all his offspring will have a normal phenotype.
More recently, other patients have been reported with imprinting defects but no detectable IC deletion or mutation (Bürger et al., 1997; Buiting et al., 1998). It has been suggested that these cases may arise from a developmental or stochastic failure to switch the imprint in the germline. This theory is supported by the observation that all non-deletion cases are sporadic, whereas the microdeletion cases are familial.

Multiple imprinted genes have been identified within the PWS/AS region (Morison and Reeve, 1998). All are paternally expressed with the exception of UBE3A. Imprinting of this gene has been shown to be restricted to brain where only the maternal allele is active (Rougeulle et al., 1997; Vu and Hoffman, 1997). Mutations in UBE3A have recently been identified in a small number of AS patients (Kishino et al., 1997; Matsuura et al., 1997). No single gene mutations have been identified in PWS, indicating that it is probably a contiguous gene syndrome.

Analysis of the PWS/AS region has shown loss of differential methylation in all PWS and AS patients with deletions, UPD or imprinting mutations (Kubota et al., 1996). Methylation analysis therefore detects all the most frequent molecular defects with the exception of UBE3A mutations. It is currently the best single diagnostic tool for AS and PWS.

1.5.2 Beckwith-Wiedemann syndrome

BWS is characterised by pre- and postnatal overgrowth, macroGLOSSIA, organomegaly, exomphalos, hemihypertrophy, neonatal hypoglycaemia, a predisposition to embryonal tumours such as Wilms tumour, and other minor anomalies. The phenotype results from disruption of a second cluster of imprinted genes, located on chromosome 11p15.5. The molecular pathology of this condition has been comprehensively reviewed by Reik and Maher (1997) and Li et al., (1997). Attempts to generate a unifying model for the pathogenesis of BWS have so far been frustrated by the heterogeneity of molecular abnormalities seen (Table 1.5). The majority of patients are sporadic. Some have been shown to have segmental pUPD arising from somatic recombination (Henry et al., 1993; Slatter et al., 1994). Others have maternally inherited rearrangements, paternal duplications or, most commonly, normal karyotypes. Loss of imprinting of IGF2 is the most frequent
Chapter 1. Introduction

molecular defect seen (Weksberg et al., 1993). In patients where disruption of other genes (H19, p57Kip2 (CDKN1C), KvLQT1 (KCNQ1)) is observed, IGF2 expression may be indirectly affected. The BWS imprinting cluster has been extensively investigated, providing evidence for both local and regional control of imprinted genes (Section 1.6.3, below).

Table 1.5. Genetic groups and their frequency in Beckwith-Wiedemann syndrome, adapted from Li et al. (1997).

<table>
<thead>
<tr>
<th>Genetic Group</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic, normal karyotype</td>
<td>85%</td>
</tr>
<tr>
<td>Autosomal dominant pedigree</td>
<td>10-15%</td>
</tr>
<tr>
<td>Segmental pUPD11</td>
<td>10-20% of sporadic cases</td>
</tr>
<tr>
<td>p57Kip2 mutations</td>
<td>5-20%</td>
</tr>
<tr>
<td>Maternal 11p15 translocations and inversions</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Paternal 11p15 duplications</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Loss of imprinting of IGF2</td>
<td>~50%</td>
</tr>
</tbody>
</table>

1.6 Imprinting mechanisms

An imprinted region may encompass many hundreds of kilobases within which lie multiple imprinted and also non-imprinted genes (Lee et al., 1999). The imprinting process is undoubtedly complex and, as yet, poorly understood. Proposed mechanisms need to meet a minimal set of criteria. Firstly, the imprint marks the parental origins of the gene resulting in a functionally hemizygous gene. Secondly, it is erasable, allowing it to be reset in the next generation. Thirdly, it is epigenetic, involving no change in the actual nucleotide sequence of the gene. Three distinct biological stages are involved: establishment of the imprint in the gamete; its maintenance throughout development and adult life; and erasure in the germline. Clues as to the mechanism by which parental alleles are marked have come from studying differences between parental alleles of imprinted genes. Two properties shown to differ according to parental allele origin are methylation and chromatin structure.
The connection between these and the imprinting process itself is currently the subject of intense investigation.

1.6.1. DNA methylation

1.6.1.1 Developmental regulation of methylation

5-methylcytosine is found exclusively at cytosine-guanine (CpG) dinucleotides. Shortly after fertilisation most genes are actively demethylated in a process thought to allow erasure of epigenetic information originating in the highly differentiated gametes (Razin and Shemer, 1995). The pluripotent state which results precedes differentiation and the creation of specific cell lineages. Following implantation a wave of de novo methylation then occurs (Figure 1.7). The majority of CpGs become modified but all CpG islands associated with 'housekeeping' genes remain unmethylated (Bird, 1986). During development tissue-specific genes are demethylated in those tissues in which they are expressed. Thus in adult cells there is a clear correlation between undermethylation of those genes that are active and full methylation of those that are inactive. Primordial germ cells escape the global preimplantation de novo methylation but undergo a similar de novo methylation process in the gonads to produce gamete specific methylation patterns (Razin and Shemer, 1995).

Methylation is clearly necessary for normal development since mice lacking a functional DNA methyltransferase gene fail to develop properly and die in midgestation (Li et al., 1992). Further evidence links methylation directly to control of gene expression. Methylation of genes in vitro prevents their expression following transfection into fibroblasts (Yisraeli et al., 1988). Conversely, treatment of cells with 5-azacytidine, an inducer of demethylation, leads to the activation of several endogenous genes (Jones, 1984).
Figure 1.7. Temporal changes in DNA methylation levels during development, adapted from Jaenisch (1997). The DNA of the zygote is substantially methylated. During cleavage there is global demethylation of the genome. After implantation a wave of de novo methylation occurs. Throughout subsequent organogenesis and differentiation the overall level of methylation is maintained. During germ cell development DNA becomes de novo methylated; however, some sequences are demethylated at later stages of gametogenesis. Sex-specific de novo methylation or demethylation of imprinted genes occurs during gametogenesis. PGC: primordial germ cells.

1.6.1.2 Differential methylation of imprinted genes

DNA methylation is an epigenetic event stable enough to be propagated through many cell generations but also easily altered by de novo methylation or demethylation at specific sites during development. These properties make it ideal as a potential means of marking parental alleles in imprinted genes.

The first evidence that parental allele specific expression is associated with differential methylation came from an unexpected observation in mouse transgenic experiments designed to determine the effect of an activated c-myc oncogene. (Swain et al., 1987). The transgene used was not derived from an endogenously imprinted gene. However, it was found to be expressed only when inherited from the father. When
Chapter 1. Introduction

inherited from the mother it was completely silent. This parent-specific expression pattern correlated exactly with differential methylation of the transgene. Silent maternal copies of the transgene were highly methylated whereas active paternal copies were unmethylated. Methylation of the transgene was acquired by its passage through the female parent and eliminated during gametogenesis in the male. It was clear that a similar mechanism could underlie parent-of-origin-specific expression of imprinted genes.

Allele-specific methylation of many imprinted genes has since been observed in late embryo and adult somatic cells. The differential methylation varies considerably in extent from gene to gene. Allelic differences have been observed in promoters, introns, exons and regions distal to the transcription unit. In general, methylation is associated with the inactive allele, as has been observed for non-imprinted genes. However, there are notable exceptions to this rule. For example, the Igf2r gene is methylated at the start of transcription on the silent paternal allele but also in an intron on the expressed maternal allele (Stöger et al., 1993).

Where the inactive chromosome is methylated, the mechanisms of transcriptional repression are likely to be similar to those proposed for non-imprinted genes. Methylation may prevent binding of methylation-sensitive transcription factors to their target sequences. Alternatively, proteins such as MeCP1 and MeCP2, which themselves block the actions of transcription factors, may bind specifically to methylated sequences (Boyes and Bird, 1991; Nan et al., 1997). Binding of MeCPs to methylated DNA reduces the accessibility of chromatin to endonucleases such as Dnase I. Some imprinted genes show marked reduction in DNase I hypersensitivity in regions of extensive methylation (Feil and Kelsey 1997). This observation supports a role for such proteins in allelic silencing. It seems that it is assembly of methylated DNA into a stable chromatin structure which results in loss of transcriptional activity (Kass et al., 1997).

For genes such as Igf2r and Igf2, where the active chromosome is methylated, other mechanisms need to be postulated. In these genes methylation may be interfering with binding of transcriptional repressors and/or specific transcription factors may be binding preferentially to methylated DNA (Constancia et al., 1998).
1.6.1.3 Evidence for methylation as a genomic imprint

To assess the role of DNA methylation in the imprinting process, Li et al. (1993) examined the expression of three imprinted genes H19, Igf2 and Igf2r in DNA methyltransferase mutant mice. Expression of all three genes was affected. In the case of H19, the normally inactive paternal allele was activated. In contrast, the same mutation resulted in silencing of the normally active paternal allele of Igf2 and maternal allele of Igf2r. DNA methylation was therefore required to maintain monoallelic expression of all three of these imprinted genes during embryonic development. These results provided the first demonstration of a causal link between DNA methylation and parental allele activity of imprinted genes. Functional studies using transgenic mice support the importance of differentially methylated sites in maintaining the imprint. For example, yeast artificial chromosome (YAC) transgenes of Igf2r can reproduce the imprinted methylation and expression pattern of the endogenous gene (Wutz et al., 1997). Deletion of a CpG island in the second intron (region 2) results in loss of imprinting and biallelic expression. This differentially methylated region therefore seems to be involved in maintenance of the Igf2r imprint.

If differential methylation of imprinted genes does have a role in determining allele-specific expression then it would be predicted to resist the wave of demethylation which occurs after fertilisation. Evidence for this has been found at loci within several imprinted genes. Methylated sequences within the intron of Igf2r, the upstream region of H19 and the promoter and 5' region of Xist all retain their methylation status in preimplantation embryos (Stöger et al., 1993; Tremblay et al., 1997; Ariel et al., 1995). These differentially methylated residues may function as ‘imprinting signals’ marking the parental origin of alleles in the embryo and adult. However, strong methylation differences are not maintained at all imprinted loci. For example, close to the promoter of H19, both parental alleles are relatively hypomethylated in the blastocyst although highly methylated in sperm. The higher level of methylation of the paternal allele is established postimplantation (Tremblay et al., 1997).
1.6.2 Chromatin structure

Another common feature of imprinted genes is their asynchronous replication. For most genes the two parental homologues replicate simultaneously during S phase. However, studies of replication timing using fluorescence in situ hybridisation (FISH) have demonstrated differential replication timing of the paternal and maternal chromosomes at the imprinted \textit{IGF2-H19}, \textit{IGF2R} and PWS/AS regions in mouse and human cell lines (Kitsberg, 1993; Knoll, 1994). Asynchronous replication appears to involve large regions of 1-2 Mb that can extend beyond the region containing imprinted genes.

Asynchronous replication is likely to reflect differences in chromatin structure between the two parental chromosomes. The potential for imprinting to confer different chromosomal states on the two parental chromosomes is also suggested by the observation that both the PWS/AS region and the \textit{IGF2-H19} region in humans display much higher rates of recombination in males than females. By comparison, rates are fairly similar in other genomic regions (Paldi \textit{et al.}, 1995). It is unclear whether differences in chromatin structure are actually involved in regulating the transcriptional activity of each allele or merely reflect the activity of a chromosomal domain. The fact that imprinted regions also contain non-imprinted genes implies that at least one additional control mechanism must be at work.

LaSalle and Lalande (1996) recently used three-dimensional FISH analysis to show that in late S phase the nuclear distance between the two parental alleles is shorter for imprinted than non-imprinted genes. Allelic association was not seen in cells from PWS and AS patients implying that the imprinted region is involved in this interaction between the chromosomes. It is suggested that “kissing” of oppositely imprinted domains at a specific stage in the cell cycle may play a role in the maintenance of allele-specific methylation and transcription during cell division.

Direct sequence repeats are frequently observed in differentially methylated regions of imprinted genes (Neumann \textit{et al.}, 1995). Repetitive elements within genomic DNA are thought to induce gene silencing by pairing and heterochromatin formation (Dorer and Henikoff, 1994). It is thought that these repeats may regulate transcription of imprinted genes by altering chromatin structure. Monoallelic methylation within the region could restrict the effect to one parental chromosome. Whatever the mechanism, it is likely that the
imprinting process involves some interaction between differentially methylated sequences around imprinted genes and longer range alterations in chromatin structure within imprinted regions.

1.6.3 Local and regional imprinting control mechanisms

Clustering in imprinted regions appears to be an important organisational component of the imprinting process. As described in Section 1.5, clusters of imprinted genes are associated with both the BWS and PWS/AS regions. These clusters have several properties in common including interspersion of both maternally and paternally expressed genes and distribution of imprinted genes over a large distance. It is clear from studies of the control of allele-specific expression that regional, as well as local, mechanisms have a part to play within these clusters.

1.6.3.1 Local control mechanisms

Evidence for local, cis-acting control elements comes from the observation that even relatively small genomic fragments injected artificially into mouse zygotes can become differentially methylated. As described in Section 1.6.3, when Igf2r was introduced on a YAC into transgenic mice, parent-of-origin specific expression was observed suggesting that local signals are sufficient for imprinting (Wutz et al., 1997). Mutation of the critical 'region 2' in Igf2r resulted in loss of differential methylation and allele-specific expression of the transgene. Thus imprinting signals can be mapped by reducing the size of the transgene injected. Ainscough et al. (1997) used a similar YAC approach to study Igf2-H19 imprinting. They found that although imprinting was conserved in transgenic mice, Igf2 expression was affected by both position and copy number of the YACs. It is therefore likely that additional, regional control mechanisms are needed to achieve perfect imprinting.

One of the most difficult aspects of imprinting to explain is the presence of closely linked but reciprocally imprinted genes such as H19 and Igf2. Bartolomei and Tilghman (1992) have proposed an enhancer competition model in which the two genes compete with each other for access to shared enhancers. Although this model cannot account for some
Chapter 1. Introduction

recent experimental findings (Tilghman, 1999), it is still widely accepted as one possible mechanism for gene silencing.

Recent studies have provided evidence for an alternative mechanism involving reciprocally imprinted antisense transcripts. Antisense RNA transcripts have been detected for both \( \text{Igf2r} \) (Wutz et al., 1997) and \( \text{UBE3A} \) (Rougeulle et al., 1998) genes. The antisense transcript of \( \text{Igf2r} \) is thought to be responsible for monoallelic expression of the gene by repressing the paternal allele in \( \text{cis} \) (Wutz et al., 1997).

1.6.3.2 Regional control mechanisms

'Imprinting mutations' which disrupt imprinting in both the PWS/AS and BWS clusters provide evidence for regional control of imprinted genes. As mentioned in Section 1.5.1, small deletions of an IC within the PWS/AS region can alter in \( \text{cis} \) the appropriate imprinting of a number of imprinted genes over a 2 Mb domain (Sutcliffe et al., 1994; Buiting et al., 1995). The IC has been mapped to the \( \text{SNRPN} \) locus (Buiting et al., 1995; Dittrich et al., 1996). Immediately upstream of the \( \text{SNRPN} \) promoter lie a series of alternative 5' exons that are spliced to exons 2-10 (Dittrich et al., 1996). In AS, IC mutations map within these upstream BD exons; in PWS, the deletions map further 3' to \( \text{SNRPN} \) exon 1. Based on these findings, Dittrich et al. (1996) have proposed a model for imprint switching during gametogenesis. They suggest that the alternative \( \text{SNRPN} \) transcript (the imprinter) acts in \( \text{cis} \) on the exon 1 region (the imprint-switch initiation site) to bring about the parental switch. In the female germline a \( \text{trans} \)-acting maternal-specific factor allows the paternal -> maternal switch. In the male germline the maternal -> paternal switch occurs, probably by default (Buiting et al., 1998). The model does not make any predictions about the fate of the paternal chromosome in the male germline and the maternal chromosome in the female germline. These imprints may be retained or erased and reestablished (Ferguson-Smith, 1996).

Imprinting mutations have also been implicated in BWS. Reik et al. (1995) identified patients with normal biparental inheritance of the 11p15.5 region but altered allelic methylation of \( \text{H19} \) and \( \text{IGF2} \). Translocation breakpoints in this disorder have been mapped to two clusters that do not disrupt the coding sequences of known imprinted genes. One of these results in activation of the repressed \( \text{IGF2} \) allele while expression from \( \text{H19} \)
remains unaffected (Brown et al., 1996). Distant rearrangements are therefore capable of affecting the imprinting process within this region.

Many features of the PWS/AS and BWS imprinting domains are reminiscent of X chromosome inactivation. The process of X inactivation has recently been reviewed by Goto and Monk (1998). Although in most cells X inactivation is random, in some species, including mice, the paternal allele is preferentially inactivated in extraembryonic cells during early embryogenesis (Takagi and Sasaki, 1975). X inactivation spreads throughout the entire chromosome from a single centre (XIC) in a fashion analogous to the proposed action of ICs in PWS/AS. The process involves the action of XIST (X-inactive specific transcript), a gene within the XIC which is uniquely transcribed from the inactive chromosome alone. XIST codes for an RNA molecule with several conserved tandem repeats which coats the inactive X chromosome. It is likely that X inactivation and imprinting of autosomal genes share at least some mechanistic components.

1.6.3.3 Implications for other imprinted regions

Control of imprinting is undoubtedly complex and our understanding of the process is still rapidly evolving. It is clear from studies so far that both methylation and chromatin structure have central roles to play. Imprinted genes tend to be localised in clusters and regulated by ICs. Multiple control mechanisms seem to be involved, some local and others regional. Experimental findings in PWS, AS and BWS have led to several mechanistic models. However, we are still far from being able to incorporate all the existing evidence into one coherent scheme. It is generally assumed that the mechanisms underlying other imprinting disorders such as SRS will eventually prove similar to those described for PWS, AS and BWS. Characterisation of the imprinted regions responsible in these disorders should help to formulate a unified model for the control of imprinting.
1.6.4 Imprinting plasticity

1.6.4.1 Developmental, tissue and species-specific imprinting

The original assumption that imprinted genes would show parent-of-origin-specific expression in every tissue throughout development has, perhaps inevitably, proved oversimplified. There are an increasing number of reports of tissue and developmental stage-specific imprinting. For example, the maternal allele of \( IGF2 \) is normally silent but becomes active in the choroid plexus and leptomeninges of mice (DeChiara et al., 1991) and humans (Ohlsson et al., 1994), resulting in biallelic expression in these tissues. \( IGF2 \) expression has also been shown to become biallelic during postnatal development in the liver (Ekström et al., 1995). More recently, \( UBE3A \) has been recognised as the AS gene (Kishino et al., 1997; Matsuura et al., 1997). Earlier studies had demonstrated biallelic expression of this gene in cultured fibroblasts and lymphoblasts, leading to suggestions that it was not imprinted (Nakao et al., 1994). However monoallelic expression has since been demonstrated in the brain (Rougeulle et al., 1997). Relaxation of imprinting has also been described for several imprinted genes in cancer cell lines (Rainier et al., 1993).

All these observations suggest that the imprinting process must allow some lability in allelic usage. Use of alternate promoters/enhancers is one means by which parental allele expression can be regulated. The \( IGF2 \) gene has four different promoters and can produce several different transcripts. Studies of the expression of these different transcripts by Ekström et al. (1995) suggest that complex promoter-specific allele usage may explain changes in developmental stage-specific imprinting of \( IGF2 \). It remains to be seen whether this will prove to be a more widespread control mechanism employed by other imprinted genes.

Since methylation is thought to play a role in the maintenance of imprinting, allelic methylation patterns would be expected to mirror changes in parental allele expression. As predicted, tissue and stage-specific variation in methylation of some imprinted genes has been found. For example, a differentially methylated region in the 3' part of \( Igf2 \) is virtually unmethylated on the silent maternal allele. Methylation on the paternal allele is tissue-specific and correlates with expression of the gene (Feil et al., 1994). In fetal brain, where \( Igf2 \) is only weakly expressed, the paternal allele is almost completely unmethylated.
In contrast, in fetal liver, which highly expresses Igf2, it is almost completely methylated. The gene is still differentially methylated in adult liver, where it is not expressed, but at a significantly lower level than in the fetus. In general, however, methylation tends to remain unchanged despite variation in expression throughout development (Constância et al., 1998). In fetal tissues MEST is monoallelically expressed from the paternal allele; in adult blood lymphocytes the gene is expressed biallelically. Despite this, differential methylation patterns appear identical in both adult and fetal tissues (Riesewijk et al., 1997). The role of methylation in developmental and tissue-specific 'relaxation' of imprinting is therefore unclear.

Imprinting is usually but not always conserved between species. In mouse, Igf2r is expressed exclusively from the maternal allele. In contrast, the human IGF2R gene is biallelically expressed in the majority of the human population (Kalsheuer et al., 1993). This may be a polymorphic trait since a small proportion seem to have monoallelic expression from the maternal allele (Xu et al., 1993). Mouse U2afbp-rs is also imprinted whereas no evidence has been found for imprinting of its human homologue, U2AFBPL (Pearsall et al., 1996).

1.6.4.2 Somatic allele switching

Occasional tissue-specific switching of allele usage has been demonstrated for a number of genes. For example, WTI (Wilms tumour 1) is maternally expressed in placenta and brain of some (but not all) individuals (Jinno et al., 1994). However, both biallelic and paternal-specific expression has been documented in fibroblast and lymphocyte samples (Mitsuya et al., 1997). Zhang et al. (1993) observed expression of H19 from the 'silent' paternal allele alone in cerebellar cells from one individual. H19 allele switching has also been described by Douc-Rasy et al. (1995) in two of 14 invasive cervical carcinomas studied. Biallelic expression of H19 was detected in a further two cervical tumours. Interestingly, the expressing H19 allele, and to a lesser extent also the silent allele, were hypomethylated in most of the tumours studied. Allele switch was accompanied by a switch in allele methylation status, with the expressing allele showing the greatest degree of hypomethylation. These observations suggest that demethylation of both H19 alleles may be associated with an early step in imprinting alteration and support the theory that
methylation is involved in maintenance of the imprint. Reversal of \textit{IGF2} methylation has been observed in one BWS patient (Brown et al., 1996). In fibroblasts \textit{IGF2} was methylated, as expected, on the paternal allele. However, surprisingly, in placenta and amniocytes the maternal allele was methylated. Although expression from the two alleles in the different tissues studied was not described, it is tempting to speculate that allele usage had also been reversed as a result of alteration in the methylation status of the gene.

The findings for \textit{H19} (Douc-Rasy et al., 1995) are in apparent conflict with maintenance of \textit{MEST} differential methylation in tissues where it is biallelically expressed (Section 1.6.4.1). There is no clear role for methylation of \textit{MEST} in the maintenance of the imprint. It is possible that only a small number of differentially methylated CpGs, which may be some distance from the coding region of \textit{MEST}, are critical for regulation of its transcription. Alternatively, different genes may employ different means of maintaining their imprint. For \textit{MEST}, stage and tissue-specific differences in chromatin structure and/or transcription factors may allow transcription from a normally silent allele. Another possibility is the use of alternate promoters, as has been seen with \textit{IGF2} (Cuisset et al., 1997).

It is currently unclear why somatic allele switching should occur in some, but not all, individuals. This question is of great interest to those studying imprinting mechanisms. It may be possible to identify crucial epigenetic or sequence differences by comparing DNA from individuals in which alternate alleles are being transcribed. The importance of the phenomenon in cancer and other human diseases also remains to be determined.

1.6.4.3 Differential usage of parental alleles

When imprinted genes were first described it seemed that the imprinting process was an all-or-nothing phenomenon. Expression was noted from one parental allele alone with no expression from the opposite, silent allele. However, it is increasingly recognised that for some imprinted genes transcription can occur from the ‘silent’ allele, albeit at low levels. For example, \textit{MEST} is expressed predominantly from the paternal allele in human chorions and early embryos (Kobayashi et al., 1997). However, expression from the maternal allele can also be detected. Quantitative analysis has demonstrated that the maternal allele is expressed at levels more than 20 times lower than those from the paternal allele.
Partial imprinting has also been described for \(p57^{KIP2}\) (CDKN1C), IPL, UBE3A and \(IMPT1\) (Chung et al., 1996; Qian et al., 1997; Vu and Hoffman, 1997; Cooper et al., 1998).

The mechanism of imprinting maintenance in these genes must permit some 'leaky' expression from the inactive allele. In the promoter region of \(Igf2\), the repressed maternal allele is unmethylated at numerous assayable CpGs (Sasaki et al., 1992). The overall methylation pattern is not obviously different from the active allele. DNaseI hypersensitive sites are consistently present on both parental chromosomes, suggesting that the chromatin of the repressed allele is potentially active for transcription. In agreement with the methylation and chromatin state, transcripts from the maternal \(Igf2\) allele are detectable at low but significant levels. Thus, once again, there is evidence that methylation status and chromatin structure are important factors in regulation of transcription from imprinted genes.

1.7 Imprinting: a role in growth regulation

From the earliest studies of mouse imprinting phenotypes by Cattanach and Kirk (1985) (Section 1.4.2, above), it has been obvious that imprinting has a major role to play in fetal growth. UPD for several chromosomes or chromosomal regions results in abnormal fetal growth, with paternal disomies promoting growth and maternal disomies inhibiting growth (Beechey and Cattanach, 1998). Furthermore, chimeric embryos formed from normal and gynogenetic embryos tend to be smaller than normal litter mates. Conversely, androgenetic chimeras are larger than expected (Barton et al., 1991).

Over 20 imprinted genes have now been identified (Morison and Reeve, 1998). A large number, though not all, of these are involved in growth regulation (Tilghman, 1999). Several imprinted genes act in the fetal growth pathway mediated by insulin-like growth factor II (IGFII). These include \(Igf2\) itself, \(Igf2r\), \(H19\) and \(Grb10\). Targeted mutations in \(Igf2\), \(Igf2r\) and \(H19\) have all been shown to result in abnormal fetal growth rates (DeChiara et al., 1990; Lau et al., 1994; Leighton et al., 1995). Other imprinted genes such as \(Ins2\), \(p57^{KIP2}\) (Cdkn1c), \(Gnas\), \(Rasgrf1\) and \(Mash2\), are involved in regulation of fetal growth and/or the cell cycle.
Chapter 1. Introduction

Not all imprinting effects, however, are directly related to growth control. The imprinting phenotypes in mice described by Beechey and Cattanach (1998) include behavioural abnormalities. As described in Section 1.5.1, the human imprinting disorders PWS and AS are also characterised by behavioural problems and mental retardation. A recent study of patients with Turner syndrome suggests that one or more gene(s) on the X chromosome escape X inactivation but are silent on the maternal X and involved in development of verbal and social skills (Skuse et al., 1997). Allen et al. (1995) described differences in the number and distribution of uniparental cells in tissues of gynogenetic and androgenetic chimeras. Androgenetic cells contribute disproportionately to mesodermal derivatives like heart and skeletal muscle and relatively little to the brain. In contrast, gynogenetic cells contribute little to mesoderm but are found abundantly in brain. Furthermore, behavioural studies showed an increase in male aggression when the proportion of gynogenetic cells in the brain was high. It therefore seems that imprinted genes may be involved in brain development and behaviour.

The role of imprinted genes in determining behaviour may in some instances indirectly affect growth regulation. For example, PWS patients characteristically develop severe obesity as a result of hyperphagia. An effect on maternal behaviour may also lead to abnormal growth in the following generation. Evidence for such an effect exists for at least one imprinted gene. Targeted disruption of the paternally expressed gene Peg1/Mest in mice is associated with poor maternal care (Lefebvre et al., 1998). This in turn is associated with markedly reduced survival rates for the offspring of mutant mothers, independent of their genotype. Although not tested experimentally, the failure of mutant females to care for their offspring may also affect the growth of their few surviving pups.
Chapter 1. Introduction

1.7.1 The parent-offspring conflict hypothesis

Many different theories have been proposed to explain the evolution of imprinting in mammals. Suggestions include prevention of parthenogenesis (Solter et al., 1988), restraint of placental growth (Hall, 1990), the result of host defense mechanisms (Barlow, 1993), protection against malignant germ cell tumours in females (Varmuza and Mann, 1994) and prevention of aneuploidy (Thomas, 1995). Most widely discussed, however, is the parent-offspring conflict hypothesis (Haig and Westoby, 1989). In polyandrous mammals there is a conflict between the desires of the two parents. The father must optimise the reproductive fitness of his offspring by enhancing their growth even at the expense of the mother’s future litters. The mother, on the other hand, must conserve her own resources during any one pregnancy to ensure that she also has many offspring. This situation has been referred to as a genetic ‘tug-of-war’ (Moore and Haig, 1991).

The conflict theory predicts that expression of the paternal genome in embryos should tend to increase offspring size whereas maternal genome expression should tend to reduce it. No imprinted genes had been identified at the time the hypothesis was proposed. However, the theory has since proved to be consistent with many of the imprinting effects observed. Igf2, H19 and Igf2r have growth effects which exactly match predictions. Mice carrying mutations of the paternally expressed Igf2 gene have birth weights 40% lower than normal (DeChiara et al., 1990). Conversely, mutations of both Igf2r and H19, two maternally expressed genes, result in prenatal overgrowth (Lau et al., 1994; Leighton et al., 1995). Also as predicted, mice carrying targeted mutations of the paternally expressed gene Mest are prenatally growth retarded (Lefebvre et al., 1998). However, not all imprinted genes have effects consistent with the model. Some, such as Ube3A, have no known role in growth, while the function of others is inconsistent with the model. For example, knockouts of the maternally expressed Mach2 have poor placental growth (Guillemot et al., 1995).

In a recent review of both human and mouse UPDs, Hurst (1997) concluded that, although some phenotypes are strongly consistent with the conflict hypothesis, much of the data does not match predictions. The growth effects seen in disomic SRS and BWS patients are strongly supportive of the model. Maternal UPD7 in SRS is associated with prenatal growth failure; paternal UPD11 in BWS results in overgrowth. However, pUPD6
in man is associated with growth retardation (Temple et al., 1995). Similarly, in mouse, the opposite growth effects seen with maternal and paternal UPD for proximal chromosome 11 (described in Section 1.4.2) neatly support the hypothesis. However, the fetal growth retardation associated with pUPD for proximal chromosome 7 and distal chromosome 12 is inconsistent.

The parent-conflict hypothesis is currently the theory that comes closest to accounting for the experimental and clinical data on imprinting phenotypes. However, existing evidence is not entirely consistent with the model. This could be resolved if not all imprinted genes are the direct physiological target of the imprinting process. Although this argument is a difficult one to disprove, we should be wary of using it to explain away all contradictory evidence. What is clear is that regulation of growth is a major function of imprinted genes. Whether imprinting originally evolved as a mechanism of growth control is a subject of greater contention.

1.8 Summary

Over the last few years the role of genomic imprinting in human disease has become increasingly recognised. Several disorders, including PWS, AS and BWS, have been shown to result from abnormal imprinting of gene(s). Recent evidence suggests that SRS is also a disorder of genomic imprinting. SRS is probably genetically heterogeneous. However, mUPD7 has been found in approximately 7% patients. Since no consistently isodisomic regions have been identified it is likely that one or more imprinted gene(s) are involved. The frequent association of imprinted genes with growth regulation is consistent with the phenotype of prenatal growth failure seen in SRS.
Chapter 2.
Experimental aims and general strategy

The objective of this study was to identify one or more imprinted gene(s) on chromosome 7 which play a role in SRS. Evidence for involvement of several candidates was sought. Investigation into the role of each gene had two specific objectives:

1. To determine the imprinting status of the gene, if not previously described.
2. To investigate possible involvement of the gene, if imprinted, in SRS.

2.1 Candidate genes

At the beginning of this study, there were no known imprinted genes located on chromosome 7. Candidate genes were therefore sought by comparing mouse and human linkage maps. As described in section 1.4.2 above, several regions of human chromosome 7 are homologous with imprinted regions in the mouse (Beechey and Cattanach, 1998). Of particular interest is the imprinted region on mouse proximal chromosome 11 homologous to human chromosome 7p11.2-p13 (Figure 2.1). Maternal UPD of proximal chromosome 11 in mice leads to prenatal growth retardation. Three genes with growth-related functions (IGFBP1, IGFBP3 and EGFR) map within this region and have been proposed as candidates for SRS (Kotzot et al., 1995; Preece et al., 1997). The original aim of this study was to investigate the role of IGFBP1, IGFBP3 and EGFR in SRS.

During the course of this investigation, MEST was identified as the first imprinted gene on human chromosome 7 (Nishita et al., 1996; Kobayashi et al., 1997; Riesewijk et al., 1997). MEST maps to 7q32, a region sharing syntenic homology with an imprinted region on mouse proximal chromosome 6 (Figure 1.5B). As discussed more fully in Chapter 7, targeted mutation of this paternally expressed gene leads to prenatal growth retardation (Lefebvre et al., 1998). It has therefore been suggested that MEST may play a role in the SRS phenotype (Nishita et al., 1996; Kobayashi et al., 1997; Riesewijk et al.,
Chapter 2. Experimental aims and strategy

1997; Lefebvre et al., 1997). Even more recently, interest has surrounded the possible involvement of another gene, GRB10, which maps to 7p11.2-12. As described in chapter 8, the mouse homologue of this gene, MEG1/Grb10, has recently been found to be imprinted and expressed exclusively from the maternal allele (Miyoshi et al., 1998). Although the imprinting status of the gene remains to be determined in man, its role as a growth suppressor has led to speculation that GRB10 may be involved in the pathogenesis of SRS. In light of these recent findings, the aim of the study was extended to include investigation into the role of these two, new candidates: MEST and GRB10.

The results are divided into four chapters, describing work on all five candidate genes. The general approach to investigation of their role in SRS is discussed briefly below. Background information and more specific details of the experimental strategies taken for each gene are presented in the relevant chapter.
Figure 2.1. Ideogram of human chromosome 7 illustrating regions homologous to mouse imprinted regions. The approximate locations of the five candidate genes studied are shown.
2.1.1 IGFBP1, IGFBP3 and EGFR

Since the SRS phenotype in mUPD7 patients is thought to result from disruption of one or more imprinted gene(s), monoallelic expression of a gene would warrant further investigation into its role in the disorder. Conversely, biallelic expression makes a gene an unlikely candidate for SRS. Prior to this study, the imprinting status of IGFBP1, IGFBP3 and EGFR had not been described. Evidence for imprinting of these three genes was therefore sought. A similar approach was adopted for each gene and is outlined below:

1. Investigation of expression in fetal tissues available for study;
2. Identification of expressed polymorphisms;
3. Investigation of parental allele expression.

2.1.1.1 Investigation of expression in fetal tissues

The most direct evidence that a gene is imprinted comes from demonstration that its alleles are differentially expressed dependent on their parental origin. Since SRS is associated with prenatal growth failure, the imprinting status of all three candidate genes was investigated in normal fetal tissues collected from terminated pregnancies. Preliminary work was carried out to determine the pattern of IGFBP1, IGFBP3 and EGFR expression in fetal samples available. This allowed tissues suitable for analysis of parental allele expression to be identified.

