Human GRB10 is imprinted and expressed from the paternal and maternal allele in a highly tissue- and isoform-specific fashion

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As part of a systematic screen for novel imprinted genes of human chromosome 7 we have investigated GRB10, which belongs to a small family of adapter proteins, known to interact with a number of receptor tyrosine kinases and signalling molecules. Upon allele-specific transcription analysis involving multiple distinct splice variants in various fetal tissues, we found that human GRB10 is imprinted in a highly isoform- and tissue-specific manner. In fetal brains, most variants are transcribed exclusively from the paternal allele. Imprinted expression in this tissue is not accompanied by allele-specific methylation of the most 5′ CpG island. In skeletal muscle, one GRB10 isoform, γ1, is expressed from the maternal allele alone, whereas in numerous other fetal tissues, all GRB10 splice variants are transcribed from both parental alleles. A remarkable finding is paternal-specific expression of GRB10 in the human fetal brain, since, in the mouse, this gene is transcribed exclusively from the maternal allele. To our knowledge, this is the first example of a gene that is oppositely imprinted in mouse and human.

INTRODUCTION

In mammals, both parental copies of most autosomal genes are expressed. For a small number of genes, however, either the maternally derived or the paternally derived gene copy is transcribed. To date, nearly 30 genes are known to function in the regulation of growth and development, including GRB10, IGFBP1 and IGFBP3 (20,21). The latter two are not imprinted and are therefore unlikely to play a role in SRS (22,23). GRB10 codes for growth factor receptor-binding proteins that interact with insulin receptors and insulin-like growth-factor receptors. Overexpression of at least some GRB10 isoforms inhibits tyrosine kinase activity and results in growth suppression. If imprinted and paternally silenced, GRB10 would be the strongest candidate for the growth restriction in mUPD7 patients.

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In contrast to the mouse, paternal uniparental disomy for human chromosome 7 (pUPD7) does not result in prenatal overgrowth. Uniparental isodisomy for paternal 7p and maternal 7q was reported in a child with growth retardation (24). Paternal UPD7 was found in a patient with postnatal growth retardation, complete situs inversus and immotile cilia (25) but also in a patient with normal body size and no clinically detectable abnormalities apart from congenital chloride diarrhoea (CLD) and mild high-frequency hearing loss (26). Here we report on the structural characterization and allelic expression analysis of human GRB10 as well as on the mutation scanning in 50 patients with SRS.

RESULTS

Cloning and sequence analysis of the human GRB10 gene

To date, four splice variants of the human GRB10 gene have been identified and are designated β, γ, ε and ζ (Fig. 1A) (http://www.bri.nrc.ca/thomasweb/grb7.html). As a prerequisite to assess GRB10’s imprinting status and its role in SRS, we performed a BLAST search with the longest isoform, GRB10c, as query sequence and identified the PAC clone RP4-537P9 (GenBank accession no. AC005153) that contains exons 1–10. To establish the genomic structures of the 5’ part and of the region downstream of exon 10, appropriate probes were hybridized to a chromosome 7-specific cosmid library. Subsequently, exon–intron boundaries for exons 10–16 were determined from cosmids ICRFc113M0546Q1 and ICRFc113A243Q1 (Fig. 1A) and are in good agreement with the results of Angrist et al. (27). Before we finished sequencing of all positive subclones from the cosmid ICRFc113A1917Q1, working draft sequences of the 5’ region became available in the High Throughput Genomic Sequences (HTGS) database. Sequence comparison of the GRB10 cDNA with the genomic clone RP5-898O18 (GenBank accession no. AC004920) identified two exons that comprise the 5’UTR of the GRB10c isoform (Fig. 1A). These exons are very likely not translated and were designated upstream non-coding (un)1 and un4. Recently, the 5’ read of a new GRB10 cDNA originating from a human testis cDNA library appeared in the GenBank database (accession no. AL041994) and was characterized further. Complete sequencing (GenBank accession no. AJ271366) showed that its translation start is identical to that of isoforms β and ζ. As in isoform ζ, exon 8 is present. In comparison with the GRB10ζ cDNA it contains three additional untranslated exons (un2, un3 and un4), two of which are unique and were not found in any other cDNA. Alignment of this cDNA with PAC clone RP5-898O18 showed that the first exon is part of a CpG island (CpG2 in Fig. 1A). Following RT–PCR this isoform was not detectable in RNA from various fetal tissues which may indicate that it is specifically expressed in testis, nor could we identify it by northern blot hybridizations of adult mRNAs other than testis using the unique exons un2 and un3 as probe (not shown). RT–PCR amplifications using primer sets 388–579, respectively, 756, 732–579, 755–756 and 733–579 (Fig. 1A) and direct sequencing of specific fragments showed that at least one GRB10β, γ or ζ isoform exists that contains exons un1 and un4 (Fig. 1B). In addition, we identified five new splice variants of the GRB10γ isoform (γ1–γ5) and very likely one novel isoform, which we designated GRB10α. This isoform contains exon un1, 20 bp of exon un4 and the last 155 bp of exon 3. Conceptual translation predicted the first initiation codon in exon 3, which is unique to this cDNA. In total, the human GRB10 gene contains at least 22 exons and encompasses >190 kb of genomic DNA which is much longer than the 47 kb estimated by Angrist et al. (27). Exon/intron sizes and boundaries of the new exons are given in Table 1.