2.1.1.2 Identification of expressed polymorphisms

In order for parental allele expression to be investigated, expressed polymorphisms within the gene of interest need to be identified. As described in Chapters 5 and 6, published reports of polymorphisms exist for both IGFBP1 (Luthman et al., 1989; Ehrenborg et al., 1992) and EGFR (Moriai et al., 1993). However, for IGFBP3, no published reports of polymorphisms could be found. A screen of the 3' untranslated region (3' UTR) was therefore carried out since this is usually the least conserved region of a gene. No sequence changes were detected using single stranded conformational
polymorphism (SSCP) analysis. However, an expressed polymorphism within exon 5 has recently been observed by another group (Dr I. Morison, personal communication). This polymorphism was demonstrated by direct sequencing and subsequently used to investigate the imprinting status of \textit{IGFBP3} (Chapter 5).

2.1.1.3 Investigation of parental allele expression

Where possible, paired fetal tissue and maternal blood samples were collected to allow parental alleles to be distinguished in fetal DNA. The informativity of a polymorphism was assessed in genomic DNA from each related mother and fetus. For full informativity the maternal DNA needs to be homozygous and the fetal DNA heterozygous. In this situation parental alleles can be distinguished in fetal mRNA. If both alleles are expressed then the gene is not imprinted. If it is imprinted then either only the maternal, or only the paternal allele, will be expressed. Where both maternal and fetal genomic DNA samples are heterozygous or paired maternal blood is not available it is not possible to determine whether monoallelic expression is confined to the maternal or the paternal allele. Expression was also investigated in cell lines from SRS patients with mUPD7. Absence of RT-PCR products in these patients but not in controls would suggest expression from the paternal allele alone.

Experimental design was based on knowledge of imprinting mechanisms at the start of this project. Imprinting is a rapidly developing field of research and, inevitably, advances in understanding have occurred during the course of this study. In some instances foreknowledge of these advances would have affected the strategy adopted. Investigation of parental allele expression is a case in point. In this study analysis of allelic expression was not quantitative. However, it has become increasing clear that imprinting is not an all-or-nothing phenomenon. As described in section 1.6.4.3, some imprinted genes are transcribed from their 'silent' alleles, albeit at much lower levels. Ideally, therefore, quantitative analysis would have been included. This is discussed in more detail with the results in Section 9.1.

Since no evidence for imprinting of \textit{IGFBP1}, \textit{IGFBP3} or \textit{EGFR} was found, further investigation into their role in SRS was not carried out.
2.1.2 **MEST**

*MEST* is already known to be imprinted. Its role in SRS was therefore investigated further. In common with other imprinted genes, differential methylation of *MEST* has been observed. Southern blot analysis of the 5' CpG island of *MEST* showed parent-of-origin-specific methylation with a methylated maternal allele and unmethylated paternal allele (Riesewijk et al., 1997). In other imprinting-related disorders such as PWS and AS, UPD, deletions and imprinting mutations all alter allelic methylation differences. It seems likely that disrupted expression of a paternally expressed gene in SRS would arise from similar mechanisms, also leading to altered methylation patterns. Absence of an unmethylated allele for *MEST* would be indicative of paternal deletions or imprinting centre mutations affecting this gene, providing support for its involvement in SRS. The differential methylation pattern on *MEST* was therefore studied in a cohort of non-UPD SRS patients. Details of this investigation are given in Chapter 7.

2.1.3 **GRB10**

Imprinting of *GRB10* in man remains to be demonstrated. However, as described more fully in chapter 8, considerable evidence already exists to implicate this gene in SRS. It was therefore decided to search directly for disruption of the gene in SRS patients. This strategy was adopted for an additional reason. Around the time that Miyoshi et al. (1998) proposed *GRB10* as a candidate for SRS, a patient with characteristic features of the disorder was referred for investigation of a 7p duplication (case 49, Appendix 1). Detailed cytogenetic analysis revealed a duplication of the region to which *GRB10* maps. It seemed possible that other patients might have a submicroscopic duplication of the same region, resulting in increased dosage of this growth suppressor. *GRB10* dosage was therefore analysed in a cohort of non-UPD SRS patients. Details of this investigation are given in Chapter 8.
Chapter 3. Materials

3.1 Silver-Russell Syndrome patients

3.1.1 SRS cohort

Clinical details of the 49 patients used in this study are listed in Appendix 1. The cohort of 53 SRS probands investigated by Preece et al (1999) (see Section 1.2.2.3) includes all these patients except patient 49. Five of the 53 patients studied by Preece et al. were not included in the current study since insufficient DNA was available. Forty three individuals fulfilled at least three of the following diagnostic criteria: low birth weight (>$2 SD below mean); short stature at the time of diagnosis (>$2 SD below mean); characteristic facial features; and facial, limb or trunk asymmetry. The remaining six probands had typical retarded postnatal growth and facial features but slightly higher birth weights (2.58-3.11 kg). All patients had either classical or mild, but consistent, facial dysmorphism. In the majority of cases the diagnosis of SRS was made following assessment by Dr S. Price at the Department of Clinical Genetics, Leicester Royal Infirmary. However, ten patients were referred to us after the main cohort had been examined. In these cases, the diagnosis was made on the basis of clinical details sent by the referring clinician. The mean maternal and paternal ages at the time of the proband’s birth were 27.6 and 30.5 years respectively.

All of these patients were shown to have a normal karyotype (performed at North East Thames Regional Cytogenetic Centre). Maternal UPD7 has been demonstrated in five patients using multiple microsatellite repeat markers (see Figure 1.4) and Southern blot analysis with two VNTR probes. Details of three of these cases (4, 30 and 47) have been published (Preece et al. 1997). The other two cases (5 and 19) were identified subsequently.

For analysis in this study, 10 ml blood was collected from patients either during initial assessment by Dr Price or by the referring clinician. Peripheral blood lymphocytes were used to create EBV immortalised cell lines at the European Collection of Cell Lines, Centre for Applied Microbiology, CAMR, Porton Down, Salisbury. In addition, consent was obtained from the parents of seven patients (three with mUPD7 and four without) to
perform punch skin biopsies. These have been used to generate fibroblast cell lines in the Cytogenetics Laboratory at QCCH (S. Blunt).

Ethical approval was obtained for this study by the Joint Research Ethics Committee of Great Ormond Street Hospital and the Institute of Child Health (approval no. 1278).

3.1.2 Patient 49: case report

The proband (Figure 3.1) is a five year old girl, born at 36 weeks gestation weighing 1.88 kg (> 2 SD below mean). Her height at birth was 47.5 cm (> 2 SD below mean). She has been fed intermittently via a gastrostomy tube since two years of age due to prolonged feeding difficulties. At four years her height was 90.2 cm (> 2 SD below mean). She was found to have a triangular face with a small chin, a relatively large, down-turned mouth, frontal bossing, fifth finger clinodactyly, slightly blue sclerae and mild labial hypoplasia. There was no evidence of lateral asymmetry. At the time of assessment she was noted to have mild developmental delay, persistent hypoglycaemia and increased sweating. Growth hormone levels were normal. Treatment with growth hormone was started at 5 years 1 month as her height remained below 3\textsuperscript{rd} centile (> 2 SD below mean).

Details of her family pedigree are shown in Appendix 2. Her mother (III.1) was born weighing 2.93 kg (10-25\textsuperscript{th} centile). Her final height is just 142.1 cm (> 2 SD below mean). She has a square facies with a relatively large mouth, fifth finger clinodactyly but no asymmetry. She also reports increased sweating and is being treated for insulin dependent diabetes mellitus. The proband's father (III.2) is of average height and phenotypically normal. Her sister (IV.1) was born with a birth weight of 3.34 kg (25-50\textsuperscript{th} centile). She does not have the characteristic facies associated with SRS and her current height at seven years is on 5\textsuperscript{th} centile. Both maternal grandparents are small with heights below 3\textsuperscript{rd} centile. Her maternal grandmother (II.1) is also reported to have a similar facial appearance to that of the proband and her mother. Her maternal grandfather (II.2) has mild learning difficulties and fifth finger clinodactyly.
Chapter 3. Materials

Figure 3.1. Patient 49 with her mother
3.2 Fetal tissues

Fetal tissue samples (placenta, skin, brain, liver, heart, intestine, kidney and lung) were obtained from the MRC Tissue Bank at Hammersmith Hospital (L. Wong). Further samples were collected from pregnancies terminated at QCCH. To avoid contamination of fetal tissues with maternal DNA, decidua was carefully removed. All tissues were washed in PBS to remove maternal blood. They were then snap frozen and stored at -70°C until use. For investigation of imprinting status, 5 ml of paired maternal blood was also collected and stored at -20°C. Local ethics approval for collection of maternal and fetal samples was granted by the Research Ethics Committee of the Royal Postgraduate Medical School (96/4955).

3.3 Reagents

All reagents apart from the following were Analar grade supplied by BDH Merck Ltd. Phenol (Rathbone Chemicals): agar, tryptone, yeast extract (Difco Laboratories); bovine serum albumin (BSA), Histopaque 1077, N-laurylsarcosine, xylene cyanol, bromophenol blue, ethidium bromide, orange G, X-Gal, isopropyl β-D-thiogalactopyranoside (IPTG), 2-mercaptoethanol, sodium bisulfite, hydroquinone, tetracycline, ampicillin, TEMED, ammonium persulphate, human placental DNA, thymidine, deoxycytosine (Sigma); random hexanucleotide primers, dNTPs, RNasin, guanidine thiocyanate (Promega); sephadex G50 and G25 (Pharmacia); kanamycin, colcemid, COT-1 DNA (Gibco BRL); nick translation kit, LSI hybridisation buffer (Vysis); Vectorshield/ DAPI (Vector Laboratories).

3.4 Cell culture media

Standard culture medium contained RPMI, penicillin-streptomycin and L-glutamine (Gibco BRL). The cell freezing medium used was DMSO (Sigma).
Chapter 3. Materials

3.5 Enzymes

All restriction endonucleases were obtained from Promega, Gibco BRL or New England Biolabs. The various sources used for other enzymes were as follows: Klenow large fragment DNA polymerase (Gibco BRL); Taq DNA polymerase (Bioline and Applied Biosystems); proteinase K (Boehringer Mannheim); sequenase (United States Biochemicals, Amersham); MMLV (Promega).

3.6 Radioisotopes

[α-32P]dCTP (3,000 Ci/mmol) and [α-35S]dATP (3,000 Ci/mmol) were all obtained as aqueous solutions from ICN.

3.7 Gel electrophoresis

The following materials were used for gel electrophoresis: agarose, low melting point agarose and DNA size markers (Promega); Sequagel reagents for polyacrylamide gel electrophoresis (National Diagnostics); precast CleanGel polyacrylamide gels and silver staining kits (Pharmacia).

3.8 Photography, blotting and autoradiography

Gels were photographed with Image Master gel documentation equipment from VDS using a Fujifilm thermal imaging system. Other materials were obtained from the following sources: Magna nylon transfer membrane (MSI); GeneScreen Plus (NEN); 3MM chromatography paper (Whatmann Ltd.); autoradiographic cassettes and intensifying screens (Genetic Research Instruments); X-ray film: Kodak Biomax and XB-200 (X-OGRAPH); developing solutions, phenisol and K5 emulsion (Ilford).
Chapter 3. Materials

3.9 Transformations

The \textit{E.coli} strain XL1blue MRF' (Stratagene) was used for subcloning of PCR products. This contains \textit{lacIqZΔM15}, allowing blue/white colour selection by $\alpha$-complementation of the $\beta$-galactosidase gene. The cloning vector used was pGEM-T Easy (Promega).

3.10 Fluorescence \textit{in situ} hybridisation (FISH) equipment

Axioscope 20 fluorescence microscope (Zeiss);
CCD camera (Photometrics);
Power Macintosh, 9500/132 (Apple Macintosh);
SmartCapture software (Digital Scientific).

3.11 Oligonucleotides

Oligonucleotides were obtained from the HGMP Resource Centre, Hinxton, Genosys or Gibco BRL, except those for tetranucleotide markers which were ordered from Research Genetics. Details of the primers used are given below in Table 3.1.
### Table 3.1. Primer sequences.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGFBP1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1-F</td>
<td>ggagctcttgatttcca tcccatcacaaggttagac</td>
<td>315</td>
<td>57</td>
</tr>
<tr>
<td>-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1-BF</td>
<td>gtctacccttggaatgggaatattaattgctgttctagagttat</td>
<td>167</td>
<td>61</td>
</tr>
<tr>
<td>-BR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1-BF</td>
<td>gtctacccttggaatgggaatattaattgctgttctagagttat</td>
<td>218</td>
<td>55</td>
</tr>
<tr>
<td>-ER</td>
<td>ttaggttataaaaagtagttact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1-EF</td>
<td>ggagctcttgatatattcaaatatagttact</td>
<td>533</td>
<td>57</td>
</tr>
<tr>
<td>-ER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFBP3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-F</td>
<td>ctacgagtctcagacacag ctgcagcagctgcatgctgca</td>
<td>301</td>
<td>57</td>
</tr>
<tr>
<td>-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-BF</td>
<td>gctcatgagatagggacagtcctgggtgtgcagcg</td>
<td>197</td>
<td>61</td>
</tr>
<tr>
<td>-BR</td>
<td>ggtgtgagttacggtacagc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-CF</td>
<td>gacatccaaagcatgtctgcatcagcagcaagagt</td>
<td>381</td>
<td>59</td>
</tr>
<tr>
<td>-CR</td>
<td>ctgcagtcagcagcagcaagagt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-DF</td>
<td>tcagagctccacaagtaatgggctggagtgc</td>
<td>670</td>
<td>55</td>
</tr>
<tr>
<td>-DR</td>
<td>ctagcatgacagatctgtgactgggctggagtgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-EF</td>
<td>aggtggagtctctgtggttctgggctggagtgc</td>
<td>358</td>
<td>55</td>
</tr>
<tr>
<td>-ER</td>
<td>tctgggtcagagctgggctgggctggagtgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3-GF</td>
<td>tgctacagctgcagcagcaagag</td>
<td>967</td>
<td>55</td>
</tr>
<tr>
<td>-ER</td>
<td>tctgggagctgggctgggctgggctggagtgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR-F</td>
<td>ggacggagcagtgtctgctggagtcgccg cgcagcgcgggctggctgctgc</td>
<td>208</td>
<td>61</td>
</tr>
<tr>
<td>-R</td>
<td>ggacggagcagtgtctgctggagtcgccg cgcagcgcgggctggctgctgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR-A1</td>
<td>gtttgggacctccggtcgctgtcgcagcagcgcgggctggctgc</td>
<td>205</td>
<td>59</td>
</tr>
<tr>
<td>-A2</td>
<td>gtttgggacctccggtcgctgtcgcagcagcgcgggctggctgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR-B1</td>
<td>gtctgagccgctgggtgctgcagcgcgggctggctgc</td>
<td>102</td>
<td>59</td>
</tr>
<tr>
<td>-B2</td>
<td>gtctgagccgctgggtgctgcagcgcgggctggctgc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.1 (cont.). Primer sequences.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MEST</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPIF</td>
<td>cactgatgcagaagacgctc</td>
<td>792</td>
<td>63</td>
</tr>
<tr>
<td>HPIR</td>
<td>cagcaccatattgctcatagg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEST-COM</td>
<td>aacacccctctcataaaaaa</td>
<td>347</td>
<td>59</td>
</tr>
<tr>
<td>MEST-MAT</td>
<td>cggagtggttgtagttgcttc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEST-COM</td>
<td>aacacccctctcataaaaaa</td>
<td>239</td>
<td>59</td>
</tr>
<tr>
<td>MEST-PAT</td>
<td>ttggtttttagttttagttggt</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GRB10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRB10-F -R</td>
<td>aagagctgaggtttctgtgctcataa</td>
<td>679</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>gagacatctgcgggtcataa</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tetrانucleotide markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S820-F -R</td>
<td>tgtcatagtttagaaccgaactaag</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ctgaggtatcagtaaaaaactcaggg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S821-F -R</td>
<td>aacaaaaaaagtaagctga</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tatgacaggtgtctgggtagt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S1804-F -R</td>
<td>ttcaagtgggtggtcacttcaactgt</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tgggtctagccagttagttggt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S2195-F -R</td>
<td>actgcactgctcatacaaat</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aggatgagagttccgtagatg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S1805-F -R</td>
<td>cctgctttggcttacactgta</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cccactctctgtgattacat</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH-F -R</td>
<td>ccaccctatgcaaatttcatggca</td>
<td>598</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>ccctgaagggcaggtgctggccacc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2-F -R</td>
<td>cttgacatgctcaatcgactgg</td>
<td>236</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>ctcctgtagaccttacagtgg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>gtaatacgacactctatatagggc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP6</td>
<td>attaggtgacactataag</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.12 Clones

The cDNA and genomic clones used and the source from which they were obtained are listed in Tables 3.2 and 3.3 below.

Table 3.2. cDNA clones

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clone name</th>
<th>Insert size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP1</td>
<td>phIGFBP1-103</td>
<td>1,460</td>
<td>Professor H. Luthman (Luthman et al., 1989)</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>hBP3-1.3</td>
<td>1,287</td>
<td>Professor D. Powell (Cubbage et al., 1990)</td>
</tr>
</tbody>
</table>

Table 3.3. Genomic clones

<table>
<thead>
<tr>
<th>Location</th>
<th>Clone name</th>
<th>Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEST</td>
<td>Icos53g3</td>
<td>Cosmid</td>
<td>S. Sherer, Toronto*</td>
</tr>
<tr>
<td>GRB10</td>
<td>H_DJ0108E23</td>
<td>PAC</td>
<td>S. Sherer, Toronto*</td>
</tr>
<tr>
<td>CFTR</td>
<td>H_DJ0020F22</td>
<td>Cosmid</td>
<td>S. Sherer, Toronto*</td>
</tr>
<tr>
<td>Di George region</td>
<td>A6121</td>
<td>Cosmid</td>
<td>P. Scambler, Institute of Child Health</td>
</tr>
<tr>
<td>Di George region</td>
<td>A9100</td>
<td>Fosmid</td>
<td>P. Scambler, Institute of Child Health</td>
</tr>
<tr>
<td>Chromosome 22</td>
<td>5H3</td>
<td>Cosmid</td>
<td>P. Scambler, Institute of Child Health</td>
</tr>
<tr>
<td>Chromosome 22</td>
<td>27</td>
<td>Cosmid</td>
<td>P. Scambler, Institute of Child Health</td>
</tr>
</tbody>
</table>

* http://genet.sickkids.on.ca/chromosome7/
Chapter 4. Methods

4.1 Solutions

The following solutions were used during the course of this study.

**DNA extraction:**

Lysis buffer: 0.3 M Sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1% Triton X-100

Suspension buffer: 75 mM NaCl, 25 mM EDTA (pH 8.0)

TE buffer: 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA (pH 8.0)

**Gel electrophoresis:**

1x TAE: 40 mM Tris-acetate, 1 mM EDTA (pH 8.0)

For 1 litre of 10x TAE buffer: 48.5 g Tris base

11.25 ml Glacial acetic acid

10 ml 0.5 M EDTA (pH 8.0)

1x TBE: 45 mM Tris-borate, 1 mM EDTA (pH 8.0)

For 1 litre of 10x TBE buffer: 108 g Tris base

55 g Boric acid

10 ml 0.5 M EDTA (pH 8.0)

**Southern analysis:**

Denaturing solution: 1.5 M NaCl, 0.5 M NaOH

Neutralising solution: 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5)

20x SSC: 3 M NaCl, 0.3 M Na Citrate (pH 7.0)
OLB:

Solution O: 1.25 M Tris-HCl (pH 8.0), 0.125 M MgCl₂
Solution A: 2.5 μl each of 0.1 M dATP, dTTP, and dGTP dissolved in TE and adjusted to pH 7.0, 9 μl 2-mercaptoethanol, Solution O added to 500 μl
Solution B: 2 M HEPES adjusted to pH 6.6 using KOH
Solution C: 90 OD units/ml Hexanucleotides in TE buffer
Solutions A, B and C mixed in the ratio 1: 2.5: 1.5, respectively.

20x SSPE: 3.6 M NaCl, 0.2 M Sodium phosphate, 20 mM EDTA (pH 8.0)

100x Denhardts: 2% Ficoll, 2% BSA, 2% PVP

Prehybridisation mix: 5x SSPE, 5x Denhardts, 0.1% SDS, 100 μg/ml denatured, sheared salmon sperm DNA

**Preparation of cosmid/PAC DNA:**

LB broth (1 litre): 10 g Tryptone, 10 g Yeast extract, 5 g NaCl (15 g Agar for plates)

**SSCP analysis:**

Loading dye: 80% Deionised formamide, 0.5% Xylene cyanol, 0.5% Bromophenol blue, 10 mM EDTA (pH 8.0)

Silver solution: 0.1% Silver nitrate, 0.37% Formaldehyde

Developing solution: 0.25 M Sodium carbonate, 0.37% Formaldehyde, 0.02% Sodium thiosulphate

Stop solution: 40 mM EDTA (pH 8.0)
DNA sequencing:
TES: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl

Stop solution: 95% Formamide, 20 mM EDTA, 0.05% Xylene blue, 0.05% Bromophenol blue

Sodium bisulfite treatment of DNA:
Bisulfite solution: 8.1 g Sodium bisulfite in 15 ml water, 1 ml 40 mM Hydroquinone, 600 µl 10 M NaOH

RNA extraction:
Trypsin-versine: 0.125% trypsin, 0.125% EDTA in PBS

PBS:
0.14 M NaCl, 0.003 M KCl, 0.01 M Na$_2$HPO$_4$, 0.002 M KH$_2$PO$_4$, pH 7.4

Solution D: 4 M Guanidine thiocyanate, 25 mM Na citrate (pH 7.0), 0.5% N-Laurylsarcosine, 100 mM 2-Mercaptoethanol

Northern analysis:
Sample buffer: 52% Deionised formamide, 6.2% Formaldehyde, 1x MOPS, 7% Glycerol, 0.05% Bromophenol blue

10x MOPS: 0.2 M MOPS (pH 7.0), 80 mM Sodium acetate, 10 mM EDTA (pH 8.0)

Prehybridisation mix: 0.47x Denhardt’s solution, 4.7x SSPE, 0.1% SDS, 10% Dextran sulphate, 0.18 mg/ml Microwaved salmon sperm DNA
4.2 DNA Protocols

4.2.1 Extraction of DNA from peripheral blood leucocytes

The method used to isolate DNA was essentially that described by Kunkel et al. (1985). Five millilitres of blood were collected in EDTA-treated tubes and stored at -20°C until use. After thawing, the sample was mixed with 45 ml lysis buffer. Following centrifugation at 10,000 rpm for 10 minutes at 4°C, the supernatant was discarded and the nuclei resuspended in 3 ml suspension buffer. SDS and Proteinase K were added to final concentrations of 0.5% and 200 µg/ml respectively. Samples were then incubated overnight at 50°C.

The samples were extracted three times with an equal volume of equilibrated phenol to remove any remaining protein. Centrifugation at 10,000 rpm for 10 minutes at room temperature separated the phases. Phenol was then removed by three further extractions using an equal volume of chloroform and centrifugation as before. DNA was precipitated by adding the aqueous phase to 8 ml absolute ethanol and spooled out using a hooked Pasteur pipette. Salt was removed by washing briefly in 70% ethanol and the DNA then dissolved in 0.5 ml TE buffer.

4.2.2 Extraction of DNA from fetal tissue

Fetal tissue samples were stored at -70°C following collection. Immediately before use they were allowed to thaw at room temperature and thoroughly washed in 1x phosphate buffered saline (PBS) to remove any traces of maternal blood. Approximately 100 mg fetal tissue were homogenised in 400 µl TE using an Ultraturrax homogeniser (IKA Labortechnik). SDS and Proteinase K were then added to final concentrations of 0.5% and 200 µg/ml respectively. Following overnight incubation at 50°C, DNA was extracted in phenol and chloroform as described in section 4.2.1. DNA was precipitated by adding the aqueous phase to 1 ml absolute ethanol and spinning for 5 minutes at 10,000 rpm to obtain a pellet. The pellet was washed in 200 µl 70% ethanol and left to dry before resuspending in 50 µl TE.
4.2.3 Small scale preparation of plasmid/cosmid DNA (miniprep)

The Wizard Plus SV Minipreps DNA Purification System (Promega) was used for small scale preparation of DNA according to the manufacturer’s instructions. Clones were either picked as single colonies from agar plates or from a prepared glycerol (500 ml overnight culture in 50% glycerol) using an inoculating loop. They were grown overnight in 10 ml LB broth containing the appropriate selective antibiotic at 37°C with shaking. Cells were harvested by centrifugation at 1,500 rpm for 10 minutes. The medium was discarded and the pellet thoroughly resuspended in 250 µl cell resuspension solution. Cells were then mixed gently with 250 µl cell lysis solution and left to stand until the cell suspension cleared before adding 8 µl alkaline protease solution. Samples were gently mixed again, incubated for exactly 5 minutes at room temperature and 350 µl neutralization solution added. The bacterial lysate was then centrifuged at 13,000 rpm for 10 minutes at room temperature to pellet the cell debris. The supernatant was carefully decanted into a spin column inserted into a 2 ml collection tube and centrifuged for 1 minute at 13,000 rpm. The columns were then washed twice with column wash solution and transferred to a clean 1.5 ml microcentrifuge tube. Finally, the DNA was eluted in 100 µl nuclease-free water by spinning for 1 minute at 13,000 rpm.

4.2.4 Large scale preparation of cosmid/PAC DNA (midiprep)

For large scale preparation of cosmids and PACs the Qiagen tip-100 kit was used, following the manufacturer’s instructions. Five millilitres of LB broth containing 25 µg/ml kanamycin was inoculated and grown overnight at 37°C with shaking. One hundred microlitres of this culture were then used to inoculate 100 ml LB broth with 25 µg/ml kanamycin and again incubated overnight with shaking at 37°C. Cells were harvested by centrifugation at 6,000 rpm in a GSA rotor for 30 minutes at 4°C. The pellet was resuspended in 4 ml P1 buffer. The solution was then mixed with 4 ml P2 buffer and incubated for 5 minutes at room temperature. Four millilitres of chilled P3 buffer were added and the mixture incubated on ice for 15 minutes. Cell debris was pelleted by centrifugation at 15,000 rpm for 15 minutes at 4°C. The supernatant was immediately
Chapter 4. Methods

removed to a clean tube and recentrifuged to ensure complete removal of the debris. The supernatant was added to a QIAGEN-tip 100 equilibrated with 4 ml buffer QBT. Once the sample had drained through completely, the tip was washed twice with 10 ml buffer QC. DNA was then eluted from the tip with 5 ml buffer QF. To precipitate the DNA, 3.5 ml isopropanol was added and the solution centrifuged at 15,000 rpm for 30 minutes at 4°C. The pellet was washed with 2 ml ice cold 70% ethanol, recentrifuged for 15 minutes and left to air dry. Finally it was redissolved in 200 µl TE.

4.2.5 Determination of DNA concentration

DNA concentrations and purity were determined spectrophotometrically by scanning between the wavelengths of 320 nm and 220 nm. The optical density (OD) at 260 nm was used to calculate the DNA concentration (one OD unit = 50 µg/ml for double-stranded DNA). The ratio between the readings at 260 nm and 280 nm gives an estimate of sample purity. In a pure preparation the ratio will lie between 1.8 and 2.0. Following spectrophotometry, samples were diluted to 200 µg/ml in TE buffer and stored at 4°C until use.

4.2.6 Restriction enzyme digestion of DNA

Restriction digests of genomic DNA were typically set up using 4 µg DNA, 1/10 volume reaction buffer and an excess (20 units) of restriction enzyme in a total volume of 40 µl. The choice of buffer and incubation temperature was determined by the suggestions of the manufacturer (Promega, Gibco-BRL, New England Biolabs). Digests were incubated at the recommended temperature for 6 hours. PCR product digestions were carried out in smaller volumes (usually 20 µl in total, with 16 µl of PCR product), for shorter incubation periods (60-90 minutes). Plasmid and cosmid digestions were also performed in smaller volumes with less enzyme and for less time (3 hours) depending on the amounts being digested.
4.2.7 Agarose gel electrophoresis

Agarose gels in 1x TAE buffer were used to visualise and size fractionate small DNA fragments. The percentage agarose used (0.8%-4.0%) was determined according to the size of the products being electrophoresed and the degree of fragment separation required. High percentage gels were used to achieve the best separation of small, closely-sized fragments from PCR product digestions. Lower percentage gels were used to visualise larger DNA fragments. Ethidium bromide (10 mg/ml) was added to the agarose solution at a concentration of 0.35 mg/ml to allow detection of DNA when visualised under UV light. Orange G loading dye (20% Ficoll, orange G) was added to all samples at 1/10 volume. At least one low molecular weight size marker (1 kb or 100 bp ladder) was loaded on each gel to allow estimation of product sizes.

PCR products were electrophoresed using either midi-gel or mini-gel apparatus. These were run at 100 volts (5.5 V/cm) for approximately 30-60 minutes, according to the size of fragments to be separated. Genomic digests were separated using 20 x 20 cm gels run at 50 volts (~1.25 V/cm) for 16-20 hours.

When PCR products or digested fragments were to be used as probes for Southern blot hybridisation, DNA was run out on low melting point agarose gel. The band of interest was visualised under UV light and cut from the gel. Three hundred microlitres of water were added to every 100 mg of agarose gel excised and the probe dissolved by incubating at 65 °C for 1 hour.

4.2.8 Polyacrylamide gel electrophoresis

Polyacrylamide gels allow high resolution of DNA samples of less than 500 bp length. Denaturing polyacrylamide gels were used for separating sequencing products. Non-denaturing gels were used for SSCP analysis and running microsatellite PCR products.
Chapter 4. Methods

4.2.8.1 Denaturing polyacrylamide gel electrophoresis

Gels were made using two glass plates approximately 38 cm long (one slightly shorter than the other), two 0.44 mm spacers and a 0.4 mm comb. Both plates and spacers were thoroughly cleaned with isopropanol prior to use to remove all traces of dust and grease. The shorter plate was siliconised on one side to allow easy removal of the plate from the gel surface following electrophoresis. The plates were then separated by the spacers and firmly taped together.

Six percent denaturing polyacrylamide gels were prepared using reagents from the Sequagel kit (National Diagnostics). The gel was polymerised by adding 350 µl 10% ammonium persulfate and 35 µl TEMED to 70 ml gel mix. After thorough mixing, the solution was carefully poured between the plates using a 50 ml syringe. Care was taken to ensure that no air bubbles were trapped between the plates. The comb was inserted to a depth of approximately 0.5 cm. Gels were left for at least an hour to polymerise before use.

After removing the tape, the gel was placed in a vertical electrophoresis tank and the two buffer reservoirs filled with 1x TBE buffer. The comb was removed and the surface of the gel flushed with buffer to remove unpolymerised acrylamide. A sharkstooth comb was used for sequencing. This was reinserted such that the teeth were resting on the gel surface to form wells. Before loading the samples, the gel was pre-warmed to enhance its denaturing properties by running for 45 minutes at 1,500 V. Wells were then flushed out with running buffer to remove urea and ensure even entry of samples into the gel. Samples were loaded and the gel run at 1,500 V, 80 mA and 100 W for an appropriate length of time.

After electrophoresis the gel plates were carefully separated. The gel was transferred to 3MM Whatman paper, covered with cling film and dried under vacuum at 80°C. The cling film was then removed and the gel exposed to X-ray film for 1-3 days, depending on the level of radioactivity incorporated.
4.2.8.2 **Non-denaturing polyacrylamide gel electrophoresis**

Gels were prepared using a similar technique to that described for denaturing polyacrylamide gel electrophoresis above. For single-stranded conformational polymorphism (SSCP) analysis 6% gels were made from a 10% acrylamide stock solution in 1x TBE. They were prepared both with and without 10% glycerol in an attempt to optimise detection of band shifts. The gel was not pre-run as the aim was to keep the gel cool and non-denaturing. Running conditions varied depending on the technique being used. SSCP samples were electrophoresed at constant voltage, 60 W and 150 mA overnight. The voltage used was dependent on the length of the PCR product being analysed: 360 V for a 250 bp product; 100 V for a 100 bp product. Bands were visualised in an identical manner to that described above. Microsatellite PCR products were run out at 1,500 V, 80 mA and 100 W for 2 hours and 30 minutes.

4.2.9 **Southern blotting**

This technique allows the transfer of size fractionated DNA fragments from an agarose gel to a nitrocellulose or nylon membrane. The method used was essentially that described by Southern et al. (1975). Gels were placed in denaturing solution and shaken for 30 minutes. This step denatures the double-stranded DNA, making it accessible for hybridisation. Gels were then transferred to neutralising solution and shaken for a further 30 minutes.

A blot was set up by placing the gel on a double layer of Whatman 3MM paper over an inverted gel tray in a large tray containing 20x SSC. The ends of the Whatman paper act as a wick, dipping into the buffer, allowing it to soak through both layers. Parafilm was laid on the edges of the gel to prevent shortcircuiting. Nylon transfer membrane was pre-wet in 3x SSC, carefully positioned on top of the gel and smoothed out gently to exclude bubbles. Three sheets of Whatman paper, soaked in 3x SSC were layered on top of the nylon membrane. Above these a stack of paper towels, a plastic plate and then a weight were placed, taking care to ensure even pressure over the filter. Capillary action results in movement of buffer from the tray through the gel to the layers above. DNA from the gel is
carried upwards but under the high salt conditions binds to the membrane. After a period of at least 16 hours the blot was dismantled and the positions of the wells marked on the membrane before briefly rinsing in 3x SSC. DNA was irreversibly fixed to the membrane by UV crosslinking (UV Genelinker, BioRad) or baking the membrane at 80°C for 2 hours.

4.2.10 DNA radiolabelling

4.2.10.1 Labelling of double-stranded DNA probes

DNA probes were labelled by random hexanucleotide priming. In this method denatured, double-stranded DNA fragments are annealed to random hexanucleotide primers. Addition of the Klenow fragment of DNA polymerase I and nucleotides dA, dT and dG with radiolabelled dC results in the synthesis of complementary strands incorporating the labelled nucleotide.

Between 50 and 100 ng DNA in 35 μl agarose/water were heated to 100°C for 8 minutes. Ten microlitres of oligonucleotide labelling buffer (OLB), 5 units of DNA polymerase I (Klenow fragment) and 40 μCi [α-32P]dCTP were then added giving a final volume of 50 μl. The labelling reaction was incubated at 37°C for 2-3 hours.

4.2.10.2 Removal of unincorporated nucleotides from labelled probe

Sephadex G-50 spin columns were prepared by plugging the bottom of a disposable syringe with a small amount of sterile polymer wool. The barrel was filled with Sephadex G-50 equilibrated in 3x SSC and then spun at 1,800 rpm for 5 minutes. This was repeated until the packed volume was around 0.9 ml. Finally, 200 μl 3x SSC were added to the top and the column spun again to compact the G-50. 150 μl were added to the labelled probe, loaded onto the column and spun as before. Unincorporated nucleotides remain trapped in the column matrix. The labelled probe was collected in an Eppendorf tube beneath the column.
4.2.10.3 Competition of labelled probe

Both the *GRB10* and A6121 probes used to determine *GRB10* dosage by Southern analysis were competed with human placental DNA before hybridisation to reduce background binding to repetitive sequences. Labelled probe was mixed with 300 μg human placental DNA, 170 μl 20x SSC and 140 μl distilled water, boiled for 8 minutes and then incubated for 30 minutes at 65°C. Competed probes were added directly to pre-warmed hybridisation mix without denaturing (see Section 4.2.11.2 below).

4.2.11 Southern hybridisation

Prehybridisation and hybridisation solutions are designed to minimise background hybridisation to the filter but maximise hybridisation rates. Hybridisation mix was the same as prehybridisation mix with the addition of 10% w/v polyethylene glycol (PEG) 6,000.

4.2.11.1 Prehybridisation

Filters were wetted in 3x SSC and placed in bottles or sealed plastic bags. Twenty millilitres of prehybridisation mix were pre-warmed to 65°C and then added to the bottle/bag, taking care to avoid air bubbles. Membranes were incubated for a minimum of 2 hours at 65°C.

4.2.11.2 Hybridisation

After prehybridisation, membranes were transferred to fresh bags. DNA probes were denatured by heating to 100°C for 8 minutes, quenched on ice and added to 20 ml of pre-warmed hybridisation mix. The mix was then added to the bottle/bag and the membrane incubated overnight at 65°C, agitating gently.
4.2.11.3 Removal of unbound probe (washing procedure)

Membranes were removed from the bags and washed in 3x SSC, 0.1% SDS at room temperature for 30 minutes with shaking. All subsequent washes were carried out at 65°C with the same SDS concentration. Stringency was gradually increased by decreasing the concentration of SSC from 3x to 2x, 1x, 0.5x, 0.2x and finally 0.1x. The wash was interrupted and membranes rinsed in 3x SSC when they registered approximately 10 cps using a Geiger counter.

Air dried filters were wrapped in cling film, placed in cassettes between intensifying screens and X-ray film and left at -70°C. Exposure times varied from 1 hour to 1 week depending on the intensity of the signal.

4.2.11.4 Removal of probe for re-hybridisation of membranes

Bound probe was removed from membranes following autoradiography to allow re-use. A solution of 0.1% SDS was boiled, poured onto the filter and allowed to cool to room temperature. Membranes were then rinsed in 3x SSC and stored dry, protected by cling film.

4.2.11.5 Quantitative analysis using the PhosphorImager

Quantitative analysis was carried out using a similar approach to that described by Abu-Amero et al. (1997). Incorporation of radioactivity into each band was measured using a PhosphorImager (Model 400; Molecular Dynamics). Phosphor screens were exposed to filters for 1-3 days depending on the intensity of the signal seen on X-ray film. Screens were then scanned on the PhosphorImager and the intensity measured by volume integration using ImageQuant software.

4.2.12 Polymerase chain reaction (PCR)

This technique allows selective amplification of target DNA sequences (Erlich et al., 1988). Two oligonucleotide primers are synthesised, one for each DNA strand, on either
side of the target DNA region. Template DNA is denatured by heating and then cooled to allow the primers to anneal to their targets on the single-strands. DNA polymerase then extends the primers at their 3' ends. Repeated cycles of heating and cooling result in the exponential synthesis of many copies of the specific segment spanned by the two primers.

Each 25 µl PCR reaction contained 2.5 µl 10x buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 125 ng of each primer and 1 unit of Taq polymerase. To prevent evaporation, one drop of mineral oil was layered over each sample. Samples were initially heated to 94°C for 4 minutes. The temperature of the reaction tubes was then cycled as follows: denaturing for 1 minute at 94°C; annealing for 1 minute at the calculated annealing temperature; extension for 1 minute at 72°C. The predicted optimal annealing temperature for specific primers was calculated from the formula:
\[ 4(G+C) + 2(A+T) - 5 = \text{annealing temperature (°C)} \]
This cycle was repeated 25-40 times before finishing with an 8 minute incubation at 72°C to allow for final extension of the PCR products.

Ten microlitre aliquots of each reaction were run on agarose gels to check the product size. Alteration of the reaction conditions was sometimes necessary to optimise yield and minimise spurious hybridisation. In most cases optimisation of annealing temperature and/or cycle number was all that was required. Where primer-dimers were prominent the concentration of primers in the reaction mix was reduced and/or addition of one of the primers delayed until the reaction had been incubated for at least 2 minutes at 94°C.

### 4.2.13 Single-stranded conformational polymorphism (SSCP) analysis

SSCP analysis was used to screen for polymorphisms as described by Orita et al. (1989). PCR products were generated from the region of interest, denatured to produce single-strands and separated by non-denaturing polyacrylamide gel electrophoresis. The technique relies on the fact that single-stranded DNA folds back on itself in a sequence dependent manner. A single base change can alter this secondary structure and
consequently change the electrophoretic mobility of separated single-strands, producing a band shift.

4.2.13.1 Vertical gel electrophoresis with radiolabelled PCR products

PCR reactions were set up using standard concentrations (section 4.2.12) plus 0.1 µl (1 µCi) \([\alpha^{32}\text{P}]dCTP\) per 25 µl reaction. The annealing temperature was raised by 1°C compared with that normally optimal. Reaction conditions remained otherwise unchanged. Three microlitres of loading dye were added to 7 µl radiolabelled product. Samples were heated to 95°C for 5 minutes to separate the strands and then quenched on ice. An additional, control sample was kept non-denatured to allow easy identification of the band size representing residual, non-denatured products. Products were run out on 6% non-denaturing polyacrylamide gels (section 4.2.8.2).

4.2.13.2 Horizontal gel electrophoresis

The majority of SSCP analysis was carried out using this method in conjunction with silver staining. For speed and ease of use, precast gels (Cleangel, Pharmacia) were used with a Multiphor II Electrophoresis Unit. Following PCR amplification of the region of interest, 5 µl of the reaction were run out on an agarose mini-gel to confirm the presence of amplified products. Precast gels were firstly rehydrated in 25 ml Gel Buffer for 1 hour. Kerosene was pipetted onto the cooling plate and the gel laid on top, taking care to avoid trapping bubbles underneath. Electrode strips were wetted with 22 ml Electrode Buffer and placed on the edges of the gel. Four microlitres of loading dye were added to 1.5 µl PCR product. Samples were heated to 95°C for 5 minutes to separate the strands, quenched on ice and then loaded onto the gel. Samples were run out at 200 V, 23 mA, 5 W for 10 minutes and then at 600 V, 30 mA, 18 W for 45-90 minutes, depending on the length of the fragment. A Multi Temp III Refrigerated Bath Circulator was used to control the temperature of the cooling plate. Gels were run at both 15°C and 4°C to optimise strand separation.
4.2.13.3 Silver staining

The silver staining technique used to visualise bands is estimated to be around 50 times more sensitive than detection by ethidium bromide. Staining was carried out using the Pharmacia silver staining kit. The method is based on that described by Heukeshoven and Dernick (1985). The gel was placed in a gently shaking, pyrex staining tray and covered with 25 ml 10% glacial acetic acid. After 30 minutes the fixing solution was poured off and the gel washed three times with distilled water. The gel was then covered with silver solution and left shaking for 20 minutes exactly. Next the gel was rinsed in distilled water for 30 seconds and developing solution added. After shaking for 2-5 minutes bands would become visible. Developing solution was poured off once the bands were well developed but before background staining became too prominent. Stop solution was immediately added and the gel left shaking for a further 10 minutes. The stop solution was then removed and 10% glycerol added to the tray for 20 minutes. Finally, gels were carefully dried with filter paper and wrapped in cling film for storage.

4.2.14 Subcloning of PCR products

This method utilises the tendency of Taq polymerase to extend PCR products by a single adenosine base at their 3' end (Clark, 1988). The commercial vector used (pGEM-T Easy, Promega), has been incubated with Taq polymerase and thymidine to create a 3' overhang. It therefore cannot self-ligate but will readily ligate to PCR products.

4.2.14.1 Preparation of PCR products

Amplified DNA from a 50 µl PCR reaction was extracted using an equal volume of Tris equilibrated phenol (pH 7.4). The sample was spun for 5 minutes at 13,000 rpm and the upper layer removed to a fresh tube. An equal volume of phenol/chloroform (1:1) was added, the sample spun again and removed to a fresh tube. Finally an equal volume of chloroform was added, the sample spun and the upper layer removed to a clean tube. One tenth volume 3 M sodium acetate (pH 4.8) and 2.5 volumes of absolute ethanol were added. The solution was placed at -70°C for 1 hour and then centrifuged at 13,000 rpm for
15 minutes to obtain a pellet. The pellet was washed with 200 μl ice cold 70% ethanol, air dried and resuspended in 20 μl distilled water.

4.2.14.2 Ligation reaction

Insert DNA was ligated into vectors using an insert:vector ratio of 1:2. Ligation reactions contained 25 ng insert DNA, 50 ng PGEM-T Easy vector and 1 unit T4 DNA ligase in a total volume of 10 μl 1x buffer. Control ligation reactions were set up simultaneously. Ligation of vector with no insert DNA was used to determine the background number of untransformed colonies. Ligation of vector with control insert DNA (provided with pGEM-T Easy) was used to assess transformation efficiency. Reactions were incubated overnight at 4°C.

4.2.14.3 Preparation of competent cells

A single colony of XL1blue MRF' cells (Stratagene) was inoculated in 10 ml LB broth containing 12.5 μg/ml tetracycline. The culture was grown overnight with shaking at 37°C. One hundred microlitres of the overnight culture were used to inoculate 10 ml LB broth with tetracycline and grown at 37°C for a further 2-3 hours with shaking. Cells were recovered by centrifugation at 1,800 rpm for 10 minutes. They were then gently resuspended in 5 ml ice cold 100 mM CaCl₂ and left on ice for 45 minutes. Cells were pelleted again by spinning at 1,800 rpm for 10 minutes and resuspended in 1 ml ice cold 100 mM CaCl₂. The cells were then stored at 4°C and used within 48 hours. The efficiency of the transformation reaction increases 4-6 fold during the first 12-24 hours.

4.2.14.4 Transformation of ligated products

Heat shock can be used to introduce ligated products into competent cells. Transformed cells can be selected for on the basis of acquired antibiotic resistance and detected using blue/white colour selection. X-gal is a chromogenic substrate of β-galactosidase. When intact, the β-galactosidase gene (lac-Z) is induced by IPTG, cleaving X-gal to produce a blue substrate. Multiple cloning sites within the vector are embedded in a region coding for lac-Z. If lac-Z is disrupted by incorporation of insert DNA into the
vector, X-gal fails to turn blue. Thus recombinant clones containing inserts give rise to white colonies.

Ligation reactions were mixed with 200 μl competent cells and left on ice for 40 minutes. Cells were then heat shocked at 37°C for 5 minutes and immediately placed back on ice for 2 minutes. Five hundred microlitres of LB broth were added and the cell suspension incubated for 1 hour at 37°C. Two hundred microlitre aliquots were plated onto LB broth + agar plates containing 50 μg/ml ampicillin, 40 μg/ml X-gal and 50 μg/ml IPTG. After allowing to dry, plates were incubated overnight at 37°C. Transformation controls containing uncut vector and competent cells were included.

4.2.15 DNA sequencing

The dideoxy chain termination method (Sanger et al., 1977) was used to determine DNA sequences. A sequence specific primer is annealed to DNA template. In the presence of DNA polymerase and a mixture of deoxynucleotides, a complementary strand extending from the primer is then synthesised. The reaction is terminated by the addition of a dideoxynucleotide. Four reactions are set up, each with a different dideoxynucleotide inhibitor. This generates a series of products of different lengths with 3’ ends at ddA, ddC, ddG or ddT, depending on which inhibitor was used. Incorporation of radioactively labelled [α-35S]dATP allows visualisation on polyacrylamide gels. All non-automated sequencing reactions were performed using reagents from the Sequenase version 2.0 kit (USB, Amersham).

4.2.15.1 Sequencing of PCR-amplified DNA

Biotinylated primers were used to enable single-stranded template to be generated from PCR-amplified DNA. One biotinylated and one unmodified primer were included in each PCR reaction. Following amplification, single-stranded DNA is obtained by immobilising the strand synthesised from the biotinylated primer on streptavidin-coated magnetic beads. After denaturing, the unbound complementary strand can be removed by washing, leaving single-stranded template bound to the beads.
Using a magnet, 30 µl Dynabeads in suspension were removed from solution. The beads were washed twice with 100 µl TES, removed from the magnet and mixed with 50 µl PCR product. Samples were incubated at room temperature for 5 minutes, tapping occasionally to keep the beads in suspension. Beads were again removed from solution using the magnet, washed twice with 100 µl TES and mixed with 100 µl fresh 0.15 M NaOH. Samples were left to denature for 5 minutes before removing the NaOH using the magnet. Beads were then washed once with 100 µl TES and once with 100 µl distilled water. Finally beads were resuspended in 5 µl water and stored at -20°C until use.

An annealing reaction was set up containing 7 µl single-stranded DNA (in bead solution), 1 µl of the non-biotinylated primer (5 pmol/µl) and 2 µl sequenase buffer. The mixture was incubated for 2 minutes at 65°C and then left at room temperature for at least 20 minutes (but no longer than 4 hours). Four tubes containing 2.5 µl of termination mix (ddA, ddC, ddG or ddT) were warmed to 42°C. Sequencing reactions were then set up. Annealed primer/template samples were mixed with 1 µl 0.1 M dithiothreitol, 2 µl labelling mix (0.75 µM each of dCTP, dGTP and dTTP), 0.5 µl [α-35S]dATP and 2 µl sequenase version 2.0 (1.5 units/µl). After incubating at room temperature for 3 minutes, 3.5 µl of the reaction were added to each of the four termination tubes and incubated at 42°C for 5 minutes. The reaction was stopped by the addition of 4 µl stop solution. Samples could be stored at -20°C for up to 1 week without significant loss of signal intensity. Prior to loading on polyacrylamide gels, samples were heated to 95°C for 2 minutes. Three microlitres of each reaction were run out in each lane.

4.2.15.2 Sequencing of plasmid DNA

Double-stranded plasmid DNA was denatured to generate single-stranded DNA template before sequencing. Six micrograms of DNA were made up to 36 µl with distilled water and mixed with 4 µl 2 M NaOH, 2 M EDTA (pH 8.0). The reaction was left at room temperature for 5 minutes and then neutralised by adding 4 µl 2 M ammonium acetate (pH 4.6). DNA was recovered by adding 150 µl absolute ethanol, placing at -70°C for 1 hour then centrifuging for 10 minutes at 13,000 rpm. The pellet was washed in ice cold ethanol, air dried and resuspended in 7 µl distilled water.
Chapter 4. Methods

Annealing reactions were set up by adding 1 μl primer (100 ng/μl) and 2 μl sequenase buffer to 7 μl denatured DNA. The mixture was incubated for 2 minutes at 65°C in a beaker of water which was then allowed to cool slowly to 37°C over approximately 30 minutes. Sequencing reactions were then carried out as described for single-stranded DNA.

4.2.15.3 Automated sequencing

Polymorphisms in both \textit{IGFBP1} and \textit{IGFBP3} were demonstrated using an automated sequencer at the Institute of Child Health. These sequencing reactions were performed by M. Hitchins. One hundred nanograms of RT-PCR products were sequenced by fluorescent-labelled dideoxynucleotide sequencing using 3.2 pmol primer per reaction. Reactions were carried out in 10 μl volumes using the dRhodamine AmpliTaq dye-terminator cycle sequencing kit, according to manufacturer's instructions (Perkin-Elmer). Sequencing reactions were run on a 5% 19:1 acrylamide:bisacrylamide denaturing gel on an automated ABI PRISM 377 DNA (Perkin-Elmer).

A few PCR products were sequenced on an ABI 394 RNA/DNA synthesiser (Applied Biosystems) (M. Jones, Molecular Biology Laboratory, Infectious Diseases Unit, Hammersmith Hospital).

4.2.16 'Clean-up' protocols for PCR products

Before automated sequencing of PCR products, sequencing templates were purified to remove unincorporated primers and dNTPs. Products sequenced at the Institute of Child Health were purified by passing the reaction through a S-3000 HR Sephacryl MicroSpin column (Pharmacia). Samples sent to the Hammersmith Hospital were usually cleaned up using the PEG precipitation technique described by Rosenthal \textit{et al.} (1993). However, where the product was obtained by cutting a band from agarose gel, the GeneClean III Kit (Bio 101 Inc.) was used, following the manufacturer's instructions.
4.2.16.1 **PEG precipitation**

Forty five microlitres of PCR product were added to 45 µl PEG/Mg/sodium acetate mix (26% PEG 8,000: 6.5 mM MgCl\(_2\): 0.6 M sodium acetate (pH6-7)). After thorough mixing the solution was left at room temperature for 10-15 minutes and then spun for 10 minutes at 13,000 rpm. The pellet was washed twice with absolute ethanol, air dried and resuspended in 20 µl distilled water. To estimate DNA concentration, 2 µl was run out on an agarose minigel.

4.2.16.2 **Gene Clean procedure**

Three volumes of 3 M NaI were added to the DNA band excised from agarose gel and incubated for 5 minutes at 55°C to dissolve the agarose. Five microlitres of Glassmilk were added to the suspension and mixed thoroughly. Glassmilk is a silica matrix in suspension which binds DNA but not contaminants. After incubating for 5 minutes at room temperature the Glassmilk was pelleted and washed three times with 500 µl New Wash. Finally the DNA was eluted in 20 µl distilled water.

4.2.17 **Sodium bisulfite treatment of DNA**

The methylation-specific PCR assay used to analyse methylation patterns within the 5' CpG island of MEST (Section 7.3.2) was based on sodium bisulfite treatment of DNA. Treatment converts unmethylated but not methylated cytosine residues to uracil. PCR can then performed using primers designed to distinguish methylated from unmethylated DNA, taking advantage of the sequence differences which result from bisulfite treatment (Herman et al., 1996).

The treatment protocol used was essentially that described by Zeschnigk et al. (1997). Genomic DNA (4 µg diluted to 70 µl in water) was denatured by adding 8 µl freshly made 3 M NaOH and incubating for 15 minutes at 37°C. Samples were then incubated for 3 minutes at 95°C to ensure complete denaturation and quenched on ice. Denatured DNA was mixed with 1 ml bisulfite solution. Mineral oil was layered over samples which were then incubated overnight in a 55°C waterbath in the dark. The
Chapter 4. Methods

GeneClean III Kit (Bio 101, Inc.) was used to recover treated DNA, using 5 μl glassmilk. DNA was eluted in 100 μl water, 11 μl 3 M NaOH added and the solution incubated at 37°C for 15 minutes. The sample was neutralised with 110 μl 6 M NH₄OAc, pH 7.0. DNA was precipitated by adding 550 μl absolute ethanol and spinning for 15 minutes at 13,000 rpm. The pellet was washed in 100 μl 70% ethanol, air dried and resuspended in 10 μl water. The concentration of bisulfite treated DNA was determined spectrophotometrically, as described in section 4.2.5.

4.3 Preparation of cell lines and cytogenetic analysis

4.3.1 Transformed lymphoblastoid cell lines

Transformed lymphoblastoid cell lines were obtained from the European Collection of Cell Lines (see Section 3.1.1). On receipt, cells were grown in suspension culture under sterile conditions using RPMI medium supplemented with 10% fetal bovine serum (Gibco BRL), 2 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 μl/ml). Cells sent on dry ice or stored in liquid nitrogen required initial resuscitation. After leaving at room temperature for 1 minute, cells were then placed in a 37°C water bath for 1-2 minutes until completely defrosted. The suspension was transferred to a 50 ml Falcon tube and 10 ml pre-warmed media added. Cells were centrifuged for 5 minutes at 1,000 rpm to obtain a pellet free of freezing media. The pellet was resuspended in 10 ml media and transferred to a 75 ml flask. Cells were grown at 37°C in 5% CO₂ atmosphere. Medium was added to keep the concentration of cells between 3 x 10⁵ and 2 x 10⁶/ml. Typically cells were fed 5-10 ml every 24-48 hours until the total culture volume reached 30 ml. Cells were then divided to prevent overgrowth.

To obtain cell pellets for RNA extraction, cells were transferred to a sterile Falcon tube and spun down for 5 minutes at 1,000 rpm. The supernatant was removed, the pellet snap frozen in liquid nitrogen and stored at -70°C until use.
4.3.2 Fibroblast cell lines

Fibroblast cell lines were generated from punch skin biopsies. Skin biopsies were carried out on patients and controls under sterile conditions using disposable 3 or 4 mm gauge punch biopsy needles. Biopsy material was placed in Ham's F-10 Medium (Imperial) and taken promptly to the Cytogenetics Laboratory at QCCH. Cell cultures were then set up using Ham's F-10 medium supplemented with 30% fetal bovine serum (Globepharm), 2 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml).

Before confluence was reached, cells were pelleted for RNA extraction. The medium was decanted and cells washed twice in 2-3 mls PBS. One millilitre of trypsin-versine was added and cells incubated at 37°C for 3-5 minutes until they had ‘rounded up’. Cells were then transferred to a sterile Falcon tube and the pellet prepared as described above for transformed lymphoblastoid cells.

4.3.3 Preparation of lymphocytes from blood

Ten millilitres of PBS were added to 10 ml fresh blood (in an EDTA tube). Into three separate tubes, 6 ml of the mixture were very carefully loaded on top of 2 ml ficoll (Histopaque-1077, Sigma). Samples were centrifuged at 2,000 rpm for 30 minutes at 4°C. During centrifugation, erythrocytes and granulocytes are aggregated by the ficoll and rapidly sediment. Lymphocytes and other mononuclear cells remain at the plasma-ficoll interface. The upper layer of plasma was removed to within 1 ml of the mononuclear cell layer and discarded. The mononuclear cell layer was then removed to a clean tube containing 2 ml PBS, taking great care not to disturb the lower layer of ficoll. PBS was added to a volume of 14 ml and mixed well. Samples were spun again at 2,000 rpm for 10 minutes at 4°C. The supernatant was carefully decanted and the cell pellet resuspended in 1 ml PBS. PBS then was added to a volume of 10 ml, mixed thoroughly and cells pelleted by spinning at 2,000 rpm for 10 minutes at 4°C. Cells were washed once more with PBS. This time the cell pellet was snap frozen in liquid nitrogen and stored at -70°C until use.
4.3.4 Cytogenetic analysis

Detailed cytogenetic analysis of chromosomes from case 49 and her family was carried out by T. Ballard, Cytogenetic Services, 35 Devonshire St., London W1. Chromosome preparations were obtained by treating lymphoblastoid cell cultures with 10 μg/ml ethidium bromide and 0.02 μg/ml colcemid 2 hours before harvest. Chromosome G banding was performed using a trypsin-Leishman technique and metaphase images captured with an Applied Imaging Cytovision system.

4.4 RNA protocols

4.4.1 Preparation of solutions and equipment

RNA is prone to ribonuclease (RNase) digestion throughout extraction, purification, blotting and hybridisation. As a result, gloves were worn at all times and the working area kept scrupulously clean. All solutions used for RNA were prepared with sterile deionised water and made where possible from reagents kept separate from the general chemicals in the laboratory. Handling of reagents, solutions and tissues was kept to a minimum. Pipettes, tips, Eppendorf tubes and a midi-gel tank were reserved for use with RNA samples only. All tweezers, spatulas and homogenisers were rinsed with water, cleaned with ethanol and baked overnight at 200°C before use. Non-sterile plastic-ware such as the gel tank and combs were treated with RNase Free (CLP) and rinsed with sterile deionised water immediately prior to use. Sterile plastic-ware such as tips and tubes were assumed to be RNase free.

4.4.2 Extraction of total RNA

4.4.2.1 Fetal tissue

Total RNA was isolated from fetal tissues using an adaptation of the guanidine isothiocyanate technique described by Chirgwin et al. (1979). Tissue samples were placed
on dry ice and, while still frozen, dissected into aliquots of 100 mg or less using a scalpel blade. Each aliquot was then homogenised in 700 µl solution D. Samples were transferred to Eppendorf tubes and mixed well with 90 µl 2 M sodium acetate (pH 4.0), 600 µl water saturated phenol and 150 µl chloroform. After incubating for 15 minutes on ice, they were spun at 13,000 rpm for 10 minutes at 4°C. The upper aqueous phase (600 µl) was removed to a fresh tube and mixed with 200 µl solution D, 20 µl 2 M sodium acetate, 500 µl phenol and 120 µl chloroform. Samples were then spun again at 13,000 rpm for 10 minutes at 4°C. The upper aqueous phase (400 µl) was removed to a clean tube and 500 µl isopropanol added. Samples were left at -70°C for at least 1 hour or overnight at -20°C.

RNA pellets were obtained by spinning at 13,000 rpm for 15 minutes at 4°C. Pellets were washed in 1 ml 70% ethanol, spun again briefly and left to air dry for 5-10 minutes. RNA was resuspended in 10-20 µl 1 mM EDTA. The concentration of RNA was determined spectrophotometrically as described for DNA (section 4.2.5) except that for RNA one OD unit = 40 µg/ml. Samples were stored at -70°C until use.

### 4.4.2.2 Cell pellets

Total RNA was extracted from cell pellets using a similar procedure to that described for fetal tissue. The total number of cells in suspension was estimated using a cell counting chamber prior to pellet formation. Seven hundred microlitres of solution D were used per 10⁶ cells.

### 4.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcription of RNA was used to generate cDNA for use in PCR amplification. Total RNA samples (1 µg diluted to 10.25 µl in water) were denatured at 70°C for 5 minutes and cooled to 37°C. Reverse transcription was carried out using 0.2 µg random hexanucleotide primers, 1x reverse transcriptase buffer, 10 mM dithiothreitol, 1 mM each dNTP, 40 units M-MLV reverse transcriptase (Gibco BRL) and 1 unit RNase inhibitor (Promega). These were added to a final volume of 20 µl and incubated at 37°C for 60 minutes. The reaction was then terminated by heating to 90°C for 5 minutes. Samples
were diluted with 80 μl water and stored at -70°C until needed. PCR was performed (as described in section 4.2.12) using 1/20 volume (5 μl) of the cDNA template.

4.4.4 Electrophoresis of RNA

The method used was adapted from those of Lehrach et al, (1977) and Goldberg (1980). RNA is heated to 65°C to remove any secondary structure which might interfere with the mobility of the RNA. Formaldehyde, used in the sample buffer and agarose gel, acts as a denaturant to prevent secondary structures reforming during electrophoresis.

A 1.5% agarose gel was made up in 1x morpholinopropanesulphonic acid (MOPS) buffer and 2.2 M formaldehyde in a final volume of 400 ml. As formaldehyde is toxic, the gel was poured in the fume hood and left to cool. The running buffer used was 1x MOPS. Total RNA samples (10-60 μg in 15 μl volume) were mixed with 30 μl sample buffer, denatured at 65°C for 6 minutes and then quenched on ice for 2 minutes. Prior to loading on the gel, 1.5 μl 1 mg/ml ethidium bromide was added to each sample. Samples were run at 100 V for 2-4 hours or 30 V overnight. Separated RNA was visualised and photographed under UV light.

4.4.5 Northern blotting

Size fractionated RNA species were transferred to nylon membrane using an adaptation of the Southern blotting technique (section 4.2.9). The method differed in the following respects: the gel was not denatured prior to blotting; the gel was inverted before transfer; and 10x SSC was used as the transfer buffer. Following blotting, membranes were baked for 2 hours at 80°C to permanently bind the RNA.
4.4.6 Northern hybridisation

The probes used for hybridisation with Northern blots were radiolabelled with \([\alpha-^{32}\text{P}]\text{dCTP}\) and separated from unincorporated nucleotides using identical methods to those used for Southern hybridisation (Sections 4.2.10.1 and 4.2.10.2).

4.4.6.1 Prehybridisation

Membranes were placed in sealed plastic bags or bottles containing 10 ml of Northern prehybridisation mix and an equal volume of formamide. Denatured salmon sperm DNA and formamide were added just before use and the mix pre-warmed. Membranes were incubated for a minimum of 2 hours at 42°C.

4.4.6.2 Hybridisation

The hybridisation mix was identical to that used for prehybridisation. Membranes were transferred to fresh bags. Probes were heated to 100°C for 8 minutes, quenched on ice and added to pre-warmed hybridisation mix. Membranes and mix were sealed in the bag and incubated overnight at 42°C.

4.4.6.3 Removal of unbound probe (washing procedure)

The washing procedure was essentially the same as that for Southern hybridisation except that washes were done at 42°C. Membranes were removed from the bags and washed in 3x SSC, 0.1% SDS at room temperature for 30 minutes with shaking. All subsequent washes were carried out at 42°C with the same SDS concentration. Stringency was gradually increased by decreasing the concentration of SSC from 3x to 2x, 1x, 0.5x, 0.2x and finally 0.1x. The wash was interrupted and membranes rinsed in 3x SSC when they registered approximately 10 cps using a Geiger counter.

Air dried filters were wrapped in cling film, placed in cassettes between intensifying screens and X-ray film and left at -70°C. Exposure times varied from 48 hours to 1 week depending on the intensity of the signal.
Chapter 4. Methods

4.5 Fluorescence in situ hybridisation (FISH)

The method used for FISH was based on that described by Harper et al. (1994) and the Vysis product protocol (Vysis, Richmond, UK).

4.5.1 Preparation of metaphase spreads

To ensure that cells were all synchronised at the same stage of the cell cycle, 200 μl thymidine solution (30 mg/ml) were added to 50 ml cultured lymphoblastoid cells and incubated for 18 hours at 37°C. The culture block was then released by adding 200 μl deoxycytosine (0.227 mg/ml). After 4 hours, cells were mitotically arrested by adding 200 μl colcemid (10 mg/ml) and incubated for a further 20 minutes. Cells were harvested by centrifugation at 1,000 rpm for 5 minutes and the supernatant discarded. The cell pellet was thoroughly resuspended in a small volume of remaining media and 10 ml of 0.075 M hypotonic KCl were added dropwise. After incubating for 20 minutes, the suspension was centrifuged at 1,000 rpm for 5 minutes and the supernatant discarded, leaving a small volume to resuspend the pellet. Fresh, ice cold fix (3:1 methanol:glacial acetic acid) was added dropwise and the volume made up to 10 ml. Cells were spun down at 1,000 rpm for 5 minutes, the supernatant removed and the fixation process repeated a further two times. Cells were then dropped onto slides cleaned with 70% ethanol. Metaphase chromosomes in fix were stored at -20°C.

4.5.2 Nick translation

Cosmid DNA was labelled with either spectrum red or spectrum green dUTP using the Vysis nick translation kit, according to the manufacturer’s instructions. One microgram of DNA was mixed with 2.5 μl 0.2 mM spectrum red or spectrum green dUTP, 5 μl 0.1 mM dTTP, 10 μl dNTP mix (0.1 mM of dATP, dCTP and dGTP), 5 μl buffer, 10 μl nick translation enzyme and distilled water to a final volume of 50 μl. The reaction was incubated at 15°C for 1 hour and then placed on ice while 9 μl of the mix were electrophoresed on 2% agarose gel. A smear of 200-500 bp products was preferred. If the
products were too large the reaction was reincubated at 15°C. The reaction was terminated by heating to 70°C for 10 minutes.

4.5.3 FISH

Prior to use, metaphase slides were aged in 2x SSC at 37°C for 30 minutes, dehydrated through 70%, 90% and 100% ethanol for 5 minutes each and air dried. Slides were then denatured in 70% deionised formamide/2x SSC at 70°C for exactly 5 minutes before being dehydrated again through 70%, 90% and 100% ethanol for 1 minute each and air dried.

Two micrograms of human COT-1 DNA were added to 5 μl of labelled probe to suppress repetitive sequences and precipitated. The probe was then resuspended in 7 μl LSI hybridisation buffer and 3 μl distilled water and denatured at 73°C for 5 minutes. Five to 10 μl of the suppressed probe were pipetted onto denatured metaphase chromosomes, a coverslip applied and sealed with cow gum. Probes were hybridised overnight in a humidified chamber at 37°C.

After hybridisation, the coverslip was carefully removed and the slides immediately washed in 1x SSC/0.1% SDS for 5 minutes at 65°C. This step was repeated three times. After the final wash, slides were air dried and counter-stained with 10 μl Vectashield/DAPI. A coverslip was then secured permanently with nail varnish. Prepared slides were stored in the dark at 4°C.

Hybridised slides were examined on a Zeiss Axioscope 20 fluorescence microscope under oil using a 100x objective. Images were captured and manipulated using the SmartCapture software from Digital Scientific. Signal intensity was measured using IPLab.
Chapter 5. \textit{IGFBP1} and \textit{IGFBP3}

5.1 Introduction

The insulin-like growth factor binding proteins (IGFBPs) are a family of six binding proteins which modulate the action of insulin-like growth factor-I and -II (IGF-I and -II). The IGFs are peptides with both mitogenic and metabolic activity. Both IGFs and their receptors are synthesised in a number of fetal tissues and are thought to be involved in autocrine/paracrine stimulation of cellular proliferation and differentiation in development (D’Ercole, 1991). There is abundant evidence that IGFs are important in the control of fetal growth. Correlation of umbilical cord serum IGF levels with birth weight has repeatedly been reported (Lassare \textit{et al.}, 1991; Verheaghe \textit{et al.}, 1993; Reece \textit{et al.}, 1994). Targeted mutagenesis of either \textit{Igfl} or \textit{Igf2} in mice results in profound IUGR, with birth size reduced by 40\% (DeChiara \textit{et al.} 1990; Liu \textit{et al.} 1993), suggesting that both genes have a direct role in fetal growth. Furthermore, a patient with severe IUGR due to deletion of human \textit{IGF1} has also been described (Woods \textit{et al.}, 1996).

The majority of IGFs in the circulation are bound to IGFBP3 in a 150 kDa complex with an acid-labile subunit. Formation of the complex stabilises IGFs and probably affects the biological activity of circulating IGFs by prolonging their metabolic half life (Binoux \textit{et al.}, 1988). Serum levels of IGFBP1 show marked diurnal variation, inversely related to insulin and blood glucose levels, suggesting that IGFBP1 is involved in glucose counter-regulation (Lee \textit{et al.}, 1993).