Table 1. Exon/intron sizes and boundaries of the new exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Intron</th>
<th>Size (kb)</th>
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<td>1</td>
<td>165</td>
</tr>
<tr>
<td>1B</td>
<td>2</td>
<td>349</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>164</td>
</tr>
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<td>4</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>22</td>
</tr>
</tbody>
</table>

Isoform- and tissue-specific imprinted expression of human GRB10

To investigate whether GRB10 is imprinted in humans, we searched for polymorphisms within the gene. By SSCP, we detected bandshifts in PCR products of exons 3 and 12 which both resulted from a G/A substitution. In addition we screened fetal DNAs for the microsatellite polymorphism in intron 14 (27). Among a total of 17 fetal DNA samples examined, three DNAs were heterozygous for exon 3, three for exon 12 and two for intron 14. We then analysed relative expression levels from the two alleles for exon 12 by SSCP followed by direct sequencing and for intron 14 in denaturing polyacrylamide gels. Both amplifications examine total expression of all GRB10 variants irrespective of the great splicing variability present in the 5’ region of the gene. Remarkably, in all fetal brain samples transcripts of both exon 12 (Fig. 2A) and intron 14 (not shown) were detectable from only one parental allele and when compared with the bands of the parental DNA, it became apparent that the paternal allele is transcribed. All other fetal tissues examined for these polymorphisms, including heart, muscle, skin, trophoblast, kidney, adrenal, liver and lung (the latter four are not shown) showed biallelic expression (Fig. 2A).

Paternal-specific expression of GRB10 was unexpected since in the mouse exclusively monoallelic transcription of the maternal Grb10 allele was reported. We subsequently investigated the imprinting status of the mouse Meg1/Grb10 locus and performed allelic expression analysis on fetal (day 15) brain and adult brain, heart, intestine, muscle and liver RNAs isolated from Mus musculus x Mus spretus F1 animals. RT–PCR was performed using the primer set described by Miyoshi et al. (15) that amplifies a single nucleotide C/A polymorphism. By sequencing all RT–PCR products, we found exclusively transcripts from the maternal Grb10 allele (Fig. 3).

The variability in GRB10 splicing and the biallelic expression observed in most fetal tissues prompted us to determine allelic expression of each variant separately. Therefore, we used different primer sets (388 and 579 or 756, 732–579, 755–756 and 733–579) (Fig. 1A) for RT–PCR experiments on RNAs from fetuses, fibroblasts, lymphoblastoid cell lines and blood that were heterozygous for the exon 3 polymorphism. Specific fragments were separated in agarose gels, cDNAs were isolated from single bands and sequenced. The expression level of most GRB10 variants in lymphoblastoid cell lines and blood was too low for sequencing, except for GRB10ε which is biallelically expressed (not shown). In fibroblasts, GRB10β/ζ, γ1, γ2 and ε2 variants are transcribed. Sequence analysis of the latter two showed biallelic expression (not shown). Examples of GRB10 RNAs are presented in Figure 2B. In most fetal tissues each splice variant is expressed from both parental alleles. In contrast, all splice variants detectable in
fetal brain RNAs, except for γ2 and γ3, showed expression from the paternal allele. Apparently, biallelic expression of GRB10γ2 and γ3 could be detected only by analysing individual species and was undetectable in the expression analysis of exon 12 and intron 14. Another interesting finding was monoallelic, maternal-specific expression of the GRB10γ1 isoform in both skeletal muscle RNAs examined. A summary of all GRB10 splice variants and fetal tissues investigated for this polymorphism and the parental origin of the transcribed allele is given in Table 2. Expression of sense transcripts in fetal brain and muscle was confirmed by strand-specific RT–PCR experiments.