The complex molecular mechanisms by which IGFBPs modulate IGF action have been reviewed by Jones and Clemmons (1995). Few \textit{in vivo} studies of function exist to date and results often appear conflicting. Current data suggest that IGFBPs have both stimulatory and inhibitory effects. Several studies have shown that IGFBP1 prevents IGF binding to cell surface receptors, thereby inhibiting IGF-mediated mitogenic and metabolic activity (Lee \textit{et al.}, 1993). Studies using IGF analogues with low affinity for IGFBPs suggest that expression of IGFBPs in some organs has a net inhibitory effect (Jones and Clemmons, 1995). However, IGF-I antibody studies in animals have shown that IGFBP3 in serum will potentiate IGF action (Stewart \textit{et al.} 1993).
Figure 5.1*. Schematic diagram of *IGFBP1* and *IGFBP3* showing regions of homology between the two genes. Numbered rectangles represent exons and the dotted regions their coding regions. Regions of close homology are shown in red. The positions of primers *IGFBP1*-F and -R and *IGFBP3*-F and -R, used to study gene expression, are indicated by horizontal arrows.
Like the IGFs, the IGFBPs are widely distributed in fetal tissues from the second trimester onwards (Pannier et al., 1994; Han et al., 1996). However, individual binding proteins display tissue specific expression patterns. Using Northern analysis, Han et al. (1996) investigated the expression of IGFBPs 1-6 in 10-16 week human fetal tissue. *IGFBP1* mRNA was detected in liver alone whereas *IGFBP3* mRNA was widely expressed. IGFBP1 levels in tissues surrounding the fetus are very high from early on in pregnancy and serum levels are highest during fetal life (Wang and Chard, 1992a; Wathen et al., 1993). At term, IGFBP1 and 2 are the major binding proteins for IGF-I in fetal serum (Wang and Chard, 1992b). Serum IGFBP3 levels increase throughout fetal life but are higher postnatally, peaking during puberty (Juul et al., 1995; Pirazzoli et al., 1997).

Several findings suggest that the IGFBPs, particularly IGFBP1, are involved in regulating fetal growth. There have been numerous observations of a negative correlation between both maternal and fetal IGFBP1 levels and birthweight (Lassare et al., 1991; Wang et al., 1991; Giudice et al., 1995; Hills et al., 1996). In addition, a few studies suggest that increased IGFBP1 is a primary factor in growth retardation rather than a secondary response to other biological changes. At 15-16 weeks, amniotic fluid IGFBP1 levels are significantly raised in pregnancies where the fetus is growth restricted at term (Hakala-Ala-Pietila et al., 1993). Furthermore, transgenic mice over-expressing *IGFBP1* show fetal growth retardation and impaired brain development (Rajkumar et al., 1995). It is thought that an increase in IGFBP1 reduces the unbound biologically active fraction of IGF-I, resulting in growth restriction. In contrast, levels of IGFBP3 are reduced in association with low birth weight (Giudice et al., 1995).

The IGFBPs share close sequence homology (Rechler, 1993). This is mainly due to strict conservation of 18 cysteine residues within their NH\(^{2}\) and COOH termini (Figure 5.1*). Extensive differences in the central region of the IGFBP molecules (exon 2 and the 3' end of exon 1) may explain in part their diverse regulation and function.

The genes for both *IGFBP1* and *IGFBP3* have been mapped to chromosome 7p12-p14 and are arranged tail-to-tail, 20 kb apart (Ehrenborg et al. 1992). Prior to this study, their imprinting status had not been described. However, imprinting of several genes in the IGF pathway had already been demonstrated. Moreover, *IGFBP1* and *IGFBP3* are located in the region of chromosome 7 homologous to an imprinted region on mouse chromosome 11 (see Section 1.4.2.1). It was therefore tempting to speculate that they may show
parental allele-specific expression. Both genes had previously been identified as potential candidates for SRS because of their likely role in prenatal growth (Kotzot et al., 1995; Preece et al., 1997). The imprinting status of IGFBP1 and IGFBP3 was therefore investigated.

5.2 Experimental strategy

The approach used to determine whether IGFBP1 and IGFBP3 are imprinted has been outlined in Chapter 2. The expression pattern of both genes was investigated in various fetal tissues at different stages. Polymorphisms within the two genes were then sought to enable expression of individual parental alleles to be studied.

A protein-sequence polymorphism within IGFBP1 at position I228M was described by Luthman et al. (1989). A subsequent report by Ehrenborg et al. (1992) confirmed the existence of this polymorphism, this time within the DNA sequence at nucleotide 5772 (GenBank accession no. M59316). An A to G transition was seen when the DNA sequence was compared to that reported by Cubbage et al. (1989). This sequence change does not affect a restriction site.

No published reports of polymorphisms within the transcribed region of IGFBP3 could be found. An expressed polymorphism therefore needed to be identified before further investigation of its imprinting status could be carried out. Since the 3’ untranslated region (UTR) of the gene is likely to be the least conserved, it was decided to screen this region for sequence changes.

In recent years, many different techniques for mutation/polymorphism detection have been developed (Grompe, 1993). These include SSCP analysis, denaturing gradient gel electrophoresis, heteroduplex analysis and chemical mismatch cleavage. SSCP analysis is widely used to search for small mutations and demonstrate polymorphisms due to its speed, simplicity and relative sensitivity. In contrast to other methods, no additional steps are required after PCR, no toxic chemicals are needed and the technique is easy to set up. In comparison to direct sequencing, large numbers of samples can be screened simultaneously. However, at the time, no previous reference to the use of SSCP analysis to demonstrate imprinting of a gene could be found. To validate the use of this method
preliminary work was carried out on the known imprinted gene \textit{IGF2}, using a well described polymorphism (Giannoukakis \textit{et al.}, 1993). Having established that it was possible to demonstrate monoallelic expression using this technique, SSCP analysis was used to screen for polymorphisms within both \textit{IGFBP1} and \textit{IGFBP3}.

No sequence changes were detected in either gene using SSCP. However both the \textit{IGFBP1} polymorphism described by Luthman \textit{et al.} (1989) and a polymorphism within the 3' UTR of \textit{IGFBP3} were demonstrated by direct sequencing of the genes (for details and discussion see Sections 5.5.2 and 5.6.2 below). These polymorphisms were then used to investigate parental allele expression of the two genes in normal fetal tissues. Expression of \textit{IGFBP1} and \textit{IGFBP3} was also studied in mUPD7 SRS patients.

\section*{5.3 Expression of \textit{IGFBP1} and \textit{IGFBP3} in first and second trimester tissues}

\subsection*{5.3.1 Northern analysis}

The expression of \textit{IGFBP1} was initially examined in total RNA isolated from mid-trimester fetal liver using Northern analysis. RNA samples extracted from placenta and chorion decidua collected at term were used as positive controls. These tissues were selected since high levels of expression of \textit{IGFBP1} and \textit{IGFBP3} had previously been observed in maternal chorion decidua and placenta, respectively (Pannier \textit{et al.}, 1994). In order to determine the quantity of fetal liver RNA needed to detect expression, increasing amounts of RNA, ranging from 10 to 50 \(\mu\)g, were loaded on the gel.

Expression of \textit{IGFBP1} and \textit{IGFBP3} was analysed by hybridising filters with probes for both genes simultaneously. Transcripts of the expected sizes were seen for both \textit{IGFBP1} and 3 (Figure 5.1). As expected, \textit{IGFBP1} was expressed in chorion decidua but not placenta whereas \textit{IGFBP3} was expressed in both. \textit{IGFBP1} expression in fetal liver was first detectable at a concentration of 30 \(\mu\)g per lane and \textit{IGFBP3} expression at 20 \(\mu\)g. The 18S and 28S ribosomal RNA bands were also visible due to non-specific cross hybridisation with the probes. Although the \textit{IGFBP1} and 18S ribosomal bands were close
in size (approximately 1.8 kb), signals from the two transcripts could be readily differentiated.

In their study of IGFBP expression using Northern analysis, Pannier et al. (1994) loaded 20 µg RNA per lane from a number of different fetal tissues (lung, heart, spleen, placenta, liver, kidney and brain). At this concentration, only low levels of IGFBP1 expression were seen in two out of three mid-gestation fetal liver samples. Since fetal liver is an abundant source of IGFBP1, it was predicted that a minimum of 20-30 µg RNA would be required to study expression in other fetal tissues using this technique. With RT-PCR, on the other hand, 1 µg total RNA can be used to generate enough cDNA template for 20 PCR reactions. Given the limited availability of fetal samples, this alternative method was used to analyse expression in the remaining fetal tissues.

**Figure 5.1.** Northern analysis of IGFBP1 and IGFBP3 expression in mid-trimester fetal liver. Lane 1: term placenta (TP); lane 2: term chorion decidua (TCD); lanes 3, 4, 5 and 6: increasing concentrations of fetal liver RNA. Position of IGFBP1 and IGFBP3 transcripts and 18S and 28S ribosomal RNA bands marked on left; transcript sizes indicated on right.
5.3.2 RT-PCR

Total RNA was extracted from normal human fetal tissues from both first and second trimester. Three sets of tissues (placenta, skin, brain, heart, liver, heart, intestine, kidney and lung) from both 10-12 and 16-18 week stages were analysed. Each set of tissues was derived from several different fetuses. RNA was reverse transcribed and the resultant cDNA amplified using PCR. Primers for the housekeeping gene GAPDH were used to control for the presence of cDNA. These primers gave a 598 bp product in all tissues investigated (Figures 5.2A and B). Control samples prepared without M-MLV reverse transcriptase (RT negative) were used to confirm the absence of contaminating genomic DNA. Primers for IGFBP1 and IGFBP3 were designed to enable expression of both genes to be studied. As described in Section 5.1 above, the family of IGFBPs share structural similarity, particularly in their -NH\textsubscript{2} and -COOH terminal regions. Primers for IGFBP1 and IGFBP3 were therefore designed to span the regions of lowest homology in exon 2 of both genes.

5.3.2.1 IGFBP1

Primers IGFBP1-F and IGFBP1-R were used to detect expression of IGFBP1. Sequence analysis of the resultant 315 bp PCR product confirmed that it matched the published sequence for IGFBP1 in the GenBank database (from nucleotides 3304 in exon 2 to 5663 in exon 4, accession no. M59316). The product spanned introns 2 and 3, allowing contamination of cDNA with genomic DNA to be easily distinguished. PCR using genomic DNA as template gave a faint band of 2420 bp in length, corresponding to the size predicted from the published sequence.

Using primers IGFBP1-F and -R, PCR was carried out at 35 cycles. The only fetal tissue found to consistently express IGFBP1 at both gestations was liver. PCR products of the expected size (315 bp) were seen in all liver samples analysed from both 10-12 and 16-18 weeks gestation (Figures 5.3A and B). Since the PCR cycle number used was outside the linear range, no conclusions could be drawn about the relative level of expression at first and second trimester stages.
Chapter 5  IGFBP1 and IGFBP3

Figure 5.2. Gel electrophoresis of RT-PCR products showing GAPDH expression in fetal tissues. Expression investigated at (A) 10-12 week gestation and (B) 16-18 week gestation. Tissue types are: placenta (P), liver (L), brain (Br), lung (Lg), gut (G), heart (H), skin (S) and kidney (K). M: 100 bp marker; Bl: blank (no template). RT negative (-) control samples confirm the absence of genomic contamination.

Figure 5.3. Gel electrophoresis of RT-PCR products showing IGFBP1 expression in fetal tissues. Expression investigated at (A) 10-12 week gestation and (B) 16-18 week gestation. Tissue types are: placenta (P), liver (L), brain (Br), lung (Lg), gut (G), heart (H), skin (S) and kidney (K). M: 100 bp marker; Bl: blank (no template).
Expression was also observed in other samples. For example, in Figures 5.3A and B, expression was seen in placenta and gut at both gestational ages. The band in placenta probably reflects contamination with maternal decidua, which is a rich source of IGFBP1, since expression could not be demonstrated in other placental samples studied. Expression was seen in four out of the six fetal gut samples analysed. Various explanations for this observation were considered. Contamination with maternal decidua or fetal liver seemed unlikely since both are usually easily distinguished from intestine at collection. It was therefore postulated that at early gestations all foregut-derived structures (including liver, stomach and duodenum proximal to the bile duct opening) might express IGFBP1. No distinction was made between large and small intestine when initial gut samples were collected. In order to test this theory, therefore, additional samples of stomach (foregut) and large intestine (mid/hind gut) were obtained from first and second trimester fetuses (data not shown). IGFBP1 expression was absent in tissues from both sources. It is possible that there is biological variability between samples and regional differences in mRNA expression in the tissues from which RNA was extracted. Alternatively, expression may be due to contamination with tissues highly expressing IGFBP1 such as maternal decidua or fetal liver.

The liver-specific expression pattern observed is consistent with previous reports of IGFBP1 expression in fetal tissues (Pannier et al., 1994; Han et al., 1996). Subsequent investigation of the imprinting status of IGFBP1 was carried out using liver samples alone.

### 5.3.2.2 IGFBP3

Primers IGFBP3-F and IGFBP3-R were used to detect expression of IGFBP3 in the same fetal tissues used to study IGFBP1. Sequence analysis of the resultant 301 bp PCR product confirmed that it matched the published sequence for IGFBP3 in the GenBank database (from nucleotides 5889 in exon 2 to 8328 in exon 4, accession no. M35878). This product was designed to span introns 2 and 3, allowing contamination of cDNA with genomic DNA to be easily distinguished. PCR using genomic DNA as template gave a faint band of 2440 bp in length, corresponding to the size predicted from the published sequence.
Using primers IGFBP3-F and -R, PCR was carried out for 35 cycles. As expected, products of the predicted size (301 bp) were seen in all tissues examined at both 10-12 and 16-18 weeks (Figures 5.4A and B). Since the PCR cycle number used was outside the linear range no conclusions could be drawn about the relative level of expression at first and second trimester stages or between different tissues.

![Image of gel electrophoresis](image)

**Figure 5.4.** Gel electrophoresis of RT-PCR products showing expression of *IGFBP3* in fetal tissues. Widespread expression is shown at (A) 10-12 week gestation and (B) 16-18 week gestation. Tissue types are: placenta (P), liver (L), brain (Br), lung (Lg), gut (G), heart (H), skin (S) and kidney (K). M: 100 bp marker; Bl: blank (no template).

As *IGFBP3* was ubiquitously expressed in the fetal tissues examined, its imprinting status was investigated in all tissue samples available from those fetuses identified as informative.
5.4 Demonstration of \textit{IGF2} imprinting using SSCP analysis

The imprinting status of \textit{IGF2} was investigated in fetal tissues using SSCP analysis to validate the use of this technique for imprinting studies. An \textit{ApaI} polymorphism in the 3' UTR has previously been used to demonstrate parental imprinting of the human \textit{IGF2} gene (Giannoukakis \textit{et al.}, 1993). The same polymorphism was used in this study to determine whether imprinting of \textit{IGF2} could be demonstrated convincingly using SSCP analysis. Genomic DNA extracted from maternal blood and paired fetal tissue was screened for the \textit{ApaI} polymorphism using the same primers used by Giannoukakis \textit{et al.} (1993) (IGF2-F and -R). These primers amplify a 236 bp fragment of exon 9 containing the \textit{ApaI} site (Figure 5.5A). After 35 cycles of PCR, products were digested with \textit{ApaI} for 3 hours at 37°C, run out on 3% agarose gel and visualised under UV light. In the presence of the site (+ allele) the PCR product is digested to give two fragments of 173 and 63 bp. A fully informative pair is one where the fetus is heterozygous (+/-) but the mother homozygous (+/+ or -/-). In this situation, parental origin of the expressed allele can be determined. Of 11 maternal/fetal pairs screened for the polymorphism, one proved to be fully informative with a heterozygous (+/-) fetus (F7) and a homozygous (+/+ or -/-) mother.

Total RNA was extracted from skin and brain from fetus F7 and reverse transcribed. Due to the distance of the polymorphism from the nearest intron (> 1000 bp), the primers IGF2-F and -R were not designed to cross an intron. RT negative samples were therefore included to detect possible genomic contamination. Following PCR amplification with primers IGF2-F and -R, products were digested with \textit{ApaI} and visualised as before. As expected, \textit{IGF2} imprinting was evident in both skin and brain, with expression of the paternal (-) allele only (Figure 5.5B). No bands were visible in the RT negative control samples.
Figure 5.5. Demonstration of IGF2 imprinting using ApaI polymorphism. (A) Schematic diagram of IGF2 and position of ApaI polymorphism (Tadokoro et al., 1991; Giannoukakis et al., 1993). Numbered rectangles represent exons and the black areas their coding regions. (B) IGF2 imprinting in tissues from fetus F7. Digested PCR products from homozygous (+/+) maternal (M) and heterozygous (+/-) fetal (F) genomic DNA are shown. cDNA derived from fetal skin and brain was amplified and digested. In both tissues only the paternally-derived (-) allele was expressed. RT -ve control lanes confirm absence of contaminating genomic DNA.
Chapter 5 IGFBP1 and IGFBP3

Having illustrated IGF2 imprinting in tissues from fetus F7, the same genomic and cDNA samples were used to demonstrate imprinting by SSCP analysis. PCR primers IGF2-F and -R were used to amplify the same 236 bp region of IGF2. Products were denatured and run out on precast gels using the Multiphor II Electrophoresis unit at 15°C for 10 minutes at 200 V and 100 minutes at 600 V. Bands were detected by silver staining.

Products from homozygous (+/+ and -/-) and heterozygous (+/-) DNA were used to show that the polymorphism was detectable by SSCP analysis. A band shift allowed the two alleles to be easily distinguished (Figure 5.6A). PCR products from the informative maternal/fetal pair were then examined. As shown in Figure 5.6B, monoallelic expression from the paternal allele alone was clearly demonstrated in those fetal tissues available from fetus F7.

![Figure 5.6](image)

**Figure 5.6.** Demonstration of IGF2 imprinting by SSCP analysis. (A) IGF2 polymorphism. Lane 1: heterozygote (+/-); lanes 2 and 3: opposite homozygotes (+/+ and -/-). (B) Monoallelic expression of IGF2 in tissues from fetus F7. Lanes 1 and 2: products from both heterozygous (+/-) fetal (F) and homozygous (+/+ maternal (M) genomic DNA. Lanes 3 and 4: products from cDNA derived from skin (S) and brain (Br). Arrows indicate the position of the two bands of interest. Monoallelic expression from the paternally-derived allele (-) is demonstrated in both tissues.
5.5 Investigation of IGFBP1 imprinting status

5.5.1 Screening for IGFBP1 polymorphism using SSCP analysis

Some reports of SSCP sensitivity suggest that, where the polymorphic site is known, detection rates of nearing 100% can be achieved (Michaud et al., 1992). However, overall sensitivity of the technique is probably nearer 80% (Sheffield et al., 1993). Detection rates can be improved by keeping fragment sizes shorter than 300 bp (Sheffield et al., 1993) and altering electrophoretic conditions. Different conditions, such as the presence or absence of glycerol or differences in gel temperature, seem to influence the migration of some polymorphic strands.

Primers IGFBP1-BF and -BR were designed to amplify 167 bp spanning the polymorphic site (nucleotides 5704 to 5870, Figure 5.7). Using these primers, 20 normal control DNA samples were screened for the presence of the polymorphism by SSCP analysis. No band shifts were detected, as illustrated in Figure 5.8. Several different experimental conditions were used in an effort to demonstrate the polymorphism. These included using precast gels run at both 4°C and 15°C as well as self-cast gels with and without 10% glycerol. However, band shifts were still not identified.

**Figure 5.7.** Sequence of human IGFBP1 surrounding polymorphic site in exon 4. A to G transition indicated by arrow. Codon coding for I228M underlined. Boxed areas represent primers used to demonstrate polymorphism and investigate imprinting status.

<table>
<thead>
<tr>
<th>BF</th>
<th>GTCTACC CTTGGAATGG GAA3AGGATC CCTGGGTCTC CAGAGATCAG 5 75 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>GGGAGACCCC AACTGCCAGA TATA TT T TA A TGTACAAAAC TGAAACCAGA 58 0 0</td>
</tr>
<tr>
<td>t</td>
<td>TGAAATAATG TTCTGTCACG TGAAATATTT AAGTATATAG TATATTT^ T A 58 5 0</td>
</tr>
<tr>
<td>ER</td>
<td>CTCTAGAACA TGCACATTTA TATATATATG TATA TG TA TA TATATATP^GT 5 9 0 0</td>
</tr>
<tr>
<td>A</td>
<td>A AC TA CTTTT TATACTCCAT A 5 9 2 1</td>
</tr>
</tbody>
</table>

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGAAATAATG TTCTGCACG TGAAATATTT AAGTATATAG TATATTTATA 5850</td>
</tr>
<tr>
<td></td>
<td>CTCTAGAACA TGCACATTTA TATATATATG TATATGTATA TATATAGT 5900</td>
</tr>
<tr>
<td></td>
<td>AACTACTTTT TATACTCCAT A 5921</td>
</tr>
</tbody>
</table>
5.5.2 Demonstration of IGFBP1 polymorphism by direct sequencing

As discussed above, not all base changes will be detected using SSCP analysis. Direct sequencing was therefore used to determine definitively whether this site was polymorphic in our sample population. The 167 bp PCR fragment amplified by primers IGFBP-BF and -BR was sequenced in DNA from ten control individuals using an automated ABI sequencer. The A to G transition was clearly demonstrated (Figure 5.9). Six A/A homozygotes, two G/G homozygotes and two A/G heterozygotes were identified. Protein-sequence analysis of IGFBP1 by Luthman et al. (1989) resulted in the recovery of isoleucine and methionine at position 228 in a ratio of approximately 3:2. This would correspond to a heterozygosity of 48%. The allele frequency seen in the ten controls gave a calculated heterozygosity of 42%, comparable with that predicted from protein sequence analysis.

Therefore, despite our knowledge of the location of the polymorphism and attempts to optimise running conditions, SSCP analysis failed to detect its presence. Theoretically, detection rates may vary with the position of the base change within the PCR product and
the composition of the DNA fragment itself. Since only one set of PCR primers was used to screen for the polymorphism, the role of these factors in failure to detect the polymorphism is difficult to evaluate.

**Figure 5.9.** Demonstration of polymorphism within exon 4 of IGFBP1. (A) and (B) electropherograms showing forward sequences for opposite homozygotes A/A and G/G respectively; (C) and (D) electropherograms showing forward and reverse sequences for an A/G heterozygote. Position of base substitution indicated by arrows.

**5.5.3 Investigation of IGFBP1 imprinting status in fetal liver**

Having demonstrated this sequence polymorphism, it was used to investigate the imprinting status of IGFBP1 in fetal liver. Products were sequenced using the dideoxy chain termination method and then electrophoresed on 6% denaturing polyacrylamide gels. Initially sequencing was carried out using primers IGFBP1-BF and -BR. One biotinylated and one unmodified primer were included in each PCR reaction to enable single stranded template to be generated from amplified DNA. However, the proximity of these primers to
Chapter 5 IGFBP1 and IGFBP3

the polymorphic site led to difficulties optimising sequencing conditions. An additional primer (IGFBP1-ER) was therefore designed slightly further 3' from the polymorphism (Figure 5.7). PCR using IGFBP1-BF and -ER primers gave a 218 bp fragment from which the sequence of the polymorphic site was easily determined. Since biotinylated IGFBP1-BF primer was already available, IGFBP1-ER primer was used in the sequencing reaction. As a result, reverse sequence was generated from the antisense strand.

Genomic DNA was prepared from fetal liver samples from 5 to 17 weeks gestation. The region spanning the polymorphism was then amplified by PCR using 35 cycles and the resultant 218 bp fragment sequenced. Sequencing products were run out at 1500 V, 80 mA and 100 W for 60 minutes and visualised by autoradiography. Out of a total of 16 fetuses screened, five were identified as being heterozygous for the polymorphism and thus informative. Their gestational ages were 5, 12, 13, 14 and 17 weeks. Since the primers used do not flank an intron, control samples without the addition of reverse transcriptase were included to detect possible genomic contamination (Figure 5.10A). These were negative in all cases. cDNA derived from liver in these five informative fetuses was sequenced. Although the technique used was not quantitative, both alleles were consistently detected at approximately equal levels and biallelic expression was demonstrated in all samples. One example is shown in Figure 5.10B.

To completely rule out the possibility of contamination by genomic DNA, amplification was repeated using primers IGFBP1-EF and -ER which span introns 2 and 3 (nucleotides 3304 to 5921). These primers amplify a 533 bp RT-PCR product which includes the polymorphism at nucleotide 5772. PCR using genomic DNA as template gave the predicted 2618 bp product.

Liver cDNA from all five heterozygous fetuses was amplified using these primers. No genomic contamination was observed in any of the samples. RT-PCR products were sequenced on an ABI sequencer using the reverse primer IGFBP1-ER. Both alleles were demonstrated at approximately the same level in all five samples, confirming that IGFBP1 is biallelically expressed in fetal liver between 5 and 17 weeks gestation. One example is shown in Figure 5.11.
Figure 5.10. Biallelic expression of IGFBP1 in fetal liver, demonstrated using primers IGFBP1-BF and -ER. (A) Expression of IGFBP1 in fetal liver samples (L13, L15, L17, L18 and 42L) from five heterozygous fetuses. RT-PCR products (+) were sequenced to determine imprinting status. RT negative control samples (-) confirm the absence of genomic contamination. (B) Biallelic expression in fetal liver from a 12 week heterozygous fetus. cDNA was sequenced using the reverse primer IGFBP-ER. The position of the polymorphism (C/T) is indicated by arrows.
Figure 5.11. Biallelic expression of *IGFBP1* in fetal liver, demonstrated using primers IGFBP1-EF and -ER. cDNA from a 12 week heterozygous fetus was sequenced using reverse primer IGFBP1-ER. Electropherogram shows both C and T alleles at the polymorphic site (indicated by an arrow).
5.6 Investigation of IGFBP3 imprinting status

5.6.1 Screening for IGFBP3 polymorphism using SSCP analysis

By comparing the sequence data for IGFBP3 published by different authors, two potential polymorphisms were identified within the 3' UTR (Table 5.1).

Table 5.1. Potential polymorphisms within the 3' UTR of IGFBP3.

<table>
<thead>
<tr>
<th>Genbank accession no.</th>
<th>Nucleotide no. of sequence difference</th>
<th>Potential polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>M35878</td>
<td>10180</td>
<td>...AAGGCA...</td>
</tr>
<tr>
<td>X64875</td>
<td>1911</td>
<td>...AAGACAA...</td>
</tr>
<tr>
<td>M35878</td>
<td>10228</td>
<td>...ATGTAAT...</td>
</tr>
<tr>
<td>X64875</td>
<td>1959</td>
<td>...ATGCAGA...</td>
</tr>
</tbody>
</table>

Neither of these sequence changes affects a restriction site. Instead, SSCP analysis was used to screen for these potential polymorphisms. Primers IGFBP3-AF and -AR were designed to span both sites giving a product of 236 bp in length (Figure 5.12). SSCP analysis was carried out using DNA from 20 normal individuals. Products were denatured and run out on precast gels using the Multiphor II Electrophoresis unit at both 15°C and 4°C. Samples were electrophoresed for 10 minutes at 200 V and 60 minutes at 600 V, then visualised by silver staining. No band shifts were detected at either temperature. Similar results were obtained using self-cast polyacrylamide gels with and without 10% glycerol. It is possible that the sequence differences reported in GenBank have arisen from sequencing errors rather than the presence of a true polymorphism.

The 3' UTR of a gene is usually the least conserved region and thus the most likely to include polymorphisms. In IGFBP3 the 3' UTR comprises the last few nucleotides within exon 4 (18 bp) and the whole of exon 5 (1582 bp). The search for polymorphisms was therefore directed at exon 5. It was estimated that in order to screen exon 5 with primers giving overlapping products smaller than 300 bp at least six primer pairs would be
Chapter 5  **IGFBP1 and IGFBP3**

needed. An alternative strategy was therefore adopted. Three sets of primers (IGFBP3-BF/R, IGFBP3-CF/R and IGFBP3-DF/R) were designed to span the whole exon. The products generated using these primer pairs were then digested using restriction enzymes selected to give fragments of an appropriate size for SSCP analysis. The location of all six primers and the restriction sites used to generate smaller fragments for analysis are shown in Figures 5.12 and 5.13).

PCR was used to amplify DNA from 20 normal individuals with all three primer sets (with the help of S. Abu-Amero). Products were digested, denatured and run out on precast gels using the Multiphor II Electrophoresis unit at 15°C and 4°C. Samples were electrophoresed for 10 minutes at 200 V and 90 minutes at 600 V. For primer pairs IGFBP3-BF/R, and IGFBP3-CF/R, all bands were easily visualised after this length of run. The digested fragments generated with primers IGFBP3-DF and -DR were more diverse in length (92, 255 and 322 bp). In order to detect the banding pattern for all fragments, samples were run out for varying lengths of time at 600 V.

Each digested fragment gave rise to at least two bands on the gel representing the two denatured strands. Additional bands were sometimes seen which presumably arose from alternative conformations of the same strand. Disappointingly, no band shifts representing sequence polymorphisms were seen with any of the three primer sets. This was rather surprising since the human genome is believed to contain, on average, one polymorphism every few hundred base pairs (Botstein *et al.*, 1980). It had been expected that SSCP analysis of this relatively unconserved, 1600 bp region of the gene would reveal at least one sequence polymorphism.

126
Chapter 5 IGFBP1 and IGFBP3

Figure 5.12. Sequence of human IGFBP3 exon 5 (GenBank accession no. M35878). Boxed areas represent primers used to screen for polymorphisms within the 3’ UTR; restriction sites are underlined.

\[ \text{Boxed areas represent primers used to screen for polymorphisms within the 3’ UTR; restriction sites are underlined.} \]

- \( \Delta \) Indicates polymorphic A insertion
- \( \square \) Indicates potential polymorphism based on database sequence comparison
Figure 5.13. Schematic diagram of IGFBP3 showing position of primers and restriction sites used to screen exon 5 by SSCP analysis. Position of primers represented by horizontal arrows; restriction sites indicated by vertical arrows. Fragment sizes generated given in bp. Black triangle indicates site of polymorphic adenine (A) insertion subsequently identified by direct sequencing.
5.6.2 Demonstration of IGFBP3 polymorphism by direct sequencing

While screening the 3' UTR of IGFBP3, we were made aware of an expressed polymorphism observed within exon 5 by another group (Dr I. Morison, Department of Biochemistry, University of Otago, New Zealand, personal communication). The polymorphism described was an adenine (A) insertion after nucleotide 10048 (Figure 5.14). Primers IGFBP3-EF and IGFBP3-ER were designed to flank this site, spanning nucleotides 9872 to 10230 (Figure 5.12). These primers were used to screen genomic DNA for the polymorphism by sequencing on an automated ABI sequencer. DNA from 31 normal controls was investigated. Two were homozygous for the published allele, 25 were homozygous for the A insertion and four were heterozygous. The observed heterozygosity for this polymorphism was 22%, with the A insertion being the more frequent allele in the population studied.

This polymorphism had been missed when screening by SSCP analysis. The site of the A insertion lies within the 158 bp fragment generated by ApaI digestion of the PCR product generated by primers IGFBP3-CF and -CR (Figure 5.12). This fragment length is close to the size found to give maximal sensitivity in SSCP analysis (Sheffield et al., 1993). The polymorphism is centrally located within this fragment, 66 bp from its 3' end. Although position of a sequence change within a fragment is known to influence sensitivity, optimal location of primers still seems mostly to rely on trial and error. Published studies estimating sensitivity have concentrated on the use of SSCP analysis to detect single base substitutions (Michaud et al., 1992; Sheffield et al., 1993). Insertion rather than substitution of a nucleotide may have less effect on single strand conformation, thus reducing the likelihood of detection by this technique. This remains speculation, however, since no published evidence could be found to confirm the theory.
Figure 5.14. Demonstration of an expressed polymorphism within the 3' UTR of IGFBP3. Electropherograms show (A) homozygote for the published sequence (GenBank accession no. M35878) and (B) homozygote for insertion of a single adenine at nucleotide position 10048.
5.6.3 Investigation of IGFBP3 imprinting status in fetal tissues

The polymorphism described above was used to investigate the imprinting status of IGFBP3 in normal fetal tissues. Primers IGFBP3-EF and -ER were used to amplify genomic DNA from 21 paired fetal and maternal samples with gestational ages ranging from 6-17 weeks. PCR products were sequenced on an automated ABI sequencer. Three fetuses of 8-10 weeks gestation were identified as being heterozygous on the basis of a frameshift observed in the sequence at nucleotide position 10049 of IGFBP3. Paired maternal DNA was homozygous in each case.

cDNA was prepared from placenta, brain, limb and skin from all three informative fetuses. RT-PCR was performed at 35 cycles using a forward primer (IGFBP3-GF) from exon 4 and reverse primer IGFBP3-ER from within exon 5 (nucleotides 8315 to 10230). In cDNA the expected product size is 975 bp. However, this primer pair spans intron 4, allowing genomic contamination to be detected as a band of increased size (1916 bp). Sequencing across the intron 4 boundary also indicated that no genomic contamination was present in any of the cDNA samples used. The polymorphic region was sequenced in the forward (sense) direction using primer IGFBP3-EF. A similar frameshift to that seen in the heterozygote genomic DNA samples was observed in all of the fetal cDNA samples available. This was confirmed in the reverse (antisense) sequence using primer IGFBP3-ER. Figure 5.15 shows the results of both forward and reverse sequencing for one of these samples. Both alleles were expressed at approximately the same level in all tissues. Biallelic expression of IGFBP3 was therefore demonstrated in all fetal tissues investigated.
Figure 5.15. Biallelic expression of *IGFBP3* in brain from one 10 week heterozygous fetus. Electropherograms show cDNA sequence in both forward (A) and reverse (B) directions. The site of nucleotide insertion, indicated by arrows, results in a frameshift.
5.7 Expression of IGFBP1 and IGFBP3 in mUPD7 patients

5.7.1 IGFBP1

IGFBP1 expression was not detectable in either transformed lymphoblasts, lymphocytes or fibroblasts, even when using up to 60 cycles of PCR. Expression of IGFBP1 mRNA in mUPD7 patients could therefore not be investigated.

5.7.2 IGFBP3

The expression of IGFBP3 was also investigated in fibroblast and transformed lymphoblast cells from SRS patients with and without UPD. RNA was obtained from fibroblast cells from two unaffected individuals, two SRS patients with mUPD7 (47 and 4) and four SRS patients without mUPD7 (11, 27, 2 and 25). RT-PCR was used to study IGFBP3 mRNA expression in these individuals using primers IGFBP3-F and -R. Products of the expected size (301 bp) were seen in both UPD and non-UPD patients, as well as in the normal controls (Figure 5.16). Thirty five cycles of PCR were used which was outside the linear range. Therefore no conclusions could be drawn about the relative level of expression in UPD compared with non-UPD patients. However, no gross differences in expression levels were observed.

RNA was also extracted from transformed lymphoblasts from the same individuals and expression of IGFBP3 investigated in these cells using the same primers. As for fibroblasts, products of the expected size (301 bp) were seen in both UPD and non-UPD patients, as well as in the normal controls. However, an additional band of approximately 500 bp was also seen in all samples. RT-PCR products were subcloned and sequenced to investigate the possibility that this larger band represented an alternatively spliced variant of IGFBP3. However, a Blast search showed that the sequence shared 97% homology with human G protein-coupled receptor (STRL22) mRNA (GenBank accession no. U68030). A 12 nucleotide stretch of primer IGFBP3-F was found to be complementary to the antisense strand of this mRNA. PCR reaction conditions allowed co-amplification of this gene which
is apparently expressed in transformed lymphoblasts but not cultured fibroblasts or the fetal tissues studied.