Methylation analysis of GRB10

Sequence analysis of the 5′ part of GRB10 showed a 1.3 kb GC-rich region with a G+C content of 75% and a high frequency of CpG dinucleotides. To study the methylation status of this region we first performed Southern blot hybridization of NarI, SmaI and BssHII digested genomic DNAs from peripheral blood, mUPD7 blood and pUPD7 lymphoblastoid cell line, using a probe that includes part of CpG1. Positive fragments of ~2 kb and 2.25 kb, respectively, are in good agreement with unmethylated restriction sites on both parental chromosomes (not shown). Subsequently, HindIII+MspI and HindIII+HpaII digested genomic DNAs from peripheral
DNAs showed a positive fragment of 650 bp, all other MspI blood, adult brain, stomach, lung and fetal brain and kidney one specific was absent in fetal brain DNA, indicating that methylation of HpaII was present in all fragments were too small to be detected. The same fragment of additional larger HpaII digested DNAs also showed a fragment of 1.21 kb that was absent in fetal brain DNA, indicating that methylation of one specific MspI–HpaII site varies between tissues. Absence of additional larger HpaII fragments suggests that MspI–HpaII sites of the CpG island are unmethylated on both parental chromosomes. To study parent-of-origin-specific methylation, normal blood DNA and cell line DNAs from patients with mUPD7 and pUPD7 were investigated using the same conditions (Fig. 4B). MspI digested maternal and paternal DNAs showed the expected fragment of 650 bp. HpaII digested maternal DNA showed only the 650 bp fragment and lacked the larger 1.21 kb fragment, that was visible in all blood DNA samples with biparental contribution. Conversely, HpaII digested paternal DNA showed only the 1.21 kb fragment and lacked the 650 bp fragment. The same blot was hybridized to a probe that encompasses only the differentially methylated I–MspI–II site is methylated in a parent-of-origin-specific manner. Sequence analysis of 50 kb interesting finding is the high number of mouse counterpart (15, this study). In this context, another individuals published by Angrist et al. (27). These results did not reveal any allelic association in the SRS patients, excluding the possibility of the patients being distally related.

**DISCUSSION**

We have investigated the human GRB10 gene and found that it is imprinted. Monoallelic expression is only detectable in fetal brain and skeletal muscle and is highly isoform specific. While in skeletal muscle maternal monoallelic expression is restricted to the GRB10ɛ splice variant, all but two GRB10 transcripts are monoallelically expressed in fetal brain. Remarkably, in this tissue allele-specific expression is confined to the paternally derived allele. Hence both paternal-specific expression and tissue-specific imprinting differ from the expression of the mouse counterpart (15, this study). In this context, another interesting finding is the high number of GRB10 splice variants, most of which contain a specific subset of 5′ untranslated exons. The mouse Grb10 gene produces only two alternatively spliced transcripts that have identical 5′ ends but differ in a short segment corresponding to aa 117–141 as well as in the 3′ UTR (28,29). It is likely that since the divergence of mouse and man, a more complex splicing pattern has evolved, allowing human GRB10 to escape from strong functional imprinting in most fetal tissues. To date, four Grb10 isoforms have been described in humans. We have found one GRB10 cDNA variant with the ORF starting in exon 3 (GRB10β), that could encode a new functional isoform.

Promoter-specific imprinting has been described for the human IGF2, GNAS1 and MEST genes (30–32) and only for Gnas is it conserved in the mouse (33). The distance between imprinted and non-imprinted promoter can be large as has been shown for Gnas (30–41 kb) (33). Paternal, maternal and biallelic expression of human GRB10 may be controlled in such a promoter-specific manner. Sequence analysis of 50 kb