![Gel electrophoresis of RT-PCR products showing IGFBP3 expression in fibroblasts from UPD and non-UPD SRS patients. Lanes 1 and 2: normal individuals; lanes 3 and 4: SRS patients 47 and 4 with mUPD7; lanes 5, 6, 7 and 8: SRS patients 11, 27, 2 and 25 without mUPD7; Bl: blank.]

**Figure 5.16.** Gel electrophoresis of RT-PCR products showing IGFBP3 expression in fibroblasts from UPD and non-UPD SRS patients. Lanes 1 and 2: normal individuals; lanes 3 and 4: SRS patients 47 and 4 with mUPD7; lanes 5, 6, 7 and 8: SRS patients 11, 27, 2 and 25 without mUPD7; Bl: blank.

### 5.8 Summary

*IGFBP1* and *IGFBP3* are located in a region of human chromosome 7 homologous to an imprinted region on mouse proximal chromosome 11. Since both genes are thought to play a role in fetal growth, they have been proposed as candidates for involvement in the SRS phenotype associated with mUPD7. However, until this study their imprinting status was unknown.

The imprinting status of *IGFBP1* was investigated in fetal liver alone since expression in fetal samples was confined to the liver. A previously described polymorphism within the coding region of the gene was sought, initially by SSCP analysis. The use of this method to demonstrate imprinting was validated using a known imprinted gene, *IGF2*. However, no band shifts could be detected when the polymorphic region of *IGFBP1* was investigated by SSCP analysis. The polymorphism was subsequently demonstrated by direct sequencing. Using this polymorphism, biallelic expression was observed in first and early second trimester fetal liver samples.

Widespread expression of *IGFBP3* was seen in normal fetal tissues from first and early second trimester stages. Since no published reports of expressed polymorphisms within *IGFBP3* could be found, SSCP analysis was used to screen the 3' UTR for
sequence changes. No band shifts were detected. However, unpublished observation by another group of an A insertion after nucleotide 10048 in exon 5 prompted further investigation of this region. The presence of this polymorphic site was confirmed by direct sequencing. Using this polymorphism, biallelic expression was demonstrated in a number of different, first trimester, fetal tissues. Expression was also seen in fibroblasts and lymphoblasts from SRS patients with mUPD7.

In conclusion, no evidence for imprinting of either IGFBP1 or IGFBP3 was found (Wakeling et al., in press).
Chapter 6. *EGFR*

6.1 Introduction

There is currently increasing awareness of the role the epidermal growth factor (EGF) family of peptide growth factors play in regulation of cell proliferation and differentiation during development as well as in tumorigenesis (Riese II and Stern, 1998). The receptors for these factors include epidermal growth factor receptor (EGFR) which belongs to the ErbB family of tyrosine kinases. EGFR is a 170 kDa transmembrane glycoprotein which mediates the effects of both EGF and transforming growth factor α (TGF-α) (Adamson, 1990). Extracellular binding of ligands to EGFR stimulates the intracellular tyrosine kinase domain. This, in turn, induces autophosphorylation of EGFR and phosphorylation of several downstream proteins involved in signaling pathways.

EGF is a potent mitogen for a wide variety of cell types (Carpenter and Cohen, 1979). However, studies in mice have found that EGF mRNA is not detectable at significant levels during fetal development at times when EGFR is clearly expressed (Popliker *et al.*, 1987). In contrast, there is evidence that TGF-α, which shares close homology to EGF, is expressed throughout development (Wilcox and Derynck, 1988). It has therefore been suggested that TGFα is the major ligand for EGFR during fetal and embryonic life.

*EGFR* is expressed in a wide range of murine embryonic and fetal tissues (Adamson *et al.*, 1981) from as early as the preimplantation stage (Wood and Kaye, 1989). *EGFR* expression has also been described in a limited number of human fetal tissues. These include placenta (Deal *et al.*, 1982), ovary and uterus (Yeh *et al.*, 1993), lung (Johnson *et al.*, 1990), keratinocyte, endothelial and skeletal muscle cells (Nanney *et al.*, 1990). These observations suggest that EGFR and its ligands are widely involved in embryonic and fetal development.

In man, IUGR has been found to be associated with a reduction in placental EGFR phosphorylation (Fondacci *et al.*, 1994). Unfortunately this and other similar studies are unable to distinguish cause from effect. However, experiments in rodents support a specific role for EGFR in fetal growth. Firstly, rat embryos explanted on day 9.5 and...
cultured in the presence of a specific EGFR tyrosine kinase inhibitor, show dose dependent growth retardation (Tebbs et al., 1996). Secondly, both natural missense mutations and artificial 'knockouts' of murine Egfr have been described (Luetteke et al., 1994; Threadgill et al., 1995). The phenotype of homozygote mutants varies depending on genetic background but includes IUGR in addition to abnormalities of skin, hair and eyes.

EGFR is located at 7p12 within the region on human chromosome 7 homologous to an imprinted region on mouse proximal chromosome 11 (Beechey and Cattanach, 1997). It has therefore been suggested that EGFR, like IGFBP1 and IGFBP3, may be imprinted and play a role in SRS (Kotzot et al., 1995; Preece et al., 1997). Differential parental gene expression of murine Egfr has been investigated in whole embryos with maternal/paternal disomy of proximal chromosome 11 (Cattanach et al., 1995). No evidence of imprinting was found at a number of different stages though this approach would not have detected tissue-specific imprinting of the gene. In studies of Egfr ‘knockout’ mice, heterozygotes were normal, whichever the transmitting parent (Luetteke et al., 1994; Threadgill et al., 1995). This observation also suggests that the gene is not imprinted in the mouse. However, the imprinting status of genes in mouse is not always conserved in man (Kalscheuer et al., 1993). Prior to this study, the imprinting status of human EGFR was undetermined.

6.2 Experimental strategy

The approach used to determine whether EGFR is imprinted has been outlined in Chapter 2. A polymorphism within the coding region of the gene, previously described by Moriai et al. (1993), was used to study parental allele expression in a range of normal fetal tissues. Imprinting status was determined by investigating expression in informative heterozygous fetuses. In order to assess whether the presence of maternal DNA in fetal samples was obscuring an imprinting effect, allelic expression was analysed in tissues from homozygous fetuses with heterozygous mothers. EGFR expression was also studied in cell lines from mUPD7 patients.
6.3 Expression of \textit{EGFR} in first and second trimester fetal tissues

Expression of \textit{EGFR} mRNA was investigated using RT-PCR. As for \textit{IGFBP1} and \textit{IGFBP3}, expression was studied in a number of normal fetal tissues (placenta, liver, brain, lung, gut, heart, skin and kidney). Total RNA was extracted from three sets of tissues from both 10-12 and 16-18 week stages. Following reverse transcription, PCR primers for \textit{GAPDH} were used to confirm the presence of cDNA (Figures 5.2A and B). Primers \textit{EGFR-F} and \textit{EGFR-R} were then used to study \textit{EGFR} expression. These were identical to the outer \textit{EGFR} primers described by Chia \textit{et al.} (1995) and had been designed to share minimal homology with other \textit{EGFR}-related receptors (e.g. \textit{ERBB2} or tyrosine consensus sites). Sequence analysis of the 208 bp PCR product confirmed that it matched the published sequence for \textit{EGFR} cDNA in the GenBank database (from nucleotides 3207 to 3414, accession no. X00588). The primers had been designed to span an intron. PCR using genomic DNA as template gave two products of approximately 750 bp and 1.3 kb in length, allowing contamination with genomic DNA to be easily detected. The identity of these two products was not confirmed by sequencing. However, it seems likely that they represent amplification from both \textit{EGFR} and one of the homologous \textit{EGFR}-related receptors.

Following 35 cycles of PCR, products of the expected size (208 bp) were seen in all tissues analysed from both gestational stages (Figures 6.1A and B). Since the PCR cycle number used was outside the linear range, no conclusions could be drawn about the relative level of expression in the various tissues at different stages.
Figure 6.1. Gel electrophoresis of RT-PCR products showing *EGFR* expression in fetal tissues. Widespread expression is shown in (A) 10-12 week gestation tissues and (B) 16-18 week gestation tissues. Tissue types are: placenta (P), liver (L), brain (Br), lung (Lg), gut (G), heart (H), skin (S) and kidney (K). M: 100 bp marker; Bl: blank (no template).

### 6.4 Identification of informative fetal samples using an expressed *EGFR* polymorphism

A polymorphism within the coding region of the *EGFR* gene had previously been described by Moriai *et al.* (1993). This A to G transition alters a *BstN*I restriction site. Primers described by Moriai *et al.* were used to amplify the region spanning the polymorphism by PCR (Figure 6.2). Primers B1 and B2 were used with genomic DNA as template; primers A1 and A2 were used with cDNA as template.
Chapter 6. EGFR

**Figure 6.2.** Schematic map of EGFR cDNA, adapted from Moriai et al. (1993) showing the transition site which creates a BstNI restriction site and the location of both primer pairs B1/B2 and A1/A2. ATG and TGA represent the start and stop codons. The open box represents the ligand binding domain (BD). The black box signifies the transmembrane domain (TM). The grey box represents the tyrosine kinase (TK) region which shares close homology to ERBB. Locations of the PCR primers are indicated by horizontal arrows. The grey bars represent the amplified fragments with sizes of digested products indicated by the black divisions.
Chapter 6. EGFR

Primers B1 and B2 give a 102 bp product which when digested with BstNI cuts to produce two fragments of 48 and 54 bp in length (Figures 6.2 and 6.3). PCR products were digested with BstNI for 3 hours at 37°C. The resultant fragments were then separated on 4% agarose gel and visualised under UV light. Genomic DNA from 20 unrelated controls was used to investigate the informativity of this polymorphism. Of these individuals, three were homozygous (-/-) for the uncut allele, three were heterozygous (+/-) and 14 were homozygous (+/+) for the cut allele. The calculated heterozygosity was 0.38.

![Figure 6.3. Polymorphism for BstNI restriction site within EGFR demonstrated using primer pair B1/B2. Following digestion, the 102 bp fragment is cut into 54 and 48 bp fragments. -/-: homozygote for uncut allele; +/-: homozygote for cut allele; +/-: heterozygote.](image)

In contrast to primer pair B1/B2, primer pair A1/A2 span an intron. PCR with primers A1/A2, using cDNA as template, gave a product of the expected (205 bp) size. PCR using genomic DNA as template gave a band of approximately 1.5 kb (data not shown). These primers therefore allow detection of contaminating, genomic DNA in cDNA samples. Since contamination of cDNA with heterozygous genomic DNA could potentially mask monoallelic expression, primers A1 and A2 were used to investigate expression of parental alleles. RT-PCR was also carried out using RT negative samples, again to allow detection of contaminating genomic DNA. PCR products were digested with BstNI for 3 hours at 37°C. The resultant fragments were then separated on 4% agarose gel and visualised under UV light.
Chapter 6. EGFR

The 205 bp PCR product obtained with primers A1 and A2 contains an additional, non-polymorphic BstNI restriction site (Figure 6.2). This site acts as a useful internal control for complete digestion. DNA from an individual homozygous (+/+) for the presence of the BstNI cutting site at position 1749 digests to give three bands of 50, 71, and 84 bp and an individual homozygous (-/-) for the absence of the site gives two bands of 71 and 134 bp (Figure 6.4). A heterozygote (+/-) with biallelic expression will have four bands of 50, 71, 84 and 134 bp (Figure 6.5).

![Figure 6.4](image)

**Figure 6.4.** Polymorphism for BstNI restriction site within EGFR demonstrated using primer pair A1/A2. The uncut 205 bp band is digested with BstNI to give two bands of 71 and 134 bp in a -/- homozygote (with no BstNI restriction site at position 1749) or three bands of 50, 71 and 84 bp in a +/+ homozygote (with BstNI sites at position 1749 on both alleles).

6.5 Investigation of EGFR imprinting status in fetal tissues

6.5.1 Demonstration of biallelic expression of EGFR

The polymorphism described above was used to investigate expression of individual parental alleles in fetal tissues. Genomic DNA was prepared from tissue from 9 to 18 week gestation fetuses and matched maternal blood samples. Primers B1 and B2 were then used to amplify the region spanning the polymorphism and the resultant products were digested with BstNI. Out of a total of 22 fetuses screened, five were identified as
being heterozygous for the polymorphism and thus informative. For fetuses F10 and F17, maternal DNA was homozygous for the undigested allele. (F17 is shown in Figure 6.5A). For fetuses F7, F8 and F20, maternal DNA was heterozygous. Their gestational ages ranged from 9 to 14 weeks.

Since widespread expression of EGFR had been demonstrated in fetal tissues, RNA was extracted from all samples available from these five fetuses and reverse transcribed. Primers A1 and A2 were used to investigate expression of the two parental alleles. Following 35 cycles of PCR amplification with these primers, products were digested with BstN1. The presence of both alleles was seen in all five cases in a range of different tissues (Figure 6.5 and Table 6.1). Expression of the EGFR gene is therefore biallelic in both first and early second trimester fetal tissues.

**Figure 6.5.** Biallelic expression of EGFR in placenta from one heterozygous fetus, F17. (A) Genomic DNA from the homozygous mother (M) and heterozygous fetus (F) amplified with primer pair B1/B2. (B) Placental cDNA amplified with primer pair A1/A2. Restriction digest cuts the 205 bp fragment to give four bands of 134, 84, 71 and 50 bp, as expected if both parental alleles are expressed. A primer-dimer band (pd) is visible. The RT -ve control lane confirms the absence of contaminating genomic DNA.
Table 6.1. Biallelic expression of EGFR in tissues from five heterozygous fetuses.

<table>
<thead>
<tr>
<th></th>
<th>F7</th>
<th>F8</th>
<th>F10</th>
<th>F17</th>
<th>F20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>NA</td>
</tr>
<tr>
<td>Skin</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Brain</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>NA</td>
<td>Biallelic</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Kidney</td>
<td>NA</td>
<td>NA</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Heart</td>
<td>NA</td>
<td>NA</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>NA</td>
</tr>
<tr>
<td>Gut</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Lung</td>
<td>NA</td>
<td>Biallelic</td>
<td>NA</td>
<td>NA</td>
<td>Biallelic</td>
</tr>
<tr>
<td>Liver</td>
<td>Biallelic</td>
<td>Biallelic</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fetuses F7, F8, F10, and F17: all 9-10 week gestation; fetus F20: 14 week gestation. NA: not available for study.

6.5.2 Search for contaminating maternal DNA in fetal samples

While screening genomic DNA, two maternal/fetal pairs were identified where the fetus was homozygous (-) but the mother heterozygous (+/−). Parental allele expression was investigated in fetal tissues available from these two fetuses (F1 and F15) in order to assess whether contamination of fetal samples by maternal DNA could be obscuring an imprinting effect (Figure 6.6). Expression was monoallelic (-) in skin from F1 and placenta and brain from F15, indicating that these tissues were free of contamination by maternal tissue. However, placenta from F1 and skin from F15 showed expression from + and - alleles suggesting that both these samples were contaminated with maternal tissue. In both cases the maternally derived (-) bands were fainter than those from fetal cDNA (+). In contrast, in fetal tissues from heterozygous fetuses, the two alleles were always visible with equal intensity (Figure 6.5).
Figure 6.6. Evidence for contaminating maternal cDNA in one of two tissues from fetus F15. (A) Genomic DNA from the heterozygous (+/-) mother (M) and homozygous (+/+) fetus (F) amplified with primer pair B1/B2. (B) Placental and skin cDNA amplified with primer pair A1/A2. In placenta the 205 bp fragment has been cut to give fragments of 84, 71 and 50 bp, as expected for homozygous (+/+ ) fetal samples. In skin an additional 134 bp band is seen, suggesting that maternal (+/-) cDNA is present in the sample. Primer-dimer bands (pd) are visible. The RT -ve control lanes confirm the absence of contaminating genomic DNA. L: 100 bp ladder.

6.6 Expression of EGFR in SRS patients with mUPD7

The expression of EGFR was also investigated in fibroblast and transformed lymphoblast cells from SRS patients both with and without mUPD7. RNA was obtained from fibroblast cells from two normal individuals, two SRS patients with mUPD7 (47 and 4) and four SRS patients without mUPD7 (11, 27, 2 and 25). RT-PCR was then used to study EGFR expression in these individuals using primers EGFR-F and -R.

PCR products of the expected size (208 bp) were seen in both UPD and non-UPD patients, as well as in the normal controls (Figure 6.7). Again, since the PCR cycle number used was outside the linear range, no conclusions could be drawn about the relative level of expression in UPD compared with non-UPD patients. However, no gross differences in expression levels were noted. Similar results were obtained using RNA from transformed
lymphoblast cells although at this cycle number, these cells did not appear to express 
EGFR as highly.

![Figure 6.7. Gel electrophoresis of RT-PCR products showing EGFR expression in fibroblasts from UPD and non-UPD SRS patients. Lanes 1 and 2: normal individuals; lanes 3 and 4: SRS patients 47 and 4 with mUPD7; lanes 5, 6, 7 and 8: SRS patients 11, 27, 2 and 25 without mUPD7; Bl: blank.]

6.7 Summary

Previous reports of EGFR expression in the human fetus have been limited to a small number of tissues. This study has shown that EGFR is widely expressed in a range of different tissues in both the first and second trimester. The imprinting status of this gene was also previously unknown. Using a restriction fragment length polymorphism within the coding region of EGFR, parental allele expression of the gene was investigated. Biallelic expression was demonstrated in a number of different, first and early second trimester, fetal tissues. Expression was also demonstrated in fibroblasts and lymphoblasts from SRS patients with mUPD7. Thus no evidence that EGFR is imprinted was found (Wakeling et al., 1998).
Chapter 7. \textit{MEST}

7.1 Introduction

\textit{Pegl} was recently identified in a systematic screen for imprinted genes by subtraction-hybridisation between cDNAs from normal and parthenogenetic mouse embryos (Kaneko-Ishino \textit{et al.}, 1995). This paternally expressed gene was found to be identical to a previously described “mesoderm-specific” cDNA known as \textit{Mest} (Sado \textit{et al.}, 1993). The predicted open reading frame codes for a polypeptide with sequence similarity to the \(\alpha\beta\)-hydrolase fold, a structural motif common to a number of different hydrolytic enzymes (Kaneko-Ishino \textit{et al.}, 1995). Since the enzymes in this class have very diverse substrates it is difficult to predict the precise biological function of \textit{Mest}.

\textit{Mest} maps to the proximal part of mouse chromosome 6 (Sado \textit{et al.}, 1993). As shown in Chapter 1 and Figure 2.1, this region shows imprinting effects and shares syntenic homology with human chromosome 7q21-qter (Beechey and Cattanach, 1997). Human \textit{MEST}, has been isolated and assigned to 7q32 (Nishita \textit{et al.}, 1996; Kobayashi \textit{et al.}, 1997; Riesewijk \textit{et al.}, 1997). Paternal-specific expression of the gene has been demonstrated in a wide range of human embryonic and fetal tissues (Kobayashi \textit{et al.}, 1997; Riesewijk \textit{et al.}, 1997). Interestingly, however, biallelic expression of \textit{MEST} is seen in adult blood lymphocytes (Riesewijk \textit{et al.}, 1997). \textit{MEST} transcripts have also been found in a lymphoblastoid cell line from a patient with mUPD7, in keeping with biallelic expression. Since the imprinting status of the gene in fetal blood lymphocytes is currently unknown, two possible explanations for these observations exist. Either the imprint is lost during lymphocyte development or imprinting is not established in these cells. As discussed in Section 1.6.4.1, developmental (DeChiara \textit{et al.}, 1991) and tissue-specific (Deltour \textit{et al.}, 1995) relaxation of imprinting is increasingly being recognised.

Lefebvre \textit{et al.} (1998) introduced a targeted mutation of \textit{Mest} to mouse embryonic stem cells. As expected, the targeted mutation was shown to be imprinted and reversibly silenced by passage through the female germ line. Paternal inheritance of the mutated allele resulted in prenatal growth retardation. Significant growth failure was evident in embryos from 18.5 days post-coitus. One week after birth, mutant mice had an average body weight
Chapter 7 MEST

65% of the wild-type value. Thereafter, however, affected mice grew in parallel with their wild-type littermates. In addition to growth retardation, adult females inheriting a mutant paternal allele also displayed abnormal maternal behaviour towards newborn pups. Lefebvre et al. concluded that Mest has a dual role in the regulation of prenatal growth and postnatal behaviour.

It had previously been suggested that Mest could be responsible for the imprinting phenotype of early embryonic lethality associated with maternal disomy for mouse proximal chromosome 6 (Kaneko-Ishino et al., 1995). This is now unlikely since Mest-deficient mice are viable (Lefebvre et al., 1998). Instead, it is possible that the gene may define a new cluster of imprinted genes, as has been found on other chromosomes.

Although little is known of its biological role, MEST has attracted much interest as a possible candidate for SRS for three main reasons. Firstly, it is the first imprinted gene to be identified on chromosome 7. Secondly, since studies have shown paternal-specific expression of MEST, it is inactive in SRS patients with mUPD7 in at least some stages of development. Lastly, as described above, targeted inactivation of the paternally derived mouse Mest allele results in prenatal growth retardation (Lefebvre et al., 1998). The pattern of growth retardation observed in Mest-deficient mice is strikingly similar to that described in SRS (Wollmann et al., 1995). In both cases growth failure is mainly prenatal with normal postnatal growth velocities. 'Catch-up' or disproportionate growth is not observed in either. For these reasons, it was decided to investigate the role of MEST in SRS patients further.

7.2 Experimental strategy

7.2.1 Analysis of MEST allelic methylation in SRS patients

As described in Section 1.5, other imprinting disorders such as PWS and AS arise from a number of different mechanisms. These include UPD, deletion of an imprinted gene(s) and mutation either within the gene itself or within an IC. UPD, deletions and imprinting mutations all alter allelic methylation differences. It is possible that MEST is involved in SRS and similarly disrupted in patients without mUPD7 by paternal deletions
or IC mutations. Allelic methylation of *MEST* was therefore investigated in a cohort of non-mUPD7 patients.

Over the years, a number of different techniques have been employed to study differentially methylated sequences. Until recently, the most widely used approach was that of Southern blot hybridisation. This relies on the inability of methylation-sensitive restriction enzymes to digest sequences containing one or more methylated CpG sites. The method can allow the methylation status of an entire region (often a CpG island) to be assessed. Quantitative analysis is also possible. However, large amounts of DNA (generally 5 μg or more) are required to detect methylation differences and these will only be recognised if a significant percentage of the alleles are involved. In addition, only CpG sites found within sequences recognised by methylation sensitive restriction enzymes can be analysed.

Recently, the use of methylation-specific PCR to analyse allelic methylation of imprinted genes has been described (Kubota *et al.*, 1997; Zeschnigk *et al.*, 1997). This test is based on sodium bisulfite treatment of DNA which converts unmethylated but not methylated cytosine residues to uracil. PCR is then carried out using primers designed to distinguish methylated from unmethylated DNA, taking advantage of the sequence differences resulting from bisulfite treatment (Herman *et al.*, 1996). Allelic methylation of *MEST* was investigated using both Southern blot analysis and methylation-specific PCR.

### 7.2.2 Analysis of *MEST* expression in SRS cell lines

Theoretically, another means of studying the role of *MEST* in SRS is to investigate expression of the gene in both UPD and non-UPD patients. The gene is imprinted such that expression is restricted to the paternal allele. Patients with mUPD7, paternal deletions or IC mutations should therefore all demonstrate loss of expression if the gene is implicated. However, as described in Section 7.1 above, biallelic expression of *MEST* has been observed in adult lymphocytes and *MEST* transcripts detected in a lymphoblastoid cell line with mUPD7 (Riesewijk *et al.*, 1997). These findings suggest that imprinting of the gene is lost in blood, the most readily available source of RNA from patients.
Chapter 7 MEST

The only other cells available for study from affected patients are cultured fibroblasts, derived from skin biopsies. The imprinting status of MEST in fibroblasts has not previously been described. To determine whether study of MEST expression would be informative in SRS patients, the imprinting status of normal fibroblasts was investigated. Parental allele expression was compared with that seen in both lymphocytes and transformed lymphoblasts.

7.3 Methylation analysis in SRS patients

7.3.1 Southern blot analysis

Parent-of-origin-specific methylation of the 5' CpG island of MEST had previously been demonstrated using Southern blot analysis (Riesewijk et al., 1997). The feasibility of using this method to screen the cohort of non-UPD SRS patients was therefore assessed. DNA from two normal individuals, one SRS patient with mUPD7 (30) and one non-UPD patient (32) was digested. HindIII was used, either alone or in combination with MspI or its methylation-sensitive isoschizomer HpaII. Samples were run out on 1.5% agarose gel at 30 V overnight, blotted using GeneScreen Plus membrane (NEN) and filters hybridised with a probe containing the 5' region of MEST.

The probe used for Southern blot hybridisation was obtained by digesting a cosmid clone containing MEST (Icos53g3) with HindIII. Size markers allowed the 4.3 kb fragment used as a probe by Riesewijk et al. (1997) to be identified. This fragment encompasses the CpG island, exon 1 and part of intron 1 (Figure 7.1). The probe was competed with human placental DNA and used to hybridise filters (as previously described in Sections 4.1.10 and 4.1.11). Figure 7.1 shows the position of the restriction sites analysed using the 4.3 kb HindIII fragment as a probe. The results of Southern blot hybridisation are shown in Figure 7.2 and were compatible with those described by Riesewijk et al. (1997).
As expected, DNA digested with HindIII alone gave a 4.3 kb band in all three individuals. DNA digested with both HindIII and MspI also showed identical banding patterns in two control individuals and SRS patients with and without mUPD. The 4.3 kb band was digested to give visible bands of 2.3 and 0.65 kb. Smaller fragments of 0.45, 0.3 and 0.23 kb, demonstrated by Riesewijk et al. (1997) could not be identified. This was probably due to inefficient transfer of these fragments to the filter or relatively weaker hybridisation of the probe to smaller fragments. HindIII and HpaII double digests, however, showed different banding patterns in mUPD7 DNA as compared to DNA from the two normal controls and the non-UPD SRS patient. In the latter, bands of 4.3, 2.3 and 0.65 kb size were seen. In mUPD7 DNA, the smaller, 0.65 kb band was absent. The presence of the 4.3 kb band, seen in all samples, indicates that part of the DNA is completely methylated in this region and remains undigested by HpaII. Absence of the 0.65
kb band in mUPD7 DNA alone demonstrates that this restriction site (Figure 7.1, M*) is fully methylated on the maternal but not paternal allele.

In DNA from both SRS patients and one normal control, an additional 2.0 kb band was seen. Riesewijk et al. (1997) had previously noted that this fragment, which contains the complete CpG island, was detectable in DNA from some, but not all, tissues. They described prominent bands in blood and lung, fainter bands in stomach and liver and very weak bands in cerebellum. The degree of methylation at this particular restriction site (Figure 7.1, arrowed) therefore varies between tissues. The results shown here imply that the degree of methylation on the maternal allele at this site also varies between individuals.

**Figure 7.2.** Methylation analysis of the 5′ end of MEST by Southern blot hybridisation. Digested DNA from two normal controls, one non-UPD SRS patient and one patient with mUPD7 was probed with a 4.3 kb HindIII fragment containing the 5′ CpG island of the gene. A 4.3 kb band is seen in all 4 lanes after HindIII digestion and is reduced to 2.3 and 0.65 kb fragments following digestion with MspI. Smaller fragments were not detected. Following HpaII digestion, fragments of 4.3, 2.3 and 0.65 kb are seen in control DNA and DNA from the non-UPD SRS patient. The 0.65 kb fragment is absent in mUPD7 DNA indicating specific methylation of the maternal allele at this restriction site. Fragments of 2.0 kb are also seen in both SRS patients and, more faintly, in one control (right) suggesting variable degrees of methylation at this MspI/HpaII recognition site.
The intensity of bands following HpaII and MspI digests was not quantitated. However, 0.65 kb fragments were visibly fainter following digestion with HpaII. If 50% of the restriction sites are methylated and 50% unmethylated then signal strength would be approximately half. This would be consistent with the difference in band intensity seen. Overall, these observations indicated that at least one MspI/HpaII restriction site was differentially methylated in both normal controls and the one non-UPD SRS patient studied. The maternal allele of MEST was methylated and the paternal allele unmethylated, as described previously by Riesewijk et al. (1997).

### 7.3.2 Methylation-specific PCR

Titration of the amount of DNA used for Southern blot hybridisation showed that large amounts (at least 10 µg) were required to detect methylation differences. In contrast, sodium bisulfite treatment of less than half this amount of DNA yields enough template for several methylation-specific PCR reactions. Southern blot analysis had only allowed one differentially methylated MspI/HpaII site (Figure 7.1, M*) to be analysed. With careful primer design, methylation of several CpG dinucleotides can be studied simultaneously by methylation-specific PCR. This technique is also much more rapid, allowing DNA from many patients to be analysed simultaneously over a shorter period of time. For these reasons, methylation-specific PCR was used to investigate parental allele methylation in the main cohort of SRS patients.

#### 7.3.2.1 Design of parent-specific primers

A schematic outline of the approach taken to design the primers used is shown in Figure 7.3. Test DNA was treated with sodium bisulfite which converts unmethylated but not methylated cytosine residues to uracil. After treatment, the opposite DNA strands are no longer complementary. PCR reactions on treated DNA are therefore strand-specific. Primers were designed to amplify the sense strand of the CpG island within exon 1 and intron 1 (Figure 7.1). Using Southern blot analysis, Riesewijk et al. (1997) had previously demonstrated differential methylation of all MspI/HpaII restriction sites within this region.
Chapter 7 MEST

The sequence of the differentially methylated CpG island published by these authors was used to design maternal and paternal-specific primers. It was assumed that methylation of the paternal and maternal alleles would differ at all CpG dinucleotides within the region.

By placing a CpG dinucleotide at the 3' end of both forward primers, maximal discrimination between methylated and unmethylated DNA was obtained. The 3' end of the maternal primer (MEST-MAT) was situated at an \textit{Msp I} restriction site (CCGG). This site was already known to be differentially methylated (Riesewijk \textit{et al.}, 1997). In contrast, the methylation status of the CpG dinucleotide at the 3' end of the paternal primer (MEST-PAT) was previously unknown. As expected, it also proved to be methylated on the maternal allele alone. Each of these primers encompassed at least one other CpG pair (further increasing their specificity) and other cytosines (allowing unmodified DNA to be distinguished from modified DNA). Regions of the DNA which do not include CpG dinucleotides show no parent-specific sequence differences following bisulfite treatment. A common primer (MEST-COM) was designed to lie within such a region. This was used as the reverse primer for both maternal and paternal-specific reactions. In the maternal-specific reaction, primers MEST-MAT and MEST-COM span 347 bp (nucleotides 544 to 890); in the paternal-specific reaction, primers MEST-PAT and MEST-COM span 239 bp (nucleotides 652 to 890) (see Figure 7.3).
Chapter 7 MEST

A) Maternal-specific reaction: methylated sense strand

1. 5‘CGGAGTGCTGCTAGCTGCCCCGCGG...CCGTTGCTCTGGAAGCTGCGGCG...TGCTACTTGAAGGGGGTGTC 3’

   MspI

2. Bisulfite treatment

   5‘CGGAGTGUTGATGTGTGCGCGG...UGCCTGUTUTGUAAGCTGCGGCG...TGUTAUTGAGGAGGGGTGTC 3’

   First cycle

3. MEST-MAT —►

   CCGAGTGTTGTTGTTGTC

   3’GCCTCACCAACATCAACAGCCGC...ACCCCAAACACATTGCAACCGCCG...ACAATATAACTCCCTCCCCACAA 5’

   Second cycle

4. 347 bp PCR product

B) Paternal-specific reaction: unmethylated sense strand

1. 5‘CGGAGTGCTGCTAGCTGCCCCGCGG...CCGTTGCTCTGGAAGCTGCGGCG...TGCTACTTGAAGGGGGTGTC 3’

2. Bisulfite treatment

   5‘UGCAGTTGUTGATGTGTGCGCGG...UGCCTGUTUTGUAAGCTGCGGCG...TGUTAUTGAGGAGGGGTGTC 3’

   First cycle

3. MEST-PAT —►

   TGCTGTTTGGTAATGTTGTT

   3’ACCTCACCAACATCAACAGCCGC...ACCCCAAACACATTGCAACCGCCG...ACAATATAACTCCCTCCCCACAA 5’

   Second cycle

4. 239 bp PCR product

Figure 7.3. Schematic representation of bisulfite treatment and amplification of (A) methylated and (B) unmethylated DNA in maternal and paternal-specific reactions, respectively. The sense strand sequence from the 5’ CpG island of MEST before (1) and after (2) bisulfite treatment is shown. Sequence interruptions are indicated by dots and methylated cytosine residues are marked with an asterisk. PCR primers are boxed. In the first cycle of both reactions the common primer (MEST-COM) anneals to a site identical in both methylated and unmethylated DNA after treatment, but directs the synthesis of two different antisense strands (3). In the second cycle either the maternal (MEST-MAT) or the paternal (MEST-PAT) primer binds specifically to one of the two antisense strands resulting in the synthesis of 347 bp and 239 bp products respectively (4).
7.3.2.2 Optimisation of PCR conditions

Four micrograms of genomic DNA were treated with sodium bisulfite as previously described (Section 4.2.17) using the method reported by Zeschnigk et al. (1997). PCR reaction conditions similar to those described by these authors were used. Each 25 µl PCR reaction contained 200 ng of bisulfite-treated DNA, 0.2 mM dNTPs, and 1 unit of Biotaq polymerase (Biotaq). The maternal-specific PCR reaction contained 1.5 mM MgCl₂ and 100 ng of each primer whereas the paternal-specific reaction contained 1.0 mM MgCl₂ and 25 ng of each primer. Reaction conditions were 5 minutes at 94°C, followed by 35 cycles denaturation for 15 seconds at 94°C, annealing for 15 seconds at 59°C and extension for 30 seconds at 72°C, and a final extension for 5 minutes at 72°C. Products were run out on 2% agarose gels stained with ethidium bromide.

In their study of PWS and AS, Zeschnigk et al. (1997) used paternal, maternal and common primers in a single-tube PCR reaction. In comparison to using two separate reactions this approach is simpler and more rapid. It also provides an internal control for failure of PCR amplification since one band should always be detected in each sample. However, when the three primers MEST-MAT, -PAT and -COM were used together, the paternal-specific product was always preferentially amplified, despite reoptimisation of reaction conditions and adjustment of relative primer concentrations.

Untreated DNA was not expected to amplify since primers should not anneal to unmodified DNA. In the paternal-specific reaction no products were seen when untreated DNA was used as template. This is an important observation since paternal deletions or IC mutations affecting MEST would be expected to result in loss of the paternal-specific band. Incomplete treatment of DNA with sodium bisulfite could potentially mask the loss of the unmethylated allele if untreated DNA were to amplify with paternal-specific primers. In the maternal-specific reaction, a band of similar size to that obtained in treated DNA was seen when untreated DNA was used as the template (Figure 7.4). The paternal-specific primer (MEST-PAT) differs by eight nucleotides from the untreated sequence. However, the maternal-specific primer (MEST-MAT) differs by only two nucleotides. The band in untreated DNA probably results from residual binding of this primer to sequence which is still partially complementary. In contrast to the clean, bright band seen in treated samples, however, this band was always faint and associated with smearing. The difference in
appearance of bands from treated and untreated DNA was always sufficiently marked to ensure that sodium bisulfite treatment was successful in the SRS samples analysed.