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**Table 1. Exon/intron sizes and junctions of the 5′ region of GRB10**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Exon size (bp)</th>
<th>cDNA position (nt)</th>
<th>Intron size (kb)</th>
<th>3′splice site</th>
<th>5′splice site</th>
</tr>
</thead>
<tbody>
<tr>
<td>un1</td>
<td>509</td>
<td>1–509</td>
<td>9.87</td>
<td>GGCAGC</td>
<td>GAGCAT/tgacct</td>
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<tr>
<td>un2a</td>
<td>144</td>
<td>1–144</td>
<td>1.68</td>
<td>CAGCCC</td>
<td>CTCCAG/tgacce</td>
</tr>
<tr>
<td>un3a</td>
<td>110</td>
<td>145–254</td>
<td>24.57</td>
<td>ttccag/ACTTGG</td>
<td>CTTCCG/tgatgt</td>
</tr>
<tr>
<td>un4</td>
<td>170</td>
<td>529–698</td>
<td>23.53</td>
<td>ttccag/CTTTGG</td>
<td>TGACAG/tgauge</td>
</tr>
<tr>
<td>t^b</td>
<td>97</td>
<td>3244–3340</td>
<td>21.18</td>
<td>ttccag/GTCTGT</td>
<td>TACCAG/tgatgg</td>
</tr>
<tr>
<td>la</td>
<td>116</td>
<td>699–814</td>
<td>5.57</td>
<td>cttccag/GTTCTG</td>
<td>AGTAAG/tgact</td>
</tr>
<tr>
<td>n1b^a</td>
<td>184</td>
<td>30289–30472</td>
<td>1.23</td>
<td>cttccag/ATATTC</td>
<td>TCCAGGTgca</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>815–902</td>
<td>29.16</td>
<td>tttaag/GACAAG</td>
<td>ACCAGG/tgagg</td>
</tr>
</tbody>
</table>

A complete table including all intron sizes and two splice sites conflicting with Angrist et al. (27) can be requested from the authors. All cDNA positions refer to the GRB10ε sequence (GenBank accession no. D86962), except for the exons un2 and un3 which are only present in the newly identified cDNA clone derived from testis RNA (GenBank accession no. AJ271366) and the exons 1 and n1b which refer to the genomic PAC clone RP4-537P9 (GenBank accession no. AC005153).
upstream of the first untranslated exon of GRB10 predicted several promoters. However, neither additional exons nor new transcribed sequences could be identified that would prove our hypothesis or exclude promoter-specific imprinting of biallelically expressed GRB10 variants. A search for CpG-rich regions in the same 50 kb as well as in the complete GRB10 gene resulted only in the CpG1 and the testis-specific CpG2 islands found previously. CpG1 contains the first untranslated exon of GRB10. All CpGs examined in this region are unmethylated on both alleles, which is in good agreement with the observed biallelic expression. Attempts are currently being made to obtain new sequence information further upstream.

Alternatively, imprinted GRB10 expression could be controlled by neighbouring or overlapping transcripts that are specifically expressed in the human brain and skeletal muscle. The complex, oppositely imprinted and biallelic expression of human GRB10 is reminiscent of the patterns described for GNAS1/Gnas (31,33). However, GNAS1 does not contain untranslated exons and one of the distinct proteins, Nesp, is encoded entirely by the first imprinted exon. This exon is absent in other transcripts of this unit. Finally, cis- or trans-acting factors may also be involved in tissue- and isoform-specific imprinting of human GRB10.

Maternal duplication of proximal mouse chromosome 11 leads to prenatal growth retardation, whereas paternal duplication of the same region results in increased embryonic growth. The imprinted genes known to map to the duplicated segment are U2af1-rs and Grb10. Theoretically, both genes can be