7.3.2.3 Investigation of parental allele methylation in a cohort of SRS patients

Forty eight SRS patients were included in this study: five with mUPD7 and 43 non-UPD patients. The clinical details of these patients are given in Appendix 1 (cases 1 to 48). In addition, DNA from seven normal controls was also analysed. Both maternal and paternal-specific products of the expected size (347 and 239 bp, respectively) were amplified from the control samples. Automated sequence analysis of these products gave the predicted sodium bisulfite modified sequences.

As expected, no paternal products were obtained in any of the five mUPD7 SRS samples. However, for all other SRS samples analysed both maternal and paternal-specific bands were visible (Figure 7.4). Therefore no evidence could be found for paternal deletions of the region amplified or IC mutations affecting *MEST*.

![Figure 7.4. Methylation-specific PCR analysis of patients and normal controls. Products were electrophoresed on 2% agarose gel stained with ethidium bromide. M: 100 bp marker; m: maternal PCR product; p: paternal PCR product. Analysis of 1 normal control, 2 SRS patients with mUPD7 (+mUPD), 3 patients without mUPD7 (-mUPD) and untreated DNA (UT). No paternal bands are seen in mUPD7 samples. In contrast, both maternal and paternal-specific products are seen in normal control and non-UPD SRS lanes, demonstrating normal differential methylation in these samples.](image)
7.3.2.4 Exclusion of suspected mUPD7 using methylation-specific PCR

The methylation-specific PCR assay described above has other potential applications. One such use is investigation of suspected mUPD7 in patients where parental DNA is not available but unusually extensive homozygosity (possible isodisomy) for polymorphic markers has been demonstrated.

Two patients with cystic fibrosis and short stature who had maternal isodisomy for chromosome 7 have previously been reported (Spence et al., 1988; Voss et al., 1989). A similar patient with short stature and probable cystic fibrosis was referred to us by Dr K. Friedman (University of North Carolina) for investigation of possible mUPD7. The 49 year old woman (K) was reported to have chronic sinopulmonary disease and pancreatic insufficiency for which she was being treated with pancreatic supplements. At 148 cm, her height was below the 3rd centile. Her parents were unrelated and of distinct ethnic backgrounds. Investigation had shown slightly elevated sweat chloride levels. No mutations had been detected within the cystic fibrosis transmembrane regulator gene (CFTR) although it had been completely sequenced. However, DNA was homozygous for the 5T allele in intron 8, a variant associated with predominant skipping of exon 9 and loss of CFTR function (Chu et al., 1993). Further tests showed that she was also homozygous for four other, two-allele restriction fragment length polymorphism (RFLP) markers around CFTR: M470V, XV2c, KM19 and JG3c. The probability of this genotype occurring by chance had been estimated to be around 1 in 125,000.

Inheritance of parental alleles could not be studied using microsatellite markers since neither parent was still alive and DNA was not available. It was therefore decided to investigate the origin of her two parental alleles using methylation-specific PCR. No paternal-specific PCR products had been seen in any of the five mUPD7 SRS samples studied to date. Similar loss of the paternal band using bisulfite-treated DNA from this patient would have strongly supported the diagnosis of mUPD7. DNA from one normal individual, two known mUPD7 patients (47 and 4) and patient K was treated with bisulfite and amplified using both maternal and paternal-specific primer pairs. The results are shown in Fig 7.5. As before, no paternal-specific products were seen in the mUPD7 samples. However, both maternal and paternal-specific bands were visible in samples from the
normal control and patient K. This observation suggested that mUPD7 was not the explanation for the phenotype in this patient.

**Figure 7.5.** Exclusion of mUPD7 in patient K using methylation-specific PCR analysis. M: 100 bp marker; m: maternal PCR product; p: paternal PCR product. Analysis of one normal control, two SRS patients with mUPD7 (+mUPD), patient K (K) and untreated DNA (UT). Both maternal and paternal-specific products were seen in samples from patient K and the normal control. In contrast, no paternal-specific band was seen in either mUPD7 sample.

The extent of homozygosity around the *CFTR* locus was further investigated using tetranucleotide PCR markers located along chromosome 7q. Each marker was tested in both patient K and two other individuals known to be heterozygous for the marker. This allowed bands of interest to be more easily identified. Results are shown in Figure 7.6 and Table 7.1. The genetic distance of each marker from D7S2201, located on distal 7p, is recorded in cM. Comparison of genetic and physical maps for chromosome 7 places *MEST* between D7S2847 and D7S1804. *CFTR* most probably lies between D7S821 and D7S2847 although the possibility that it lies between D7S2847 and D7S1804 cannot be excluded. No region of homozygosity was seen despite reported homozygosity for several markers around *CFTR*. Markers D7S821, D7S2847 and D7S1804 which flank both *MEST* and *CFTR*, were all found to be heterozygous.
Figure 7.6. Investigation of allele inheritance in patient K using tetranucleotide markers located on chromosome 7q. Each marker was tested using DNA from two known heterozygotes (C1 and C2) and patient K (K). Patient K is heterozygous for all markers shown, including D7S821, D7S2847 and D7S1804 which flank MEST and CFTR.
Table 7.1. Analysis of allele inheritance in patient K using tetranucleotide markers located on chromosome 7q.

<table>
<thead>
<tr>
<th>Tetranucleotide Marker</th>
<th>Genetic distance (cM)</th>
<th>Allele inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7S820</td>
<td>92.4</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>D7S821</td>
<td>105.4</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>D7S2847</td>
<td>120.8</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>D7S1804</td>
<td>132.5</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>D7S2195</td>
<td>147.6</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>D7S1805</td>
<td>167.6</td>
<td>Heterozygous</td>
</tr>
</tbody>
</table>

Genetic distance is recorded from D7S2201, a marker on distal 7p. All markers, including D7S821, D7S2847 and D7S1804 which flank both MEST and CFTR, were found to be heterozygous.

The most likely explanation of the results in this patient is coincidental homozygosity of the CFTR markers originally studied. These markers were all located very close to/within CFTR. M740V is a common intragenic polymorphism. JG3c is approximately 1.3 cM centromeric to CFTR whereas KM19 and XV2c are just 0.2 cM telomeric to the gene. Investigation with microsatellite markers has shown that the region of homozygosity is not greater than 15.4 cM since the patient was heterozygous for those markers surrounding CFTR. Methylation-specific PCR analysis demonstrated normal differential methylation of MEST, virtually excluding mUPD7. Segmental isodisomy of a small region surrounding CFTR as a result of postsomatic recombination cannot be completely ruled out. However, this must be extremely unlikely given the heterozygosity seen for all markers within the region and the proximity of MEST (7q32) to CFTR (7q31.2). Although seen on chromosome 11 in association with BWS (Henry et al., 1993), segmental isodisomy of chromosome 7 has never been reported. Finally, the possibility of a small genomic deletion encompassing all or part of CFTR requires further investigation though this is a rare cause of cystic fibrosis.
7.4 Analysis of *MEST* imprinting status in cell lines

7.4.1 *MEST* imprinting status in normal cell lines

The imprinting status of *MEST* was studied using primers HP1F and HP1R described by Kobayashi *et al.* (1997). These primers span a polymorphic *Af*III restriction site (GenBank accession no. D78611; nucleotides 1513 to 2306; Figure 7.7). These primers amplify a 794 bp product in both genomic and cDNA. In the presence of the *Af*III restriction site, the enzyme cuts this product into 388 and 406 bp fragments. Genomic DNA from 12 normal individuals was amplified and five heterozygotes identified. Skin biopsies were obtained from two of these informative individuals (G and M) and used to prepare fibroblast cultures (Section 4.3.2). Fresh blood samples from these controls were used to prepare lymphocytes (Section 4.3.3). Blood was also sent to the European Collection of Cell Lines, Porton Down to create transformed lymphoblast cell lines (Section 4.3.1).

![Figure 7.7. Schematic diagram of MEST and position of AfIII polymorphism, adapted from Kobayashi *et al.* (1997) and Riesewijk *et al.* (1998). Exons are represented by boxes with translated regions depicted as shaded areas. The AfIII restriction site used to study imprinting status of MEST lies within the 3' UTR. PCR using primers HP1F and HP1R gives a 794 bp product. AfIII digestion results in 406 and 388 bp fragments.](image)

162
RNA was extracted from subconfluent fibroblasts, lymphocytes and transformed lymphoblasts and reverse transcribed to generate cDNA. After 40 cycles of PCR, expression of *MEST* was clearly seen in all cell lines from both individuals. PCR products were digested with *AflIII* for 90 minutes at 37°C, then electrophoresed on 2% gels. Since the primers used do not flank an intron, control samples without the addition of reverse transcriptase were included to detect possible genomic contamination. These were negative in all cases.

The results from individuals M and G are shown in Figures 7.8 and 7.9, respectively. In G (Figure 7.8) digested products were visible on ethidium bromide stained gels. In M, although bands were detectable, they were fainter. For this reason gels from this individual were blotted and probed with radio-labelled PCR product amplified using the same primers (HP1F and HP1R) (Figure 7.9). In both fibroblast samples the uncut allele alone was detectable in cDNA. Expression in these cells was therefore monoallelic. In lymphocytes from control M, expression remained monoallelic (Figure 7.9). However, surprisingly, *MEST* was expressed from the opposite (cut) allele to that in fibroblasts. In lymphocytes from control G, (Figure 7.8) biallelic expression was demonstrated. Expression in transformed lymphoblasts from both individuals was biallelic. In both cases the upper, uncut allele was more intense. However, this difference in allele intensity was much more marked in control G (Figure 7.8) than M.
Figure 7.8. Ethidium bromide-stained gel showing MEST imprinting status in cell lines from heterozygous control (G). (A) fibroblasts, (B) lymphocytes and (C) transformed lymphoblasts were studied. Genomic DNA shows the presence of both cut and uncut alleles. RT-PCR shows monoallelic expression of MEST in fibroblasts but biallelic expression in both lymphocytes and transformed lymphoblasts. In lymphocytes the cut allele is preferentially amplified whereas in transformed lymphoblasts the uncut allele is more intense. RT negative control lanes (-) confirm the absence of contaminating genomic DNA.
Figure 7.9. Autoradiograph showing MEST imprinting in cell lines from heterozygous control (M). Genomic DNA shows the presence of both cut and uncut alleles. RT-PCR shows monoallelic expression of MEST in (A) fibroblasts and (B) lymphocytes. Expression occurs from the uncut allele alone in fibroblasts but the cut allele in lymphocytes. Expression in transformed lymphoblasts (C) is biallelic. RT negative control lanes (-) confirm the absence of contaminating genomic DNA.

7.4.2 Expression of MEST in SRS patients

Expression of MEST in fibroblasts from both normal individuals studied had been shown to be monoallelic. It was therefore decided to continue investigation of MEST expression in SRS patients using fibroblasts in preference to lymphoblasts. Skin biopsies were taken from three patients with mUPD7 (4, 5 and 47) and two non-UPD patients (2 and 25). Fibroblast cultures were prepared from these samples and cells harvested while still subconfluent. RNA was extracted from cell pellets and cDNA generated by reverse transcription. PCR primers for GAPDH were used to confirm the presence of cDNA. Primers HP1F and HP1R were then used to analyse expression of MEST in these samples. Despite using 40 cycles of PCR, levels of expression were low and the 794 bp product only faintly visible on an ethidium bromide-stained gel. The gel was therefore blotted and probed with a radio-labelled PCR product amplified using the same primers (HP1F and HP1R) (Figure 7.10).

Expression of MEST was seen in fibroblasts from one normal control and all SRS patients investigated, both with and without mUPD7. The PCR cycle number used was
outside the linear range so conclusions could not easily be drawn about the relative level of expression in UPD compared with non-UPD patients and the normal control. However, the signals obtained in two of the three mUPD7 samples (lanes 1 and 3, Figure 7.10) were of distinctly lower intensity than those seen in the other samples studied.

Expression of MEST in mUPD7 samples suggests that, despite the monoallelic expression seen in normal fibroblasts, some expression from the maternal allele must be occurring. This observation makes analysis of MEST expression in SRS patients difficult to interpret. Further investigation into expression in SRS patients was therefore not attempted.

![Figure 7.10](image.png)

**Figure 7.10.** MEST and GAPDH expression in fibroblasts from UPD and non-UPD SRS patients. MEST RT-PCR products (above) detected by autoradiography. GAPDH RT-PCR products (below) electrophoresed on ethidium bromide-stained agarose gel. Lanes 1, 3, and 5: SRS patients (4, 47 and 5) with mUPD7; lanes 7 and 9: SRS patients (25 and 2) without mUPD7; lane 11: normal control. RT negative control lanes (-) confirm the absence of contaminating genomic DNA.
7.4.3 Comparison of MEST expression in cell lines and fetal tissues

Expression of MEST from the paternal allele alone has been demonstrated in human fetal tissues (Kobayashi et al., 1997; Riesewijk et al., 1997). In contrast, the work described above and by Riesewijk et al. has shown that in cell lines this strict pattern of monoallelic expression is lost. Somatic allele switching or biallelic expression may occur. The biological significance of such expression is questionable. Perhaps imprinting is only tightly maintained in those tissues expressing the gene at high, biologically active levels. MEST expression in fetal tissues where the gene is known to be imprinted was therefore compared to levels in cell lines.

Expression was examined using Northern analysis. RNA was extracted from fetal tissues previously described as showing monoallelic expression from the paternal allele (Riesewijk et al., 1997). Normal skin, heart, brain, tongue and kidney samples were used. RNA was also extracted from fibroblast, lymphocyte and transformed lymphoblast cells from normal individuals. Twenty micrograms of each RNA sample were loaded on 1.5% agarose gel, electrophoresed for 3 hours at 100 V and blotted overnight. Expression of MEST was analysed by hybridising the filter with radio-labelled PCR product prepared using primers HP1F and HP1R.

MEST transcripts were seen in all fetal tissues but none of the cell lines (Figure 7.11). Transcript size was calculated to be 2.5 kb from RNA size markers loaded on the gel. This corresponds with the length of the mRNA sequence (GenBank accession no. Y11534) published by Nishita et al. (1996) The intensity of the 18S and 28S ribosomal bands on the gel clearly shows that absence of expression in adult cell lines was not due to degradation or lower quantity of RNA in these lanes. Detection of expression in fetal tissues but not adult cell lines supports the theory that imprinting is only strictly maintained in those tissues expressing the gene at high levels.
Figure 7.11. Northern analysis of MEST expression in cell lines and fetal tissues. The autoradiograph (above) shows expression of MEST is absent in adult cell lines (lymphocytes (L), transformed lymphoblasts (Lb) and fibroblasts (F)) but present in fetal tissues (skin (S), heart (H), brain (B), tongue (T) and kidney (K)). Position of the 2.5 kb transcript is shown on the right. Ethidium bromide was added to samples and the gel photographed (below) before blotting. 18S and 28S ribosomal bands are marked on the left.

7.5 Summary

Recent studies have mapped MEST, to human chromosome 7q32. A role for this paternally expressed, imprinted gene in SRS has been proposed. Southern blot analysis confirmed previous reports of differential methylation of MEST. In other disorders of genomic imprinting, such as PWS and AS, multiple mechanisms are involved. UPD, deletions and IC mutations all alter allelic methylation differences. Methylation-specific PCR was used to screen for paternal deletions or IC mutations of MEST in 43, non-UPD SRS patients. No alteration of allelic methylation patterns was observed. It therefore seems unlikely that MEST plays a major role in SRS.

Further evidence for or against the role of MEST in SRS could theoretically be gained by studying expression of the gene in cell lines from affected patients. However, expression in lymphocytes and transformed lymphoblasts has been shown to be biallelic. Using an RFLP within the 3' UTR of MEST, parental allele expression of the gene was investigated in fibroblasts, lymphocytes and transformed lymphoblasts from two heterozygous controls. Expression was also studied in fibroblasts from a small number of
SRS patients. In none of the cell lines investigated was expression found to be confined to the paternal allele alone. Further investigation into expression of the gene in SRS patients was therefore not attempted. Expression of *MEST* could be detected by Northern analysis in fetal tissues but not adult cell lines. This observation supports the theory that imprinting is only strictly maintained in those tissues expressing the gene at high, biologically active levels.
Chapter 8. **GRB10**

### 8.1 Introduction

Growth factor receptor binding protein 10 (GRB10) is a member of the emerging GRB7 family of proteins which includes GRB7, GRB10 and GRB14. These proteins are characterised by a proline-rich N-terminal motif containing a pleckstrin homology (PH) domain and a C-terminal Src homology 2 (SH2) domain (Figure 8.1). SH2 domains are found in proteins which act as signal transducers for tyrosine kinases; PH domains have been implicated in cellular signaling and cytoskeletal organisation (Shaw, 1996). Mouse *Grb10* was first cloned by Ooi et al. (1995) who mapped the gene to chromosome 11. *GRB10* was subsequently mapped to the homologous region on human chromosome 7p11.2-p12 (Jerome et al., 1997). A BAC clone containing human *GRB10* has recently been isolated and sequenced in order to determine its genomic organisation (Angrist et al., 1998). The gene was found to contain at least 16 exons and to encompass 47 kb or more.

GRB10 seems to act as a growth suppressor by binding to insulin and IGF-I receptors (IR and IGF1R) and inhibiting their tyrosine kinase activity. The gene is widely expressed but highest levels of *GRB10* mRNA have been found in insulin target tissues such as skeletal muscle and adipocytes. Using yeast two-hybrid screens, GRB10 has been shown to bind to both IR and IGF1R (Liu and Roth 1995; O’Neill et al., 1996; Morrione et al., 1996). Liu and Roth (1995) investigated the role of GRB10 using transfected Chinese hamster ovary cells over-expressing the gene. After treatment of these cells with insulin, GRB10 formed complexes with IR, inhibiting the insulin-induced increase in phosphorylation of insulin receptor substrate 1 and a GTPase-activating protein (GAP)-associated protein. In another study, the mitogenic effects of both insulin and IGF-I were blocked by microinjecting the GRB10 SH2 domain into fibroblasts (O’Neill et al., 1996).

GRB10 also binds to other, activated tyrosine kinase receptors such as platelet derived growth factor receptor and EGFR (Frantz et al., 1997). However, binding is weak in comparison to that with IR and IGF1-R. GRB10 may therefore have a more general role in receptor tyrosine kinase-mediated signal transduction though the biological significance of these interactions remains to be determined.
Chapter 8 GRB10

At least four isoforms of GRB10 are known to exist (Dong et al., 1997). Details of these isoforms are summarised in Table 8.1 and Figure 8.1. Western blot studies show differential expression of isoforms in skeletal muscle and cancer cell lines (Dong et al., 1997), suggesting that different isoforms may have distinct biological functions. This theory is supported by evidence that insulin stimulation results in serine phosphorylation of GRB10γ, but not GRB10α (Dong et al., 1997). The precise mode of action of the various GRB10 isoforms within the insulin signaling pathway remains to be investigated.

Table 8.1. Details of the four known human GRB10 isoforms, adapted from Dong et al. (1997).

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Previous Nomenclature</th>
<th>Accession No.</th>
<th>N-terminus</th>
<th>PH domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRB10α</td>
<td>GRB-IR</td>
<td>U34355</td>
<td>A</td>
<td>Shortened</td>
</tr>
<tr>
<td></td>
<td>(Liu and Roth, 1995)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRB10β</td>
<td>GRB-IR - β</td>
<td>U66065</td>
<td>B</td>
<td>Full length</td>
</tr>
<tr>
<td></td>
<td>(Frantz et al., 1997)</td>
<td>U69276</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRB10γ</td>
<td></td>
<td>AF001543</td>
<td>A</td>
<td>Full length</td>
</tr>
<tr>
<td>GRB10δ</td>
<td></td>
<td>D86962</td>
<td>C</td>
<td>Full length</td>
</tr>
</tbody>
</table>

Figure 8.1. Schematic diagram of the domain structure of human GRB10, adapted from Angrist et al. (1998). All isoforms have a proline-rich N-terminal motif, a central region containing a PH domain and a C-terminal SH2 domain. The PH domain sequence and the length and sequence of the N-terminus vary between the different isoforms.
Mouse *Grb10* has recently been shown to be identical to *Megl*. This maternally expressed gene was one of seven identified in a systematic screen for maternally expressed imprinted genes using subtraction hybridisation with androgenic and normal fertilized mouse embryos (Miyoshi *et al.*, 1998). The location of *Megl/Grb10* on proximal chromosome 11 has led to speculation that this gene is responsible for the imprinted phenotypes associated with disomy of this region. Over-expression of *Grb10* in mice with mUPD for proximal chromosome 11 could result in growth retardation by inhibiting insulin and IGF-I activity. Conversely, absence of *Grb10* expression in mice with pUPD for the same region could account for the overgrowth observed.

Human *GRB10* is located within the region on chromosome 7p homologous with the imprinted region on mouse proximal chromosome 11 (Beechey and Cattanach, 1998). Miyoshi *et al.* (1998) have suggested that the growth retardation phenotype seen in association with mUPD7 is the result of over-expression of *GRB10*. No evidence for imprinting of *GRB10* in man has been reported to date. Despite this, the gene is a strong candidate for a role in SRS. It would be interesting to study the phenotype of *Megl/Grb10* transgenic mice since growth retardation would provide further evidence for a role of *GRB10* in SRS. However, disruption of the gene in affected patients must be demonstrated to prove its involvement definitively.

### 8.2 Experimental strategy

Further support for the theory that *GRB10* may play a role in SRS has come from an exciting observation in one SRS proband. This patient (case 49; described in Section 3.1.2) was referred for further investigation by Dr V. Proud from Children’s Hospital of the King’s Daughters, Norfolk, Virginia. She has a characteristic SRS phenotype and was known to have a 7p duplication. Cytogenetic analysis demonstrated a *de novo* duplication of 7p11.2-p12, the region within which *GRB10* maps. FISH studies have been used to confirm *GRB10* duplication in this patient.

Over-expression of an imprinted gene with growth suppressing activity within the region 7p11.2-p12 could explain the phenotype both in this child and those patients with mUPD7. It is also possible that smaller, cytogenetically undetectable duplications of such a
gene may be responsible for growth retardation in non-UPD SRS patients. Although its imprinting status is not yet known in man, it was felt that GRB10 was a strong candidate for involvement in the growth retardation phenotype seen in patient 49, those SRS patients with mUPD7 and possibly other SRS cases without UPD. Evidence for duplication of the region encompassing GRB10 was therefore sought in a cohort of non-UPD SRS patients.

A number of different techniques have been used to demonstrate increased gene dosage in other disorders where duplications are involved. For example, duplication of the proteolipid protein (PLP) gene is seen in a proportion of patients with Pelizaeus-Merzbacher disease. Techniques used to identify duplication of this gene include Southern blot hybridisation (Ellis and Malcolm, 1994), densitometric RFLP analysis (Wang et al., 1997), quantitative fluorescent multiplex PCR and FISH (Woodward et al., 1998). FISH provides rapid results which can be scored by eye using a fluorescence microscope. However, using conventional FISH procedures on metaphase chromosomes, duplicated signals can only be resolved distinctly if they are at least 1 Mb apart (Trask et al., 1991). Although resolution can be improved by an order of magnitude using interphase FISH, small duplications will still go undetected. This problem can be overcome using quantitative fluorescent PCR which is also rapid and requires only small amounts of DNA. However, problems have been experienced with this technique since the accuracy and sensitivity of the assay can be affected by a number of factors, including non-uniform amplification of loci and the quality and source of genomic DNA. Results can therefore be difficult to reproduce (Woodward et al., 1998). In contrast, Southern blot hybridisation has successfully been used at QCCH to demonstrate lack of hemizygosity for IGF1R in a cohort of SRS patients (Abu-Amero et al., 1997). This method was therefore chosen to investigate GRB10 dosage in a cohort of non-UPD SRS patients.
8.3 De novo 7p11.2-p12 duplication in a SRS patient

8.3.1 Cytogenetic analysis

SRS patient 49 was referred for further investigation following detection of an abnormal karyotype. Routine cytogenetic analysis at the Eastern Virginia Medical School, USA had revealed a 7p duplication. Her clinical details and those of her immediate family are described in Section 3.1.2 and Appendix 2. Lymphoblastoid cell cultures from the patient, her mother, father, sister and both maternal grandparents were sent to Mr T. Ballard, Cytogenetic Services, 35 Devonshire St., London for detailed karyotypic analysis. Cytogenetic examination of chromosomes from the patient showed duplication of the region 7p11.2-p12 (Figure 8.2). All other family members had normal karyotypes.

Figure 8.2. Partial karyotype with ideogram showing duplication of chromosome 7p11.2-p12 in patient 49. The position of the duplication is indicated by arrows; the normal homologue is on the left.

8.3.2 Fluorescence *in situ* hybridisation (FISH) studies

FISH analysis (done by Dave Monk) was used to demonstrate duplication of *GRB10* within the duplicated segment. Chromosomes from patient 49 were prepared from a lymphoblastoid cell line. Probe DNA was obtained from PAC clone H_DJ0108E23, containing *GRB10*. This clone had previously been mapped to chromosome 7p11.2-p12 using FISH (Jerome *et al.*, 1997). DNA from cosmid H_DJ0020F22 was used as a control probe. This clone contains *CFTR* which maps to chromosome 7q31.2. Nick translation (Section 4.5.2) was used to label the *GRB10* probe with spectrum red dUTP and the *CFTR* probe with spectrum green dUTP. The two probes were then simultaneously hybridised to chromosome spreads, as described in Section 4.5.3.

In metaphase the *CFTR* and *GRB10* probes hybridised, as expected, to chromosome 7q and proximal 7p, respectively. The control *CFTR* probe appeared to give two signals of approximately equal intensity. In contrast, the *GRB10* signal from one chromosome 7 was consistently more intense than from the other (Figure 8.3). Signal intensity on the two chromosomes was measured for both probes in 50 metaphases using the Smartcapture image analysis system. Using Student’s *t* test, the mean difference in signal intensity was found to be significantly greater for the *GRB10* probe (66.9% increase) compared with the *CFTR* probe (26.0% increase) (*p* < 0.001). In interphase nuclei, where chromosomes are much more extended, it was possible to distinguish two distinct *GRB10* signals on one chromosome and one signal from the other (Figure 8.3). Together, these findings are consistent with duplication of *GRB10* in patient 49.
Figure 8.3. FISH analysis showing duplication of GRB10 in patient 49. Lymphoblastoid cells were hybridised with GRB10 (red) and control CFTR (green) probes. In metaphase (left), the duplicated chromosome is recognisable by increased signal intensity for GRB10 (arrow). In the interphase nucleus (right) the duplicated chromosome gives a dual GRB10 signal (arrowhead) and the normal chromosome a single signal.
8.4 Investigation of *GRB10* dosage by Southern blot analysis

The possibility that submicroscopic duplications of the region containing *GRB10* are a more general cause of SRS was investigated by Southern blot analysis. *GRB10* dosage was measured in 37 SRS patients without mUPD7, including patient 49, and in 17 normal individuals. In order to compare *GRB10* dosage between normal individuals and affected SRS probands, compatible control and *GRB10* probes were needed for hybridisation. Control and *GRB10* probes were generated with the following restrictions in mind:

- both probes should ideally hybridise to single bands;
- these bands should be of similar, though easily resolved sizes;
- the two bands should be close in size to reduce the variation in transfer of control and *GRB10* DNA due to uneven blotting within lanes.

### 8.4.1 Optimisation of Southern blot hybridisation

#### 8.4.1.1 *GRB10* probe

At the time at which this experiment was designed, no genomic sequence data for *GRB10* was available. Primers GRB10-F and -R were designed to amplify a 679 bp region of the gene (from nucleotides 1152 to 1830; Genbank accession no. AF001534). All known isoforms of *GRB10* are predicted to give an identical product size with these primers (Dong *et al.*, 1997; Angrist *et al.*, 1998). cDNA, reverse transcribed from normal fibroblast RNA, was amplified using these primers. Sequence analysis of the PCR product confirmed that it matched the published sequence. This product was used as a probe for dosage studies.

Attempts to amplify genomic DNA with the same primers gave multiple bands. This suggested that at least one intron was located between the primers. The length of intronic sequence could not be estimated, although failure to amplify this region suggested that it was outside the limits of standard PCR (around 2 kb). Since completion of this study, Angrist *et al.* (1998) have published a report describing details of the genomic structure of
GRB10. The information given shows that primers GRB10-F and -R span six exons (exons 10 to 15). The combined size of the five intervening introns is estimated to be 13.5 kb.

8.4.1.2 Restriction enzymes

Initially, a number of different restriction enzymes (ApaI, BamHI, EcoRI, HindIII, XbaI) were used for Southern blot analysis with the GRB10 probe. The aim was to identify a digest giving a single band on hybridisation. All enzymes selected were known not to have a restriction site within the cDNA sequence amplified. Four micrograms of DNA from three normal individuals were digested for 6 hours at 37°C, run out on 0.8% agarose gel at 50 V overnight and blotted.

The results of Southern blot hybridisation are shown in Figure 8.4. Two signals were seen with all the enzymes used. This confirmed the presence of intronic sequence within the region amplified by primers GRB10-F and -R. A single restriction site within this intronic sequence would result in hybridisation of the probe to two bands, as seen.

Although a single band had originally been sought, the presence of two GRB10 bands was felt to be potentially useful. The intensity of both bands could be compared simultaneously to that of a control to give two, complementary sets of dosage data. Since a suitable control probe for use with XbaI digest was already available, further investigation was carried out using this enzyme. Two GRB10 bands were seen at approximately 5.0 and 11.0 kb (Figure 8.4). The control probe, a 6.9 kb XbaI fragment (see Section 8.4.1.3, below), hybridised to give a band of intermediate size.
Figure 8.4. Southern blot showing hybridisation of *GRB10* probe. Two bands are visible following digestion with all five restriction enzymes shown. The probe hybridises to *XbaI* fragments of approximately 5.0 and 11.0 kb in length.

8.4.1.3 Control probe, A6121-1

To generate a control probe, four clones located on chromosome 22 were used, the details of which are given in Table 3.3. Two micrograms of DNA from one fosmid (A9100) and three cosmids (A6121, 5H3 and 27) were digested with 10 U *XbaI* for 3 hours at 37°C. Digested DNA was then run out on 0.8% agarose gel overnight at 40 V (Figure 8.5A). For a restriction fragment to be used as a control probe it must give a single band on Southern blot hybridisation. Fragments containing repetitive sequences are therefore unsuitable. In order to identify suitable fragments, DNA from the gel was transferred to nylon membrane and hybridised with 100 ng human placental DNA. Almost all >5.0 kb *XbaI* fragments from the four clones hybridised strongly suggesting they contain repetitive sequences (Figure 8.5B). However, one 6.9 kb fragment from cosmid A6121 gave a signal which was faint in comparison with its relative intensity on agarose gel, suggesting that it contains little repetitive sequence (Figure 8.5A).
Figure 8.5. Identification of Xbal fragment containing non-repetitive sequence for use as control probe in GRB10 dosage study. (A) Gel electrophoresis of Xbal digests of fosmid A9100 and cosmids A6121, 5H3 and 27. (B) Southern blot hybridisation of XbaI digests with human placental DNA. The 6.9 kb fragment from cosmid A6121 (arrowed) hybridises weakly in comparison to other bands, suggesting that it contains little repetitive sequence.

DNA from cosmid A6121 was digested with XbaI, electrophoresed on low melting point agarose gel and the 6.9 kb fragment (A6121-1) cut out. When this fragment was used to probe XbaI digested control DNA, a single 6.9 kb band was detected. The same filter was then hybridised with both A6121-1 and GRB10 probes simultaneously. All three expected bands (approximately 11.0, 6.9 and 5.0 kb) were readily detectable. In order to exclude the possibility of a common polymorphism at the XbaI sites under investigation, a filter with DNA from ten normal individuals was hybridised simultaneously with GRB10 and A6121-1 probes. Three bands were seen in all lanes and there was no evidence of RFLP.
8.4.2 Quantitative analysis of \textit{GRB10} dosage in SRS patients

\textit{GRB10} dosage was studied in 37 non-UPD SRS patients, including patient 49. The clinical details of these patients are listed in Appendix 1. Patients 7, 23, 25, 28, 36, 45 and 48 were excluded from the study since insufficient DNA was available. Six micrograms of total genomic DNA from normal individuals and SRS probands were digested with 30 units of Xba I for 6 hours at 37°C and electrophoresed on 0.8% agarose gels overnight. DNA from six normal individuals was interspersed with DNA from 10 SRS patients to allow for differences in blotting efficiency across the filter. Filters were then hybridised simultaneously with \textit{GRB10} and A6121-1 probes (Figure 8.6).

8.4.2.1 Calculation of gene dosage

For each filter, incorporation of radioactivity in each band was measured by volumetric analysis using a PhosphorImager (Model 400; Molecular Dynamics). Following the first reading, filters were stripped and re-probed. The average of two readings for each proband was taken. The mean readings for \textit{GRB10} and A6121-1 bands for all normal individuals on a filter were represented as GN and CN respectively. The readings for \textit{GRB10} and A6121-1 bands for each proband were represented as GP and CP respectively. The ratio of GP/CP in each proband to the mean value GN/CN for normal individuals was calculated using the formula: (GP x CN)/(GN x CP). Where there is one copy of \textit{GRB10} for each copy of A6121-1, the ratio will approximate to one. A duplication of the gene will result in three copies of \textit{GRB10} for every two copies of the control, and hence a ratio of 1.5 is expected. Since the \textit{GRB10} probe used hybridised to two \textit{XbaI} fragments, separate ratios were calculated for upper and lower \textit{GRB10} bands.

For a few patients, where upper and/or lower \textit{GRB10} band ratios seemed unusually high or low, DNA was loaded onto a second gel. Average readings taken from both filters were used to determine the final ratios. The data used to calculate these values is shown in Appendices 3a and b.
8.4.2.2 Gene dosage in SRS cohort

The ratios of *GRB10* upper and lower band intensities to A6121-1 band values in patients, normalised against the mean ratios for normal controls, are shown in Figures 8.7A and B. Both sets of ratios followed a normal distribution with almost all values lying close to one. Mean patient ratios were calculated excluding readings for proband 49 with known 7p11.2-p12 duplication. The mean value obtained for the upper *GRB10* band was 1.01 (SD 0.15) and for the lower *GRB10* band was 0.98 (SD 0.13). With the exception of probands 1 and 49, the range of values for the upper band was 0.74- 1.28 and for the lower band was 0.73- 1.29. Ratios generally fell within, or just outside, 95% confidence limits.