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**Figure 2.** Allelic expression analysis of human GRB10 in five heterozygous fetuses. Arrows in DNAs point to the heterozygous status and in RNAs to biallelic and monoallelic GRB10 expression. (A) Sequence analysis of fetuses heterozygous for the exon 12 polymorphism. The primer set amplifies all GRB10 isoforms. Except for paternal-specific expression in fetal brain, all tissues show transcripts from both parental alleles. (B) Sequence analysis of PCR and RT–PCR products of fetuses heterozygous for the exon 3 polymorphism. Except for brain γ2 and muscle γ1, all RT–PCR products were amplified with exon 3 primers that do not discriminate between GRB10 isoforms. However, the same biallelic expression was detected in individual GRB10 splice variants. Fetal brain RNAs of fetuses 2 and 3 show a single peak at the respective position indicating transcription from only one parental allele. In contrast, predominant monoallelic expression from the opposite allele is evident for γ1 isoform in muscle RNAs.
regarded as candidates for these effects. However, homozygous U2af1-rs knockout mice do not have a conspicuous phenotype [Sunahara et al. (34) in ref. 15]. Human U2AFBPL maps to chromosome 5q23–q31 (35) and is biallelically transcribed in placenta (2). Therefore, several authors speculated that maternal-specific expression of human GRB10 and thus an excess of its product in mUPD7 patients could result in growth retardation and SRS. Although point mutations in human GRB10 may be in SRS, our finding of a non-imprinted gene in most fetal tissues together with absence of mutations in a large panel of SRS patients, both argue against a major role of this gene in the aetiology of SRS. However, we cannot exclude the possibility that alterations in regulatory elements or epigenotype contribute to the SRS phenotype. Likewise, another imprinted gene with growth-suppressing function may be involved. This is corroborated by the strict parental bias in the origin of all uniparental disomies and partial trisomies that are associated with SRS. Moreover, recent reports on two unrelated SRS patients who both have a 10 cM duplication of partial 7p on their maternally derived chromosome (20,21) and the report of Miyoshi et al. (36), who describe a girl with characteristic features of SRS and partial maternal trisomy for 7p13–q11 support this assumption. It is also possible that the phenotype in mUPD7 probands results from undetected trisomic mosaicism for chromosome 7p and increased dosage of a non-imprinted gene. Several mUPD7 cases have been shown to have arisen by trisomy rescue (37). Though less likely, involvement of an imprinted and paternally expressed gene from chromosome 7 cannot be excluded.

The biological role of Grb10 adapter proteins is not well understood and the effect of Grb10 on mitogenesis is discussed controversially in the literature. Liu and Roth (38) found an inhibitory effect on insulin receptor and IGF1R signalling while the studies of O’Neill et al. (39) gave opposite results. Overexpression of mouse GRB10α isoform has an inhibitory effect on IGF-I mediated mitogenesis but has no effect on insulin stimulation of cell proliferation (40). One possibility to explain these discrepant results is that the experiments were performed with different isoforms that might have unique, opposite functions. In addition, distinct Grb10 domains may help to define specific functions in different signalling pathways (41). Nothing is known about the importance of Grb10 protein in the brain during fetal development and other proteins of the Grb family may compensate for its absence in mUPD7 patients.

Identification of imprinted genes that map proximal and distal to GRB10 is in progress and should shed more light on their role in the aetiology of SRS.

**MATERIALS AND METHODS**

**Fetal and patient material**

For imprinting studies, DNA was extracted from blood samples, lymphoblastoid cell lines and tissues according to standard techniques. With informed consent of the parents, biopsies from therapeutic and spontaneous abortions (11–25 weeks’ gestation) were taken within 15 h of delivery. Maternal

<table>
<thead>
<tr>
<th>Fetus</th>
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<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
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<td>biall.</td>
<td>nd</td>
<td>biall.</td>
<td>patern.</td>
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<td>biall.</td>
<td>nd♂</td>
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<td>nd♀</td>
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<td>nd♀</td>
<td>nd♀</td>
<td>nd♀</td>
</tr>
</tbody>
</table>

*Isoform numbering is identical to that in Figure 1B. nd, allelic expression was not determined.

*RT–PCR products are not separable in agarose gel.

*Isoform is very poorly expressed.*

![Figure 3](Image)

**Figure 3.** Allele-specific expression analysis of mouse Megl/Grb10 on tissues from Mus musculus × Mus spretus F1 adult animals. All RNAs investigated show monoallelic expression from the maternally derived allele.
repeat typing. Nineteen unrelated probands of German origin were used as controls.

**Genomic structure of GRB10**

A human chromosome 7-specific cosmid library was hybridized with PCR products covering exon 10 and the first 250 bp of the 3′UTR as well as with a 446 bp long PCR product containing exon un1. Hybridizations were performed according to standard protocols. DNA from positive cosmids was isolated using QIAprep Spin Plasmid Kit (Qiagen, Hilden, Germany). EcoRI digested, phenol/chloroform purified and directly sequenced on ABI 377 automated sequencer using primers located in exons. The complete genomic structure of GRB10 was determined using BLAST (http://www3.ncbi.nlm.nih.gov/BLAST/).