![Gene dosage analysis using Southern blot hybridisation. DNA from normal controls (lanes 1, 4, 7) and SRS patients (lanes 2, 3, 5, and 6) was digested with XbaI. The position of upper and lower *GRB10* and A6121-1 bands is indicated on the left. DNA from proband 49 in lane 5 shows 7p11.2-p12 duplication and increased *GRB10* dosage. DNA from proband 1 in lane 6 shows XbaI RFLP (arrowed) and hemizygosity for upper *GRB10* band.](image-url)
Figure 8.7. Histograms representing the GRB10 to control signal ratios determined by quantitative analysis of Southern hybridisation. (A) Upper GRB10 band; (B) lower GRB10 band. Sample standard deviation (SD) and mean are given for both sets of ratios. Ratios for the proband with duplication of 7p11.2-12 (patient 49) lie well above the normal range, confirming increased dosage of GRB10. No other SRS patients have similarly increased ratios.
DNA from patient 49 with duplication of 7p11.2-p12 is shown on the Southern blot in Figure 8.6. Ratios of 1.85 and 1.44, for upper and lower bands respectively, clearly demonstrated increased dosage of \textit{GRB10} associated with duplication of the gene (Figures 8.7A and B).

\textit{GRB10} dosage was also investigated in the family of patient 49. Since the presence of the duplication was confined to the patient herself, it was predicted that \textit{GRB10} dosage should be normal in other family members. DNA from the proband, her sister, mother, father, maternal grandmother, maternal grandfather and four controls was digested with \textit{XbaI} and hybridised with \textit{GRB10} and A6121-1 probes, as described above (Figure 8.8). As expected, her karyotypically normal relatives all had ratios close to 1.00. Ratios for upper and lower bands ranged from 0.89 to 0.97 and 0.86 to 0.94, respectively (Appendix 3c). In contrast, patient 49 again had high ratios of 1.33 and 1.34 for upper and lower \textit{GRB10} bands, consistent with increased gene dosage.

\textbf{Figure 8.8.} Gene dosage analysis in patient 49 and her family using Southern blot hybridisation. The position of upper and lower \textit{GRB10} and A6121-1 bands is indicated on the left. Proband (P) shows increased dosage of \textit{GRB10} whereas the maternal grandmother (GM), maternal grandfather (GF), mother (M), father (F), sister (S) and four normal controls (C) all have normal gene dosage.
Proband 1 had a normal ratio of 1.09 for the lower GRB10 band but a ratio of just 0.63 for the upper band. This was the lowest value obtained (-2.5 SD) and suggested that this patient was hemizygous for the upper band. Southern blot analysis using DNA from patient 1 gave an additional band of approximately 15.0 kb (Figure 8.6, arrowed). XbaI digestion of parental DNA showed that this additional band was inherited from his phenotypically normal father (Figure 8.9). To exclude the possibility that the patient had inherited a small insertion within GRB10, EcoRI and HindIII restriction digests of DNA from the patient and his father were hybridised with the GRB10 probe. Fragment sizes were identical to those seen in normal individuals (data not shown). The most likely explanation of the findings in this patient is therefore heterozygosity for a rare XbaI polymorphism. Since this additional band was seen in just one SRS patient out of 37 and none of the 17 unrelated controls, the heterozygosity for this polymorphism was estimated to be around 2%.

![Southern blot demonstrating paternally inherited XbaI RFLP in patient 1.](image)

**Figure 8.9.** Southern blot demonstrating paternally inherited XbaI RFLP in patient 1. DNA from the patient (C), his mother (M), father (F) and a normal individual (N) was digested with XbaI and hybridised with the GRB10 probe. In the patient and his father, the intensity of the 11.0 kb band was reduced and an additional band of approximately 15.0 kb seen.
In conclusion, Southern blot hybridisation demonstrated increased \textit{GRB10} dosage in one individual with known duplication of 7p11.2-p12. However, no evidence for duplication of this region was found in any of the 36 other SRS patients investigated.

\textbf{8.5 Summary}

The role of \textit{GRB10} in SRS deserves investigation for a number of reasons. Firstly, the mouse homologue of this gene (\textit{Meg1/Grb10}) has recently been found to be imprinted and expressed exclusively from the maternal allele. Secondly, the gene codes for a growth suppressor and maps to 7p11.2-p12. Finally, during this study, \textit{de novo} duplication of chromosome 7p11.2-p12 was observed in one patient with features characteristic of SRS. FISH and gene dosage analysis by Southern blot hybridisation confirmed that \textit{GRB10} is duplicated in this patient. However, no evidence was found for increased \textit{GRB10} dosage in 36 other, non-UPD SRS probands, suggesting that duplication of this gene is not a common cause of the SRS phenotype.
Chapter 9: Discussion

SRS is an excellent model for investigation of the molecular mechanisms underlying fetal growth retardation. Until recently, however, relatively little was known about its genetic basis. Studies have been hampered by its genetic heterogeneity. Familial inheritance and different karyotypic abnormalities have been described in a few patients but the majority are sporadic. The discovery of mUPD7 in a proportion of patients has led to the suggestion that SRS is an imprinting disorder. When this study was initiated, a few genes on chromosome 7 had already been proposed as candidates for SRS, although their role had not been further investigated (Kotzot et al., 1995; Preece et al., 1997). The recent identification of at least one imprinted gene on chromosome 7 has sparked further interest in the genetic basis of SRS.

Investigation of candidate genes in imprinting disorders is complex in comparison to investigation in single gene disorders with classical Mendelian inheritance. In particular, excluding genes as candidates for imprinting disorders can be very difficult for a number of reasons. Imprinted genes are not necessarily imprinted in all tissues or at all stages, making results from patient cell lines difficult to interpret. In addition, epigenetic as well as sequence changes may be responsible for abnormal gene expression. Finally, imprinted genes are often clustered and share regional control mechanisms. Disruptions at considerable distances from an imprinted gene may therefore affect its function. Imprinting disorders such as PWS, AS and BWS have been found to result from multiple mechanisms, some of which are still incompletely understood. It seems reasonable to speculate that abnormal imprinting in SRS will also result from several different mechanisms, perhaps involving more than one gene as in PWS and BWS. Five candidates for SRS have been examined in this study. The results and their implication for SRS are discussed below.
Chapter 9 Discussion

9.1 IGFBP1, IGFBP3 and EGFR

When work on this project started, no imprinted genes had been identified on chromosome 7. *IGFBP1*, *IGFBP3* and *EGFR* had, however, been proposed as candidates on the basis of their location within a region of chromosome 7 homologous to a mouse imprinted region and their known involvement in fetal growth (Kotzot et al., 1995; Preece et al., 1997). The imprinting status of these three genes was therefore investigated.

Expression analysis demonstrated transcription of *IGFBP3* in a wide range of tissues. In contrast, *IGFBP1* was expressed almost exclusively in the liver. These results are similar to those previously described (Pannier et al., 1994; Han et al., 1996). In the current study, fetal tissues from as early as five weeks gestation were examined. Expression of both genes was observed by Han et al. (1996) in late first trimester samples (from 10 weeks gestation). Expression of both *IGFBP1* and *IGFBP3* has therefore been demonstrated from even earlier on in pregnancy than previously reported. *EGFR* has been detected in a large number of tissues in the mouse embryo (Adamson et al., 1981). However, work on human fetal tissues has been limited. Previous studies have described expression in placenta (Deal et al., 1982), ovary and uterus (Yeh et al., 1993), lung (Johnson et al., 1990), keratinocyte, endothelial and skeletal muscle cells (Nanney et al., 1990). This study has shown that *EGFR* mRNA is detectable in a wide range of fetal tissues during both first and second trimester in man. Overall, the results of these expression studies support the view that all three gene products have important, and probably numerous, roles to play in normal human fetal growth and development.

The imprinting status of *IGFBP1*, *IGFBP3* and *EGFR* was examined directly by investigating expression of their individual parental alleles. In order to distinguish parental alleles, polymorphisms for all three genes were identified. A previously described polymorphism within the coding region of *EGFR* (Moriai et al., 1993) was demonstrated using BstNl restriction enzyme digestion. At the start of this study, an expressed polymorphism had been reported for *IGFBP1* (Luthman et al., 1989; Ehrenborg et al., 1992) but not *IGFBP3*. SSCP analysis was used to detect the previously described *IGFBP1* polymorphism and to screen for sequence changes within the 3' UTR of *IGFBP3*. This method was chosen for its simplicity, speed and ability to screen multiple
samples simultaneously. However, since no previous studies could be found in which SSCP analysis had been used to investigate parental allele expression, the technique was first validated by demonstrating paternal-specific expression of *IGF2*. More recently, SSCP has also been used to show maternal-specific expression of the *IPL* (imprinted in placenta and liver) gene (Qian *et al.*, 1997). As has been discussed in Section 5.5.2 and 5.6.2, attempts to demonstrate sequence changes in either gene using this technique were unsuccessful. Instead, direct sequencing was used to screen for polymorphisms within *IGFBP1* and *IGFBP3* and then investigate their parental allele expression.

Biallelic expression of *IGFBP1*, *IGFBP3* and *EGFR* was observed in all fetal tissues investigated. The approach used for all three genes was based on previous studies such as that by Kalsheuer *et al.* (1993) which showed biallelic expression of human *IGF2R*. Parental allele expression was not assessed quantitatively. However, it is increasingly recognised that imprinting is not always an all-or-nothing phenomenon. Several recent reports have described 'leaky' expression from the inactive allele of imprinted genes (Chung *et al.*, 1996; Kobayashi *et al.*, 1997; Qian *et al.*, 1997; Vu and Hoffman, 1997; Cooper *et al.*, 1998). As a result, it has now become usual to analyse the relative levels of expression from the two alleles. Quantitative analyses of restriction enzyme digests (Chung *et al.*, 1996; Kobayashi *et al.*, 1997), SSCP analysis (Qian *et al.*, 1997) and single-nucleotide allele-specific (SNAS) primer extension (Vu and Hoffman, 1997) have all been used to demonstrate preferential, but not exclusive, transcription of one allele in various imprinted genes. Since the relative levels of expression from the maternal and paternal alleles were not measured in this study, it is difficult to rule out the possibility of partial imprinting. However, for all three genes investigated, the PCR cycle number used was kept to a minimum and both alleles were found at approximately the same level in all tissues, suggesting that *IGFBP1*, *IGFBP3* and *EGFR* are biallelically expressed rather than partially imprinted.

Tissue-specific imprinting has been repeatedly described (DeChiara *et al.*, 1991; Ohlsson *et al.*, 1994; Rougeulle *et al.*, 1997; Lee *et al.*, 1997). For this reason, exclusion of a gene as a candidate, on the basis of lack of observable imprinting, is difficult. For example, it was initially suggested that *UBE3A*, recently identified as the AS gene (Kishino *et al.*, 1997; Matsuura *et al.*, 1997), was an unlikely candidate for this disorder as biallelic expression had been demonstrated in cultured fibroblasts and lymphoblasts. It has
now been shown that this gene is imprinted in brain alone (Rougeulle et al., 1997; Vu and Hoffman, 1997). It is therefore important to study parental allele expression in those tissues likely to be involved in the pathogenesis of the disease being investigated. Since \textit{IGFBP1} expression was only consistently detected in fetal liver samples, analysis of its imprinting status was necessarily confined to this tissue. \textit{EGFR} and \textit{IGFBP3}, however, are widely expressed. For these two genes biallelic expression was demonstrated in as many informative tissues as possible. In particular, examination of both genes in placental tissues was felt to be crucial since adequate placental function is known to be an important determinant of fetal growth. In reality it is difficult to know how important a role placental insufficiency plays in SRS. No published data exists on placental weights in SRS and patients do not show the postnatal ‘catch-up’ growth usually seen in IUGR due to placental failure (Wollmann et al., 1995).

In addition to tissue-specific imprinting, the imprinting status of some genes alters during the course of development (Ekström et al., 1995). Since the fetal samples examined were obtained from termination of pregnancies, this investigation was limited to first and second trimester tissues. Unfortunately, although fetuses up to 18 weeks gestation were screened, the latest stage informative fetuses were 17, 10 and 14 weeks gestation for \textit{IGFBP1}, \textit{IGFBP3} and \textit{EGFR}, respectively. Definition of imprinting status in all three genes was therefore confined to first and/or early second trimester fetal tissues. Intrauterine growth retardation in SRS has been noted on routine, 20 week antenatal scans in some patients but is usually detected during the last trimester. Of the 49 SRS patients included in this study, six were known to have IUGR at/before 20 weeks (S. Price, personal communication). The onset of growth failure may therefore occur at a slightly later stage in pregnancy than that of the tissues examined. However, this difference in timing was felt to be insufficient to affect the conclusions drawn from the study.

Where feasible, paired maternal blood was collected at the same time as fetal tissue. Unfortunately, no maternal DNA was available for the fetal liver samples used to investigate \textit{IGFBP1} since these came from the MRC Tissue Bank at Hammersmith Hospital. Samples used to investigate \textit{IGFBP3} and \textit{EGFR} were collected from QCCH where it was generally possible to obtain paired maternal blood. Investigation of maternal-fetal pairs is particularly informative for two reasons. Firstly, where the fetus is heterozygous but the mother homozygous, the parental allele expressed can be determined
if a gene is imprinted. For example, in this study, paternal specific expression of $IGF2$ was demonstrated using an Apal polymorphism in a heterozygous fetus with a homozygous mother (Section 5.4). Secondly, as described below, analysis of paired maternal and fetal samples allows contamination of fetal tissues by maternal decidua to be detected.

The presence of maternal DNA in fetal samples could potentially obscure an imprinting effect and as a result be misinterpreted as biallelic expression. However, maternal-specific expression cannot be masked in those fetuses whose mothers are homozygous for the undigested allele. For two heterozygous fetuses (F10 and F17) maternal DNA was homozygous for the $EGFR$ polymorphism. Biallelic expression was demonstrated in both. Similarly, for $IGFBP3$, all three fetuses identified as being heterozygous had homozygous mothers and biallelic expression in all tissues. It is therefore possible to exclude the possibility that contaminating maternal DNA is masking maternal-specific expression of both $EGFR$ and $IGFBP3$. Maternal/fetal pairs where the mother was heterozygous and the fetus homozygous for the $EGFR$ polymorphism were examined to look for evidence of amplification from contaminating maternal mRNA. This was found in two out of five tissues investigated. In both, the maternally derived bands were significantly fainter. It therefore seems unlikely that the widespread biallelic expression of $EGFR$ observed is due to contaminating maternal tissue. Biallelic expression of both $IGFBP1$ and $IGFBP3$ was also seen in a number of tissues from several different individuals. Both alleles were seen at approximately equal levels in all samples. These observations make it unlikely that monoallelic expression of either $IGFBP1$ or $IGFBP3$ was disguised by amplification of maternal DNA.

An alternative strategy for identifying maternal contamination of fetal tissues was adopted by Kobayashi et al. (1997). They used a DNA polymorphism for $WT1$ to show that no maternal DNA was present in samples where there was apparent 'leaky' imprinting from the inactive maternal allele of $MEST$. In the current study, one of the fetuses (F7) heterozygous for the $EGFR$ polymorphism was the same fetus used to investigate $IGF2$ imprinting by SSCP analysis. The mother was heterozygous for the $EGFR$ but homozygous for the $IGF2$ polymorphism. Since expression of $IGF2$ was seen exclusively from the paternal allele in both skin and brain, these samples must be free of maternal contamination. Biallelic expression of $EGFR$ was demonstrated in these same samples,
Chapter 9 Discussion

providing further evidence that paternal-specific expression of \textit{EGFR} is not being obscured by incomplete removal of maternal tissue.

Expression of \textit{IGFBP3} and \textit{EGFR} mRNA was demonstrated in fibroblasts and transformed lymphoblasts from SRS patients with mUPD7. It seems, therefore, that neither of these genes are expressed from the paternal allele alone, supporting the conclusion that they are biallelically expressed throughout development.

The results from patient cell lines must, however, be interpreted with caution for two main reasons. Firstly, imprinting of several genes has been found to alter postnatally as well as during cell culture and transformation. For example, \textit{MEST} is expressed from the paternal allele alone in several different human fetal tissues (Kobayashi \textit{et al.}, 1997; Riesewijk \textit{et al.}, 1997). However, biallelic expression of this gene has been demonstrated in adult blood lymphocytes and transformed lymphoblasts (see results in Section 7.4.1 and Riesewijk \textit{et al.}, 1997). \textit{EGFR} and \textit{IGFBP3} expression was therefore analysed in fibroblasts as well as lymphoblasts to address concerns about artifactual loss of imprinting in transformed cell lines. Secondly, since levels of mRNA were not measured quantitatively, only paternal-specific expression can be excluded. For maternal-specific expression to be detected, the level of expression in mUPD7 cells would need to be compared to that in cell lines from normal individuals. Levels at least double those in normal samples would be expected. Although quantitative analysis is theoretically possible, the number of mUPD7 cell lines available for study is small, making statistical analysis difficult.

An alternative strategy would be to study cell lines from pUPD7 individuals. If expression were confined to the maternal allele, no transcripts should be detected. Only two cases of pUPD7 have been reported (Höglund \textit{et al.}, 1994; Pan \textit{et al.}, 1998) and, although requested, pUPD7 samples were not available to use in collaboration. Complete hydatiform moles have an entirely paternally-derived genome (Lawler \textit{et al.}, 1982) and would also be expected to show absent expression of a maternally-expressed gene. However, the expression of imprinted genes in hydatiform moles does not always correlate well with their parental-allele expression in normal tissues (Walsh \textit{et al.}, 1994; Zaragoza \textit{et al.}, 1997).

Although it is virtually impossible to completely rule out tissue or stage-specific monoallelic expression, analysis in normal fetal tissues and mUPD7 cells failed to provide
any evidence for imprinting of IGFBP1, IGFBP3 or EGFR. It therefore seems unlikely that any of these genes play a direct role in the pathogenesis of SRS. This conclusion could have been strengthened by searching for mutations within their promoter and coding regions. However, in the absence of evidence for imprinting it was decided not to investigate IGFBP1, IGFBP3 or EGFR further. Instead, efforts were concentrated on MEST and GRB10 since MEST has recently been shown to be paternally expressed in both mouse and man and GRB10 is known to be imprinted in mouse. The imprinting status of GRB10 in man is currently under investigation.

9.2 MEST

During this study, several reports were published demonstrating that human MEST is imprinted, expressed from the paternally-derived allele (Nishita et al., 1996; Kobayashi et al., 1997; Riesewijk et al., 1997) and maps to chromosome 7q32 (Jerome et al., 1997). Recently, targeted mutagenesis of the mouse homologue, Mest, has been shown to give rise to prenatal growth retardation (Lefebvre et al., 1998). These observations make MEST an excellent candidate for SRS and its role was therefore investigated.

9.2.1 Allelic methylation in SRS patients

Absent expression of the paternally expressed MEST gene may account for the SRS phenotype in patients with mUPD7. However, evidence for this can only be provided by demonstrating disruption of the gene itself in non-UPD SRS patients. In common with other imprinted genes, MEST has been shown to be differentially methylated. The CpG island at the 5' end of MEST is methylated on the silent maternal allele but unmethylated on the active paternal allele (Riesewijk et al., 1997). Differential methylation of the imprinted PWS/AS region has been used to diagnostic advantage since all the most common molecular abnormalities, with the exception of UBE3A mutations in AS, result in altered allelic methylation (Kubota et al., 1996). In PWS, mUPD15, paternal deletions and IC mutations all result in loss of the unmethylated paternal allele. In AS, pUPD15, maternal deletions and IC mutations result in loss of the methylated maternal allele. Since it is likely
that SRS results from similar mechanisms to those described in PWS and AS, abnormal methylation due to paternal deletions and/or IC mutations would be expected in at least a few non-UPD patients. In order to determine whether *MEST* plays a significant role in SRS, differential methylation of the critical 5' region of the gene was investigated by methylation-specific PCR. The optimisation of this assay has been discussed in Section 7.3.2.2. Normal allelic methylation patterns were demonstrated in 43 non-UPD cases of SRS. Therefore no evidence was found for IC mutations causing aberrant methylation within the 5' CpG island of *MEST*. In addition, there was no evidence for paternal deletions encompassing the 5' CpG sites investigated in these patients.

A role for *MEST* in SRS is not, however, entirely ruled out by this approach for several reasons. Firstly, the frequency of IC mutations may be low in SRS. In PWS and AS, IC mutations have been detected in ≤5% of patients (Nicholls et al., 1998) and only 5-10% of BWS patients have altered methylation of *IGF2* (Reik and Maher, 1997). Secondly, differential methylation of only six CpG dinucleotides within the 5' CpG island was analysed. It is possible that IC mutation may result in loss of differential methylation of some, but not all, CpG dinucleotides. Altered methylation at CpGs within the amplified PCR fragment, other parts of the CpG island or even additional differentially methylated regions in other parts of the gene would not be detected. Paternal deletions outside the region amplified or within the promoter or coding regions of the gene would also go undetected. Finally, *MEST* may be involved in SRS via mechanisms seen in other disorders of genomic imprinting which would not be expected to alter allelic methylation. Gene function may be disrupted directly by mutations within the coding region as have been found in *UBE3A* for some AS patients (Kishino et al., 1997; Matsuura et al., 1997). Alternatively, abnormalities in other, neighbouring imprinted genes may indirectly affect *MEST* expression. In the same way, translocations into the *KVLQTL* gene are associated with biallelic *IGF2* expression in BWS (Brown et al., 1996; Lee et al., 1997). In conclusion, no evidence was found to implicate *MEST* in SRS but it cannot be completely excluded as a candidate on the basis of these findings alone.

While this study was in progress, Riesewijk et al. (1998) published a report describing their investigation into the role of *MEST* in SRS. They used Southern hybridisation to demonstrate normal methylation patterns in the 5' region of *MEST* in 35 patients with SRS and nine with primordial growth retardation. Since methylation analysis
Chapter 9 Discussion

will not detect point mutations within the coding region of \textit{MEST}, they also screened a total of 58 patients for sequence changes using SSCP analysis. Apart from one silent mutation and two novel polymorphisms, nucleotide changes were not detected in any of these patients. Together, the results described by Riesewijk \textit{et al.} (1998) and those obtained in this study argue strongly against a major role for \textit{MEST} in SRS.

The paternal-specific PCR reaction gave bands with DNA from all of the normal individuals but none of the mUPD7 SRS patients tested, demonstrating the specificity of the paternal primer (MEST-PAT) for unmethylated DNA. This technique can therefore be used to confirm suspected mUPD7 in patients where parental DNA is not available but unusually extensive homozygosity (possible isodisomy) for polymorphic markers has been demonstrated. So far one such case has been referred to QCCH for investigation (Section 7.3.2.4). This patient had short stature in combination with symptoms of cystic fibrosis and homozygosity for a rare variant of \textit{CFTR} and four other RFLP markers around this gene. However, methylation-specific PCR suggested that this patient did not have mUPD7 since both parental alleles of \textit{MEST} were detected. This conclusion was supported by the observation that the region of homozygosity was not greater than 15.4 cM since the patient was heterozygous for several tetranucleotide markers surrounding \textit{CFTR}. It should be noted that segmental UPD arising from postzygotic somatic recombination is well documented in BWS (Henry \textit{et al.}, 1993; Slatter \textit{et al.}, 1994) and cannot be excluded by this approach. However, segmental UPD7 has not been reported to date.

Theoretically the assay could also be used to detect pUPD7. However, since pUPD7 DNA was not available, the specificity of the maternal primer (MEST-MAT) for methylated DNA could not be tested. Since pUPD7 is associated with a normal phenotype (Höglund \textit{et al.}, 1994), this investigation is unlikely to be requested for clinical diagnosis.
9.2.2 MEST expression in cell lines

The role of MEST in SRS was also studied by investigating its expression in cell lines from UPD and non-UPD patients as well as normal individuals. The findings obtained in this part of the study were unexpected and at times apparently contradictory. The explanations for these results and the wider implications for use of patient cell lines to investigate imprinting disorders are discussed below.

MEST expression was found to be monoallelic in fibroblasts from two normal individuals. Since no parental DNA was available from these two individuals, it was not possible to determine which parental allele was transcribed. It seems likely that expression is paternal-specific, as it is in fetal tissues, including skin (Riesewijk et al., 1997). However, maternal-specific expression of in these cells cannot be ruled out, especially since altered allelic expression of other genes in cell lines has been observed. For example, a study of WTI imprinting status in cultured fibroblasts and lymphoblasts showed paternal or biallelic expression in contrast with previous findings of maternal or biallelic expression in human placenta and fetal brain (Mitsuya et al., 1997). Paternal-specific expression of MEST in fibroblasts could be confirmed by analysis of MEST expression in fibroblasts from further normal heterozygous individuals with informative parental DNA.

Biallelic expression of MEST was demonstrated in transformed lymphoblasts from both normal controls studied as well as in lymphocytes from one of these individuals. These findings are consistent with those reported previously by Riesewijk et al., (1997) who described expression from both parental alleles in adult blood lymphocytes.

Interestingly, in the second lymphocyte sample investigated in this study there was monoallelic expression of MEST. Moreover, there was an apparent switch in allele usage with transcription of the opposite allele to that in fibroblasts from the same individual. Somatic allele switching in some individuals has been described for a few other genes (Zhang et al., 1993; Douc-Rasy et al., 1995; Brown et al., 1996; Mitsuya et al., 1997). However, this is the first time that the phenomenon has been observed for MEST. Additional heterozygous individuals would need to be identified to allow the extent of the variation in allelic expression of MEST in different cell lines to be evaluated. It would also be interesting to determine whether the allele switch observed was associated with alteration
Chapter 9 Discussion

in allelic methylation. *H19* somatic allele switching in cervical carcinomas has been shown to be accompanied by a switch in methylation status (Douc-Rasy *et al.*, 1995).

The methylation status of the 5′ CpG island of *MEST* was not examined in any of the cell lines used. However, in a previous study it was found that parent-of-origin-specific methylation was not lost in lymphocytes biallelically expressing the gene (Riesewijk *et al.*, 1997). Several explanations for this observation are possible. Loss of monoallelic expression may result from demethylation of specific, as yet untested CpGs. Methylation analysis has so far been limited to CpGs within *MspI* restriction sites (Riesewijk *et al.*, 1997). Other CpGs could be examined in this and other regions using the technique of bisulfite genomic sequencing described by Frommer *et al.* (1992). Alternatively, specific transcription factors in lymphocytes and/or transformed lymphoblasts may allow transcription of the methylated maternal allele. Finally, alternate promoters may be used in those cells which escape imprinting, as has been described for *IGF2* (Ekström *et al.*, 1995). The findings from one study of *MEST* expression in mUPD7 patients support this explanation (Cuisset *et al.*, 1997). Expression was investigated in mUPD7 leukocytes by RT-PCR using several different primer pairs. Exon 1 repeatedly failed to amplify, although mRNA from the distal coding region of the gene could be detected. As six independent database sequences showed that exon 2 was joined to a common sequence unrelated to exon 1, the authors hypothesised that the maternal allele was being transcribed from an alternate upstream promoter.

Since monoallelic expression had been seen in fibroblasts from both normal individuals, expression of *MEST* in SRS patients was studied in these cells. Rather surprisingly, expression of the gene was seen in all three mUPD7 samples, although the signals obtained in two of the three mUPD7 samples were of distinctly lower intensity than those seen in non-UPD and control samples (Figure 7.10). The most likely interpretation is that the maternal allele is active in cell lines from these patients and that ‘leaky’ expression is occurring from the normally silent allele of *MEST*. Similar relaxation of imprinting of some, but not all, imprinted genes within the PWS/AS region has been reported in cells from two patients with PWS (Rogan *et al.*, 1998).

In all five mUPD7 SRS patients investigated at QCCH, centromeric markers were found to be heterodisomic. This suggests that the UPD in these cases has arisen as a result of maternal meiosis I nondisjunction and subsequent trisomic rescue (Preece *et al.*, 1999;
Chapter 9 Discussion

Section 1.3.2.2). Theoretically, mosaicism for UPD and trisomy 7 in the mUPD7 patients studied could result in the expression of *MEST* from the paternal allele in a small percentage of trisomic fibroblast cells. To investigate the possibility of mosaicism for trisomy 7, parental allele inheritance of one VNTR marker was studied by Southern blot hybridisation in fibroblast DNA from two of the mUPD7 patients shown to express *MEST* (4 and 5) (work done by D. Monk). No paternally-derived bands were visible even when X-ray film was exposed for two weeks in order to detect a low level of mosaicism.

The findings in both normal and mUPD7 cell lines can be explained if strict imprinting of *MEST* is lost in cells expressing the gene at low levels. To test this theory *MEST* expression was investigated using Northern analysis. The gene was not expressed at significant levels by adult fibroblasts, lymphocytes or transformed lymphoblasts. In comparison, expression was clearly demonstrated in several fetal tissues (skin, heart, brain, tongue and kidney) in which monoallelic expression had previously been demonstrated (Riesewijk *et al*., 1997). These findings are consistent with the developmental changes in *Mest* expression observed in mice (Lefebvre *et al*., 1998). The gene is transcribed widely and at high levels in newborn mice but expression levels decrease shortly after birth. In adult mice expression is confined almost exclusively to the nervous system. In the current study, 40 cycles of RT-PCR were needed to generate signals strong enough to detect *MEST* parental allele expression in both normal and SRS cell lines. Even then bands were not always visible using ethidium bromide staining and for some experiments autoradiographic detection was required. The high degree of sensitivity needed to detect expression in cell lines calls its biological significance into question. Overall the findings support the hypothesis that imprinting is only strictly maintained in those tissues expressing the gene at high levels.

Using RT-PCR and RFLP analysis, *MEST* expression in human chorions and early embryos was shown to be predominantly paternal in origin (Kobayashi *et al*., 1997). However, expression from the maternal allele could also be detected, albeit at levels more than 20 times lower than those from the paternal allele. These results can be explained if there is a low level of basal transcription from both alleles. Transcription of the active paternal allele predominates in tissues expressing the gene at high levels. RT-PCR at relatively low cycle numbers will detect a signal from this allele alone and the gene appears to be fully imprinted. At higher cycle numbers partial imprinting is observed. In 'non-
expressing' tissues, such as adult lymphocytes and lymphoblasts, transcripts can still be detected if the cycle number used is high enough since RT-PCR is extremely sensitive. However, both alleles will be amplified at roughly equal, though very low, levels. To test this hypothesis, quantitative RT-PCR could be used to determine the relative levels of expression from the two parental alleles in fetal tissues and various cell lines.

A low basal level of transcription from both alleles may explain why expression of MEST was detected in fibroblasts from mUPD7 patients despite the fact that monoallelic expression was seen in normal individuals. Southern hybridisation and autoradiography was required to detect expression of MEST in all three mUPD7 patients. In contrast, monoallelic expression of MEST in normal fibroblasts could be demonstrated using ethidium bromide staining in both normal individuals. Direct comparison of allelic expression levels in normal individuals and non-UPD and mUPD7 patients was not attempted since sample numbers were small. However, it is tempting to speculate that if parental allele expression was analysed to the same degree of sensitivity in normal individuals and mUPD7 patients, maternal allele expression would be detectable in both. Again, this could be put to the test using quantitative RT-PCR.

It is also possible that instead of a generalised low level of transcription from the 'silent' allele in all cells there may be clonal relaxation of imprinting. It would be interesting to examine the differentially methylated region of MEST in fibroblasts from mUPD7 patients and normal lymphoblasts. Relaxation of imprinting may reflect the fact that not all CpG dinucleotides are strictly differentially methylated in all cells. If this is the case then bisulfite sequencing of the 5' CpG island in several clones should reveal mosaic methylation of some CpGs.

Loss of imprinting in cell lines may reflect changes in the methylation status of the gene. Alterations in DNA methylation have been observed in culture, although in most cases this process is slow and occurs over many generations (Razin and Cedar, 1991). CpG islands are usually unmethylated even when the associated gene is transcriptionally silent. However, studies by Antequera et al. (1990) have shown that CpG islands at several tissue-specific, but not housekeeping, genes become methylated in culture. This phenomenon is thought to explain the loss of cell type-specific functions in cell lines. Fibroblast cells used in the current study were subjected to no more than one passage in order to minimise the risk of cell culture artefacts. Lymphoblasts were also investigated
Chapter 9 Discussion

after as few passages as possible. Relaxation of imprinting status has also been found in cells from 'post-confluent' cultures where growth is restricted (Ungaro et al., 1997). For this reason care was taken not to grow fibroblast cells beyond subconfluence.

Cell lines are often the only source of tissue available from patients with imprinting disorders. They have proved extremely useful for investigation of those genes whose imprinting status is strictly maintained in cultured cells. For example, imprinting of the paternally expressed genes PAR-1 and PAR-5 from the PWS/AS region was first indicated by the observation that transcripts from both genes are detected in cultured cells of AS but not PWS deletion patients (Lalande, 1994). However, similar studies have also led to misleading conclusions being drawn about candidate genes which do not maintain strict imprinting. As described above, UBE3A was recently identified as the AS gene (Kishino et al., 1997; Matsuura et al., 1997) despite earlier suggestions that it was an unlikely candidate based on its biallelic expression in cell lines (Nakao et al., 1994). Monoallelic expression of this gene in brain has since been demonstrated (Rougeulle et al., 1997). The results in the current study also show that the imprinting status of some genes in cell lines may not reflect their actual status in relevant tissues at critical stages of development. Consequently, in this study cultured cells were not used to investigate whether abnormal imprinting of MEST results in SRS. Overall, findings suggest that the use of patient cell lines has important limitations which must be recognised by those investigating candidate genes in SRS and other imprinting disorders.
Chapter 9 Discussion

9.3 GRB10

One of the main factors hindering identification of imprinted gene(s) on chromosome 7 responsible for the mUPD7 phenotype has been the absence of SRS patients with karyotypic abnormalities of this chromosome. Such patients have proved invaluable in the delineation of other imprinted regions. The recognition of a large number of PWS and AS patients with chromosome 15q deletions has allowed precise mapping of the PWS/AS region (Buiting et al., 1993). Similarly, BWS patients with segmental UPD11, 11p15 duplication and translocation breakpoints have helped to narrow the critical region for BWS (Hoovers et al., 1995). Paternal UPD6 is associated with transient neonatal diabetes mellitus (TNDM) (Temple et al., 1995). Other patients with paternal duplication of 6q23 have been identified, providing further evidence for a paternally expressed imprinted gene for TNDM localised to this region (Temple et al., 1996).

Until very recently, however, the only clinical clue to the location of the SRS gene(s) on chromosome 7 was a child described by Eggerding et al. (1994). The patient had postnatal, but not prenatal, growth retardation and some features of SRS including a typical facial appearance, mild asymmetry and bilateral clinodactyly. On karyotypic analysis she was found to have paternal UPD7p and maternal UPD7q. Since pUPD7 is associated with a normal phenotype, this case report is often cited as supporting evidence for exclusion of the SRS gene(s) from 7p. However, it is also possible that intrauterine growth control requires normal expression of genes on chromosome 7p whereas genes on 7q are involved in regulation of postnatal growth.

Another case report has just been published in which a girl with characteristic features of SRS and 47XX,UPD(7)mat+r(7)pat/46XX,UPD(7)mat mosaicism is described (Miyoshi et al., 1999). Microsatellite marker analysis detected only one maternal allele at each of 16 telomeric loci examined on chromosome 7 but both maternal and paternal alleles at four centromeric loci (D7S2552, D7S499, D7S494 and D7S2503) spanning the region 7p13-q11. Together these results indicated that the patient had mUPD7 in all cells and mosaicism for ring chromosome 7 (r(7)(p13q11)) of paternal origin. The authors state that “if the putative SRS gene is imprinted, it can be ruled out from the 7p13-q11 region, because biparental alleles contribute to the region”. However, there are several problems with this conclusion. Firstly, there is no evidence to show that genes on the ring
Chapter 9 Discussion

chromosome are active. Follow up of patients with supernumerary marker chromosomes has found that they are frequently not associated with any phenotypic effect (Warburton et al., 1991). Secondly, even if the ring chromosome is active, it can only be used to infer exclusion of a paternally expressed gene from the region. If the SRS gene is transcribed exclusively from the maternal allele, it will be over-expressed in this patient, whether or not the gene is located within the ring chromosome, since she has two maternal alleles. Lastly, the genetic distance spanned by the four markers used to define the extent of biparental inheritance is small (2.26 cM between 64.81 and 67.07 cM) (URL: http://cedar.genetics.soton.ac.uk/pub/chrom7/map.html). Furthermore, UPD was demonstrated using marker D7S506 which is estimated to lie less than 0.1 cM distal to D7S2552 on 7p. The trisomic region is therefore likely to be considerably smaller than 7p13-q11. Two of the biparentally inherited markers (D7S499 and D7S494) have been mapped more precisely to 7p11.2 and 7p11.2-q11.1, respectively (Tsui et al., 1994). It is interesting to note that IGFBP1, IGFBP3 and GRB10 lie between two markers (D7S2497 and D7S506) which are outside the potentially excluded region. EGFR lies between the UPD marker D7S506 and the biparental marker D7S2552. It is therefore not clear whether EGFR is located within the ring chromosome.

Since so few clues currently exist as to the location of the SRS gene(s) on chromosome 7, additional patients with karyotypic abnormalities of this chromosome are of great interest. In the current study, 7p11.2-p12 duplication has been demonstrated for the first time in a child with characteristic features of SRS. Arguably, this finding is the most useful cytogenetic clue to the location of the SRS gene(s) on chromosome 7 to date. It seems likely that an imprinted gene within the duplicated segment 7p11.2-p12 is responsible for the SRS phenotype in this patient. The duplication may cause disruption of a critical gene at one of its breakpoints. Alternatively, increased dosage of a gene with growth suppressing activity may result. Over-expression of a maternally expressed, imprinted gene could account for the phenotype in both this patient and those with mUPD7.

Although many members of the patient’s family have short stature, none have characteristic features of SRS (Appendix 2). Family photographs show that only the patient herself has the typical facial appearance of SRS. Detailed cytogenetic analysis found no evidence of 7p11.2-p12 duplication in either parent. In addition, in this study, normal GRB10 dosage was demonstrated in all family members investigated other than the patient.
Chapter 9 Discussion

Therefore, although germline mosaicism in the parents cannot be excluded, the duplication probably arose *de novo*. Its parental origin is currently being investigated using microsatellite markers. If the phenotype in this patient is due to over-expression of a maternally expressed imprinted gene(s), the origin of the duplicated allele should be maternal.

It is interesting to note that involvement of a maternally expressed gene(s) would also account for the rarity of reports of paternal transmission of SRS. The pedigrees of imprinting disorders are characteristic (Hall, 1990). An imprinted gene is transmitted in a Mendelian manner but its expression is determined by the sex of the transmitting parent. Thus, disruption of a maternally expressed gene will result in a disease phenotype only if transmitted by the mother. If the father transmits the abnormal gene it is effectively silenced and his children will be unaffected. However, if, in turn, his non-manifesting daughter transmits the gene, her children will be affected. In an imprinting disorder equal numbers of affected and unaffected males and females are seen in each generation and the pedigree can look like autosomal dominant, autosomal recessive or multifactorial inheritance, depending on which part of the family is being observed. The SRS families reviewed by Escobar *et al.* (1978) and Duncan *et al.* (1990) fit well into this pattern of inheritance (see Section 1.2.2.1).

The duplication patient described in this study is of particular interest since the potentially imprinted gene *GRB10* maps to the region 7p11.2-p12 (Jerome *et al.*, 1997). This gene has an inhibitory effect on the growth promoting activities of insulin and IGF-I (O’Neill *et al.*, 1996). The mouse homologue of *GRB10* (Grb10/Megl) is imprinted and expressed exclusively from the maternal allele (Miyoshi *et al.*, 1998). *GRB10* is therefore a good candidate for SRS. Patients with mUPD7 are expected to over-express *GRB10* if it is transcribed from the maternal allele alone. However, this does not prove that the gene is responsible for the phenotype. Instead, specific evidence that the gene is disrupted in SRS is needed. Dosage of the gene in those SRS patients without mUPD7 was therefore analysed.

Duplication of *GRB10* was clearly demonstrated in patient 49 both by FISH and gene dosage analysis. *GRB10* must therefore be located within her duplicated region. However, no further SRS patients with increased *GRB10* dosage were identified. Several possible explanations for this need to be considered. All are largely dependent on whether
Chapter 9 Discussion

GRB10 is, or is not, maternally expressed in man as well as in mouse. This remains to be determined.

If GRB10 is imprinted then mechanisms other than duplication of the gene may be causing its over-expression in SRS patients. In BWS, paternal 11p15 duplications are seen in less than 1% patients (Li et al., 1997). Biallelic expression of IGF2 is the most common molecular abnormality in BWS patients without cytogenetic abnormalities. By analogy, over-expression of GRB10 in SRS without UPD7 could result from loss of imprinting. The level of GRB10 expression needs to be investigated in SRS patients both with and without mUPD7 and compared to that in normal controls. Over-expression of GRB10 in patients with mUPD7 compared to normal controls would be indicative of its maternal-specific expression in man. Over-expression of the gene in non-UPD patients would provide evidence for loss of GRB10 imprinting in SRS and suggest direct involvement of this gene in the disease phenotype.

The role of GRB10 was investigated since this gene had already been identified as a good candidate for SRS. However, other genes may be involved. Since many imprinted genes have been found to lie within clusters (Reik and Maher, 1997), it is tempting to speculate that GRB10 lies within a similar cluster of imprinted genes, all or some of which are duplicated in patient 49. One or more of these may be involved in SRS, in addition to or instead of GRB10. Further work needed to investigate the region surrounding GRB10 is discussed more fully in Section 9.5.

Another explanation for the phenotype in patient 49 is over-expression of a non-imprinted gene within the trisomic region. It is also theoretically possible that the phenotype in mUPD7 patients is due to mosaicism for trisomy 7 and increased dosage of a non-imprinted gene. However, no evidence to support this was found when two mUPD7 patients were investigated for low level mosaicism in fibroblast cells (see Section 9.2.2 above). Instead, the molecular mechanism in patient 49 may prove to be unique and distinct from that in mUPD7 patients where imprinting has been implicated. The lack of additional SRS probands with duplications in this region and the heterogeneity associated with the syndrome make this difficult to rule out.

Nearly 90 reported cases of chromosome 7 deletion and duplication are listed on the cytogenetic databases available at the Institute of Child Health. Prenatal short stature, skeletal asymmetry and/or triangular facies are described in association with several of
these. However, no region consistently gives rise to characteristic SRS-like features when deleted or duplicated.

In addition to the patient described by Miyoshi et al. (1999), at least five other reports of trisomy for a region overlapping 7p11.2-p12 have been published. The clinical details of all these patients are summarised in Table 9.1. Four of the cases involved much larger segments of duplicated chromosomal material. Three had duplications of the entire short arm of chromosome 7 (Zeres et al., 1989; Odell et al., 1987; Carnevale et al., 1978), two of which were due to familial translocations. Another child had duplications of both 7p13-q21 and 5q35-qter (Wahlström et al., 1976). In all four cases, the extent of the duplication was much greater than that found in patient 49 and, as expected, the phenotypes described were markedly more severe. In addition, since other autosomes were involved in some of these patients it is difficult to attribute specific features of their phenotypes to duplication of chromosome 7.

The most interesting case report, however, describes a 10 month old child with a familial inverted duplication of 7p12.2-p13 (Schaefer et al., 1995). Some of the features seen in the proband are reminiscent of SRS. She had feeding difficulties, failure to thrive, bilateral fifth finger clinodactyly and developmental/speech delay. However, her birth weight was thought to be normal (25th centile), her facial features were not characteristic of SRS and she showed significant 'catch-up' growth once feeding via a gastrostomy tube was initiated. At the time of the report her height was 81 cm (3rd-10th centile) and weight 9.9 kg (just below 3rd centile). Furthermore, although her brother, mother and grandmother were all cognitively delayed and had the same inverted duplication of chromosome 7, none are described as having growth retardation. Further definition of the phenotype and duplication in all four affected individuals would be interesting since the trisomic region in this family apparently overlaps that in patient 49. Phenotypic differences, in particular presence/absence of growth failure, may result from differences in extent, position, and/or orientation of the two duplications.
Table 9.1. Clinical features of patients with partial trisomy 7p involving the region 7p11.2-p12

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>III-1 F</td>
<td>III-3 M</td>
<td>II-2 F</td>
<td>I-2 F</td>
<td>F</td>
</tr>
<tr>
<td>Antimongoloid-slanting eyes</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaked nose</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue sclerae</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular anomalies</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choanal atresia</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS anomalies</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Craniofacial anomalies</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Developmental/speech delay</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 days 7 months</td>
</tr>
<tr>
<td>Down-slanting eyes</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down-turned mouth</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feeding difficulties</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fifth finger clindactyly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal anomalies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genitourinary anomalies</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>High narrow palate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertelorism</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoplastic maxilla/mandible</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lateral asymmetry</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-set/ posterior rotated ears</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low birth weight (&gt; -2 SD)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mental retardation</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prominent anterior fontanelle</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Redundant neck skin</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative macrocephaly</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short stature (&gt; -2 SD)</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Skeletal anomalies</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triangular shaped face</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Chapter 9 Discussion

Routine karyotypic analysis was performed on all the patients included in the SRS cohort used in this study, including those for whom insufficient DNA was available to analyse GRB10 dosage. The karyotype was normal in all cases except patient 49. In light of the findings in this study and the recent case report by Miyoshi et al. (1999) careful cytogenetic analysis of all SRS patients is imperative, paying particular attention to the region around 7p11.2-p12. Discovery of further SRS patients with karyotypic abnormalities of chromosome 7 may prove to be crucial in identifying the imprinted gene(s) involved in this disorder.

9.4 Conclusion

Interest in SRS as a new imprinting disorder is gathering pace as the number of reported cases of mUPD7 increases. However, investigation into the molecular basis of SRS is far from straightforward. Not only is there evidence for genetic heterogeneity within the syndrome but there are also very few reports of patients with karyotypic abnormalities. Cytogenetic clues have been central to the investigation of other imprinting disorders such as PWS, AS and BWS but are notably rare in SRS.

The discovery of mUPD7 in association with approximately 7% of SRS cases has led to the suggestion that one or more imprinted gene(s) on chromosome 7 are responsible for the phenotype in these patients. However, until recently no imprinted genes on chromosome 7 had been described. The only clue as to the location of imprinted genes on the chromosome was known homology of some regions to imprinted regions in the mouse. The three candidates IGFBP1, IGFBP3 and EGFR were identified on the basis of their location in these regions and their known role in fetal growth. However, this study has found no evidence for imprinting of any of these genes.

During this investigation MEST became the first imprinted gene on chromosome 7 to be described. This gene has attracted much interest as a candidate for SRS. However, work done in this study and by Riesewijk et al. (1998) has failed to provide evidence to support a major role for MEST in the disorder. Even more recently another potentially imprinted gene, GRB10, has been identified as a good candidate. Although the results described have failed to provide evidence for a role for GRB10 in SRS, investigation into
its potential involvement is ongoing. In addition, since imprinted genes are commonly found in clusters it seems likely that other candidates will be identified by mapping the regions surrounding both \textit{MEST} and \textit{GRB10}. The region surrounding and including \textit{GRB10} is now of particular interest following the observation of 7p11.2-p12 duplication in one patient with characteristic features of SRS.

It is probable that several different molecular abnormalities underlie the SRS phenotype which is clinically heterogeneous. This study has focused exclusively on chromosome 7. However, other chromosomes, including 15 and 17, have been implicated and regions elsewhere within the genome may yet prove to be involved in significant numbers of patients. Those SRS patients with mUPD7 included in this study all had mild facial features and no evidence of asymmetry. It could therefore be argued that the association of mUPD7 and SRS is merely due to the resulting prenatal growth retardation. However, other patients with mUPD7 and classical features of SRS have been described by other groups (Eggermann \textit{et al.}, 1997). In addition, demonstration during this study of 7p11.2-p12 duplication in one patient with characteristic features of SRS has provided further support for involvement of chromosome 7 in this condition. It will be interesting to see whether further patients with abnormalities of this chromosome will now be identified.

SRS may arise as a result of several different disruptions within a single pathway of growth control. Identification of the gene(s) on chromosome 7 responsible in a proportion of cases, may suggest other, functionally related genes as candidates for SRS. More generally, this work is expected to provide valuable insights into the molecular mechanisms underlying fetal growth retardation.
9.5 Future Work

As has been discussed, the results of this study indicate that IGFBP1, IGFBP3, EGFR and MEST are all unlikely to be involved in SRS. However, despite negative findings so far, it remains possible that GRB10 plays a role in this disorder. The observation of 7p11.2-p12 duplication in one SRS patient suggests that one or more gene(s) within this region are responsible for the phenotype. Future work will concentrate on GRB10 and other potentially imprinted genes within the surrounding region.

The imprinting status of GRB10 in man will be determined. Essentially the same approach can be taken as was used to investigate IGFBP1, IGFBP3 and EGFR in this study. A transcribed polymorphism within the 3’ UTR of the gene has already been identified by direct sequencing of this region (M. Hitchins). This will be used to examine parental allele expression of GRB10 in both normal fetal tissues and cell lines from heterozygous individuals. Evidence for imprinting of the gene will strengthen the case for its involvement in SRS.

If monoallelic expression of GRB10 is demonstrated in adult cell lines, it will be interesting to compare expression levels of the gene in samples from both mUPD7 and non-UPD SRS patients with those in normal controls. If GRB10 is maternally expressed in cell lines then mUPD7 samples should over-express the gene. Increased expression in non-UPD patients would suggest loss of imprinting in SRS, as has been seen for IGF2 in BWS. This possibility could also be investigated directly by determining GRB10 imprinting status in heterozygous, non-UPD SRS patients.

The parental origin of the duplication in patient 49 is currently being examined. Unfortunately, investigation of parental alleles using a polymorphic dinucleotide repeat within GRB10 (Angrist et al., 1998) proved uninformative. This work will be continued using other microsatellite markers located within the duplication. The extent and orientation of the duplication also needs to be defined. Proximal and distal breakpoints can be mapped by FISH using cosmid and PAC probes from the region around GRB10. In the first instance cosmid clones containing IGFBP1, IGFBP3 and EGFR will be used. Additional probes are available from Dr S. Sherer at the Hospital for Sick Children, Toronto. Two-colour interphase FISH with two probes can be used to determine whether the duplication lies in an inverse or tandem orientation.
Preliminary work has shown that $GRB10$ replicates asynchronously in common with other imprinted genes (D. Monk). Interphase FISH of cells arrested in S phase, using probes from a PAC contig around $GRB10$ will be used to determine the extent of this asynchronously replicating region. Efforts will then be made to identify imprinted genes within the region of interest, initially by determining the imprinting status of expressed sequenced tags (ESTs) mapping to the region. Although, mUPD7 cell lines could theoretically be used to detect paternal-specific expression, problems experienced in this study with loss of imprinting in cell lines suggest that this approach is not reliable. Similar difficulties are likely to be experienced interpreting expression in cell lines from pUPD7 patients. Moreover, samples from pUPD7 individuals are not currently available to us. Parental allele expression will therefore be determined directly in fetal tissues using polymorphisms identified within the ESTs. Another cluster of imprinted genes is also thought likely to exist around $MEST$. Similar investigation of this region will aim to identify additional imprinted candidates.

Any imprinted genes identified will be treated as candidates for SRS and their role in the disorder investigated. Given the evidence for genetic heterogeneity in SRS it is likely that multiple mechanisms involving several genes are responsible for the phenotype. If one or more imprinted genes(s) on chromosome 7 can be implicated in the disorder, it may be possible to identify other genes within the same biochemical pathway as candidates for SRS.
Summary

- Maternal UPD7 has been found in approximately 7% of SRS and a region of common isodisomy ruled out, suggesting that one or more imprinted gene(s) on chromosome 7 are involved in SRS. The role of five candidates (IGFBP1, IGFBP3, EGFR, MEST and GRB10) has been investigated.

- Since the imprinting status of IGFBP1, IGFBP3 and EGFR was previously undescribed, their parental allele expression was studied in both normal first and second trimester fetal tissues and mUPD7 patients. No evidence was found for monoallelic expression of any of these three genes. Although recent reports of tissue and stage-specific imprinting make it difficult to completely rule out imprinting of these genes, it seems unlikely that these genes play a role in SRS.

- The role of the paternally expressed, imprinted gene MEST was investigated using a methylation-specific PCR assay specifically designed to detect differential methylation of its 5' CpG island. Normal allelic methylation patterns were found in 46 non-UPD SRS patients. These findings support those described in a study by Riesewijk et al. (1997) in which methylation was analysed by Southern hybridisation. The evidence suggests that MEST does not have a major role in SRS.

- Loss of imprinting of MEST was demonstrated in both lymphocytes and transformed lymphoblasts from normal adult controls. Expression of the gene was also detected in mUPD7 fibroblasts. It is hypothesised that a low level of basal transcription from the 'silent' maternal allele is detectable at the sensitivity required to detect transcription of MEST in cell lines. In one of two normal individuals investigated somatic allele switch in MEST expression was observed for the first time. These results illustrate the limitations of using patient cell lines to investigate candidate genes in imprinting disorders.
A novel 7p11.2-p12 duplication has been described in a patient with characteristic features of SRS, suggesting that the gene(s) on chromosome 7 responsible for the SRS phenotype lies within this region.

The growth suppressing gene GRB10 maps to 7p11.2-p12 and has a maternally expressed homologue in the mouse (Grb10/Megl). Increased dosage of this gene has been demonstrated in the patient with 7p11.2-p12 duplication but not in 36 other non-UPD SRS patients investigated. Although no evidence was found for a role for GRB10 via duplication of the gene in this SRS cohort, further work is needed to determine whether GRB10 is involved in the disorder. Alternatively, other gene(s) within the region surrounding GRB10 may be imprinted and responsible for the SRS phenotype.
References


References


Beechey CV, Cattanach BM. Genetic imprinting map. Mouse Genome 1997;95:54-55.

Beechey CV, Cattanach BM. MRC Mammalian Genetics Unit, Harwell, Oxfordshire. World Wide Web Site - Mouse Imprinting Data and References 1998;
URL: http://www.mgu.har.mrc.ac.uk/imprinting/implink.html.


References


References


Chu C-S, Trapnell BC, Curr GST, Cutting GR, Crystal RG. Genetic basis of variable exon skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nat Genet 1993;3:151-156.


References


References


Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methyl cytosine residues in individual DNA strands. Proc Natl Acad Sci USA 1992;89:1827-1831.


References
References


Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, Winter PD. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 1991;303:1019-1022.


References


221
References


References


Lalande M. In and around SNRPN. Nat Genet 1994;8:5-7.


References


Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 1993;75:59-72.


Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA, Lee DC. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev 1994;8:399-413.


References


Miyoshi O, Kondoh T, Taneda H, Otsuka K, Matsumoto T, Niikawa N. 47,XX,UPD(7)mat,+r(7)pat/46,XX,UPD(7)mat mosaicism in a girl with Silver-


O'Neill TJ, Rose DW, Pillay TS, Hotta K, Olefsky JM, Gustafson TA. Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like
References


Qian N, Frank D, O'Keefe D, Dao D, Zhao L, Yuan L, Wang Q, Keating M, Walsh C, Tycko B. The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51, implicated in Fas expression and apoptosis. Hum Mol Genet 1997;6:2021-2029.


References


230


References


232
References

Tebbs CA, Cumberland PFT, Pratten MK. Inhibition of the epidermal growth factor receptor during organogenesis of the rat embryo: a role in growth retardation? 51st Meeting of the Developmental Pathology Society: Programme and Abstracts 1996:3.3.


Varmuza S, Mann M. Genomic imprinting- defusing the ovarian time bomb. Trends Genet 1994;10:118-123.
References


References


References


Appendix 1.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>DOB</th>
<th>Sex</th>
<th>B.Wt. &gt; -2 SD</th>
<th>Classic face</th>
<th>Asymmetry</th>
<th>Clinodactyly</th>
<th>GH Tx</th>
<th>Pat. age</th>
<th>Mat. Age</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23/3/88</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>37</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31/7/90</td>
<td>M</td>
<td>3.11</td>
<td></td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>44</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16/5/91</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>25</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2/2/94</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>30</td>
<td>29</td>
<td></td>
<td>mUPD7</td>
</tr>
<tr>
<td>5</td>
<td>1/9/94</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>41</td>
<td>40</td>
<td></td>
<td>mUPD7; (1)</td>
</tr>
<tr>
<td>6</td>
<td>21/6/83</td>
<td>F</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>30</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>22/10/81</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>34</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>23/11/85</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>33</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3/12/81</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>30</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>14/1/84</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>34</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>16/11/60</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>35</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>27/8/84</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>31</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>12/4/88</td>
<td>F</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>21</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>21/1/91</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>29</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>17/6/88</td>
<td>F</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>33</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5/11/86</td>
<td>F</td>
<td>2.58</td>
<td></td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>24</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>19/1/85</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>31</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>10/8/74</td>
<td>F</td>
<td>2.68</td>
<td></td>
<td></td>
<td>Y</td>
<td>31</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>26/5/94</td>
<td>F</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>32</td>
<td>28</td>
<td></td>
<td>mUPD7</td>
</tr>
<tr>
<td>20</td>
<td>22/1/83</td>
<td>F</td>
<td>2.89</td>
<td></td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>NK</td>
<td>23</td>
<td>Non-paternity</td>
</tr>
<tr>
<td>21</td>
<td>18/3/93</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>NK</td>
<td>30</td>
<td>30</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>22</td>
<td>11/12/74</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>NK</td>
<td>30</td>
<td>22</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>23</td>
<td>8/12/93</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>27</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>14/8/87</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>31</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>23/3/88</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>30</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>20/5/92</td>
<td>M</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>26</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>13/1/68</td>
<td>F</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
<td>33</td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GH Tx: growth hormone treatment
NK: not known

Referring clinician (where not examined by Dr S. Price):
(1) Dr R. Newbury-Ecob, Bristol Royal Hospital for Sick Children
(2) Dr C. Garrett, Kennedy-Galton Centre, Northwick Park Hospital, Harrow
## Appendix 1 (cont.). Clinical details of patients in SRS cohort

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>DOB</th>
<th>Sex</th>
<th>B.Wt. &gt; -2 SD</th>
<th>Classic face</th>
<th>Asymmetry</th>
<th>Clinodactyly</th>
<th>GH Tx</th>
<th>Pat. age</th>
<th>Mat. Age</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>16/6/83</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>41</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>17/12/60</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>29/10/90</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>sm5</td>
<td>Y</td>
<td>Y</td>
<td>31</td>
<td>31</td>
<td>mUPD7</td>
</tr>
<tr>
<td>31</td>
<td>18/11/89</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>NK</td>
<td>NK</td>
<td>29</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>15/12/82</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>sm5</td>
<td>Y</td>
<td>Y</td>
<td>28</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>28/9/90</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>33</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>27/5/92</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>29</td>
<td>26</td>
<td>(4)</td>
</tr>
<tr>
<td>35</td>
<td>12/11/88</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>29</td>
<td>26</td>
<td>(4)</td>
</tr>
<tr>
<td>36</td>
<td>13/3/94</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>24</td>
<td>21</td>
<td>(5)</td>
</tr>
<tr>
<td>37</td>
<td>14/7/95</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>20/6/79</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>NK</td>
<td>NK</td>
<td>(6)</td>
</tr>
<tr>
<td>39</td>
<td>2/4/81</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>26</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>31/1/77</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>18</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>5/7/84</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>29</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>1/10/90</td>
<td>F</td>
<td>2.72</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>34</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>31/8/89</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>34</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>24/9/86</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>23</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>7/10/93</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>27</td>
<td>26</td>
<td>(7)</td>
</tr>
<tr>
<td>46</td>
<td>23/9/89</td>
<td>F</td>
<td>2.78</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>34</td>
<td>34</td>
<td>mUPD7</td>
</tr>
<tr>
<td>47</td>
<td>1/6/90</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>35</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1/2/84</td>
<td>M</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>35</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>10/2/93</td>
<td>F</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>30</td>
<td>31</td>
<td>(8)</td>
</tr>
</tbody>
</table>

GH Tx: growth hormone treatment
NK: not known
sm5: small fifth finger

Referring clinician (where not examined by Dr S. Price):
(3) Dr J. Patel, Addenbrooke’s Hospital, Cambridge
(4) Dr F. J. Gareis, Children’s Hospital, Oakland, California
(5) Dr L. Brueton, Kennedy-Galton Centre, Northwick Park Hospital, Harrow
(6) Professor M. A. Preece, Great Ormond Street Hospital, London
(7) Dr M. Barrow, Leicester Royal Infirmary
(8) Dr V. Proud, Children’s Hospital of the King’s Daughters, Norfolk, Virginia
Appendix 2. Family pedigree for patient 49

N  Normal phenotype

Short stature

Height > 2 SD below mean

Proband with SRS phenotype
### Appendix 3a.

**GRB10 upper band: A6121-1 band intensities in SRS cohort**

<table>
<thead>
<tr>
<th>Patient</th>
<th>1st reading Filter 1</th>
<th>2nd reading Filter 1</th>
<th>1st reading Filter 2</th>
<th>2nd reading Filter 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.73</td>
<td>0.67</td>
<td>0.55</td>
<td>0.59</td>
<td>0.63</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
<td>0.72</td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>3</td>
<td>1.11</td>
<td>0.97</td>
<td></td>
<td></td>
<td>1.04</td>
</tr>
<tr>
<td>6</td>
<td>1.12</td>
<td>0.95</td>
<td></td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>8</td>
<td>0.96</td>
<td>0.81</td>
<td>0.95</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td>9</td>
<td>1.22</td>
<td>1.21</td>
<td></td>
<td></td>
<td>1.21</td>
</tr>
<tr>
<td>10</td>
<td>1.22</td>
<td>1.09</td>
<td></td>
<td></td>
<td>1.15</td>
</tr>
<tr>
<td>11</td>
<td>1.28</td>
<td>1.27</td>
<td></td>
<td></td>
<td>1.28</td>
</tr>
<tr>
<td>12</td>
<td>1.05</td>
<td>1.04</td>
<td></td>
<td></td>
<td>1.05</td>
</tr>
<tr>
<td>13</td>
<td>1.08</td>
<td>0.73</td>
<td></td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>14</td>
<td>0.98</td>
<td>0.96</td>
<td>0.64</td>
<td>1.09</td>
<td>0.92</td>
</tr>
<tr>
<td>15</td>
<td>1.06</td>
<td>1.14</td>
<td></td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td>16</td>
<td>0.90</td>
<td>0.87</td>
<td></td>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td>17</td>
<td>1.14</td>
<td>1.11</td>
<td></td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>18</td>
<td>0.86</td>
<td>0.93</td>
<td></td>
<td></td>
<td>0.90</td>
</tr>
<tr>
<td>20</td>
<td>1.07</td>
<td>0.75</td>
<td></td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>21</td>
<td>1.20</td>
<td>ND</td>
<td></td>
<td></td>
<td>1.20</td>
</tr>
<tr>
<td>22</td>
<td>1.14</td>
<td>1.03</td>
<td></td>
<td></td>
<td>1.08</td>
</tr>
<tr>
<td>24</td>
<td>0.90</td>
<td>0.88</td>
<td></td>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td>26</td>
<td>1.05</td>
<td>1.36</td>
<td></td>
<td></td>
<td>1.21</td>
</tr>
<tr>
<td>27</td>
<td>1.14</td>
<td>1.26</td>
<td></td>
<td></td>
<td>1.20</td>
</tr>
<tr>
<td>29</td>
<td>1.03</td>
<td>1.21</td>
<td></td>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td>31</td>
<td>1.23</td>
<td>1.27</td>
<td></td>
<td></td>
<td>1.25</td>
</tr>
<tr>
<td>32</td>
<td>0.94</td>
<td>1.15</td>
<td>0.79</td>
<td>0.69</td>
<td>0.89</td>
</tr>
<tr>
<td>33</td>
<td>1.02</td>
<td>1.25</td>
<td></td>
<td></td>
<td>1.14</td>
</tr>
<tr>
<td>34</td>
<td>0.94</td>
<td>0.99</td>
<td></td>
<td></td>
<td>0.96</td>
</tr>
<tr>
<td>35</td>
<td>1.01</td>
<td>0.78</td>
<td></td>
<td></td>
<td>0.90</td>
</tr>
<tr>
<td>37</td>
<td>1.30</td>
<td>1.27</td>
<td>1.09</td>
<td></td>
<td>1.22</td>
</tr>
<tr>
<td>38</td>
<td>0.79</td>
<td>1.12</td>
<td></td>
<td></td>
<td>0.96</td>
</tr>
<tr>
<td>39</td>
<td>ND</td>
<td>0.99</td>
<td>1.28</td>
<td>1.03</td>
<td>1.10</td>
</tr>
<tr>
<td>40</td>
<td>1.12</td>
<td>1.14</td>
<td></td>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td>41</td>
<td>1.36</td>
<td>1.41</td>
<td>0.85</td>
<td>1.17</td>
<td>1.19</td>
</tr>
<tr>
<td>42</td>
<td>0.86</td>
<td>0.62</td>
<td>0.75</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>43</td>
<td>0.88</td>
<td>0.88</td>
<td></td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>44</td>
<td>1.04</td>
<td>1.21</td>
<td></td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>46</td>
<td>1.10</td>
<td>1.21</td>
<td></td>
<td></td>
<td>1.16</td>
</tr>
<tr>
<td>49</td>
<td>1.98</td>
<td>1.86</td>
<td>1.87</td>
<td>1.69</td>
<td>1.85</td>
</tr>
</tbody>
</table>

ND: Not done due to excess background hybridisation
Appendix 3b.

**GRB10 lower band: A6121-1 band intensities in SRS cohort**

<table>
<thead>
<tr>
<th>Patient</th>
<th>1st reading Filter 1</th>
<th>2nd reading Filter 1</th>
<th>1st reading Filter 2</th>
<th>2nd reading Filter 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.14</td>
<td>1.07</td>
<td>1.00</td>
<td>1.13</td>
<td>1.09</td>
</tr>
<tr>
<td>2</td>
<td>1.04</td>
<td>0.88</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.12</td>
<td>0.34</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.86</td>
<td>0.87</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.30</td>
<td>1.36</td>
<td>1.09</td>
<td>0.97</td>
<td>1.18</td>
</tr>
<tr>
<td>9</td>
<td>0.98</td>
<td>0.92</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.86</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.15</td>
<td>1.00</td>
<td>1.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.78</td>
<td>0.94</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.10</td>
<td>1.06</td>
<td>1.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.36</td>
<td>1.43</td>
<td>1.25</td>
<td>1.15</td>
<td>1.29</td>
</tr>
<tr>
<td>15</td>
<td>0.87</td>
<td>0.84</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.16</td>
<td>1.02</td>
<td>1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.80</td>
<td>0.78</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.02</td>
<td>0.95</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.16</td>
<td>1.21</td>
<td>1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1.25</td>
<td>ND</td>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.93</td>
<td>1.00</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1.19</td>
<td>1.16</td>
<td>1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>0.83</td>
<td>1.00</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>0.99</td>
<td>0.96</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>0.89</td>
<td>1.16</td>
<td>1.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>0.98</td>
<td>1.13</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>1.25</td>
<td>1.46</td>
<td>1.03</td>
<td>0.69</td>
<td>1.10</td>
</tr>
<tr>
<td>33</td>
<td>0.97</td>
<td>0.98</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>1.03</td>
<td>1.19</td>
<td>1.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>1.40</td>
<td>1.12</td>
<td>0.75</td>
<td>0.88</td>
<td>1.03</td>
</tr>
<tr>
<td>38</td>
<td>1.19</td>
<td>0.70</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>ND</td>
<td>0.87</td>
<td>1.25</td>
<td>0.85</td>
<td>0.99</td>
</tr>
<tr>
<td>40</td>
<td>0.84</td>
<td>ND</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>1.36</td>
<td>1.44</td>
<td>0.67</td>
<td>1.13</td>
<td>1.15</td>
</tr>
<tr>
<td>42</td>
<td>1.09</td>
<td>0.65</td>
<td>0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>0.95</td>
<td>0.91</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>0.87</td>
<td>1.11</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>0.92</td>
<td>1.04</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>1.44</td>
<td>1.36</td>
<td>1.51</td>
<td>1.44</td>
<td>1.44</td>
</tr>
</tbody>
</table>

ND: Not done due to excess background hybridisation.
Appendix 3c.

*GRB10*: A6121-1 band intensities in family of patient 49

<table>
<thead>
<tr>
<th>UPPER BAND</th>
<th>1st reading</th>
<th>2nd reading</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grandmother</td>
<td>0.91</td>
<td>1.00</td>
<td>0.96</td>
</tr>
<tr>
<td>Grandfather</td>
<td>0.98</td>
<td>0.96</td>
<td>0.97</td>
</tr>
<tr>
<td>Mother</td>
<td>0.84</td>
<td>0.97</td>
<td>0.91</td>
</tr>
<tr>
<td>Father</td>
<td>0.93</td>
<td>0.95</td>
<td>0.94</td>
</tr>
<tr>
<td>Sister</td>
<td>0.81</td>
<td>0.97</td>
<td>0.89</td>
</tr>
<tr>
<td>Patient 49</td>
<td>1.30</td>
<td>1.35</td>
<td>1.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LOWER BAND</th>
<th>1st reading</th>
<th>2nd reading</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grandmother</td>
<td>0.89</td>
<td>0.85</td>
<td>0.87</td>
</tr>
<tr>
<td>Grandfather</td>
<td>0.92</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>Mother</td>
<td>0.95</td>
<td>0.86</td>
<td>0.91</td>
</tr>
<tr>
<td>Father</td>
<td>0.84</td>
<td>0.88</td>
<td>0.86</td>
</tr>
<tr>
<td>Sister</td>
<td>0.89</td>
<td>0.99</td>
<td>0.94</td>
</tr>
<tr>
<td>Patient 49</td>
<td>1.36</td>
<td>1.31</td>
<td>1.34</td>
</tr>
</tbody>
</table>