**Identification of a testis-specific GRB10 splice variant**

The human EST (GenBank accession no. AL041994) originating from a testis cDNA library was identified by BLAST using part of the genomic clone RPS-898018 (GenBank accession no. AC004920) as query sequence. The corresponding cDNA clone (DKFZp434P0119) was obtained from the Resource Center of the German Human Genome Project (Berlin, Germany). DNA was isolated using QIAprep Spin Plasmid Kit (Qiagen), sequenced with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) and analysed on an ABI 377 automated sequencer. The complete sequence of this cDNA clone (accession no. AJ271366) is deposited in the EMBL database (http://www.ebi.ac.uk/embl/).

**Polymorphism and expression analysis of GRB10**

PCR was carried out on 100 ng of genomic DNA in a 25 µl reaction mixture using 10 pmol of each primer, 1.5 mM MgCl₂, 250 µM dNTPs and 0.5 U of Taq polymerase (Perkin Elmer, Weiterstadt, Germany). Amplifications with primers located in adjacent introns consisted of a total of 40 cycles. Exon 3 was amplified with primer set 5′-GCTCTTGTGCTTTTTCTGTG-3′, 5′-ACAGCTCTACATCTTTGAGG-3′, exon 12 with primer set 5′-CTGGCCCTTTTCCTTTTCCTTT-3′, 5′-GGCTAACCACCTTTT-3′ and the microsatellite repeat with primer set 5′-ACTTAAATGCGAAGCCTG-3′, 5′-GAATGAAAAACAGAAAACAAAC-3′.

Total RNA was isolated as described previously (4). First strand cDNA was synthesized from 250–500 ng of total RNA by MMLV reverse transcriptase (Gibco BRL, Eggenstein, Germany) for 90 min at 37 °C using random hexamer primers and was subjected to PCR with a total of 40 cycles under the same conditions as genomic DNA. Exon 3 was amplified with primers 5′-GCCCTGGTGAACGATGAA-3′ and 5′-CTGACAGGGAGATGTC-3′. For each reaction, a negative control without the addition of reverse transcriptase was amplified in parallel. For polymorphism analysis, non-radioactive SSCP was performed as described (12). PCR and RT–PCR products were analysed by direct sequencing on an ABI automated sequencer using corresponding primers.

PCR and RT–PCR for the microsatellite repeat in intron 14 were carried out on unspliced hnRNA in the presence of the [α³²P]dCTP (Amersham, Little Chalfont, UK). Products were

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**Figure 4.** Methylation analysis of the CpG1 region of human GRB10 by MspI-HpaII Southern blot analysis. (A) Genomic DNA from fetal brain and kidney, adult brain, stomach, liver, lung and peripheral blood was digested with HinIII-MspI (left lanes) and HindIII-HpaII (right lanes) and probed with the 446 bp PCR product covering exon un1 of the longest GRB10 isoform. Specific signal of 650 bp is visible in MspI and HpaII digests and of 1.2 kb in HpaII digests only (see detailed description in the text). (B) Lymphoblastoid cell line DNA from patients with mUPD7 and pUPD7 digested with HindIII, HindIII-MspI and HindIII-HpaII and hybridized to a 446 bp PCR product. MspI digested maternal and paternal DNAs show the expected fragment of 650 bp. In contrast, only the 1.21 kb fragment is present in paternal DNA after HpaII digestion, indicating methylation of one specific MspI-HpaII site on the paternal chromosome. (C) Schematic drawing of the 5′ CpG1 region and its methylation pattern in different tissues. The putative transcription start is indicated by the arrow. The open box represents the CpG1 region, bold lines at the top indicate probes and thin lines chromosomes. Circles represent methylation-sensitive restriction sites analysed (N, NruI; S, Smad; B, BssHII and unmarked are MspI–HpaII sites). H, HindIII site is indicated by arrowhead. Unmethylated CpGis are shown as open circles, methylated CpG as black circle.

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The studied SRS population consists of 50 patients, ascertained as part of ongoing molecular investigations on SRS. The patients were referred from the Children’s hospital, University of Tübingen. The diagnosis of SRS was based on the criteria according to Eggermann et al. (18). G-banding from peripheral blood lymphocyte cultures of the patients showed no chromosomal abnormalities. UPD7 was excluded by short tandem repeat typing. Nineteen unrelated probands of German origin were used as controls.

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separated on standard 6% denaturing polyacrylamide gel at 60 W for 3 h. Gels were dried and exposed to X-ray films overnight at room temperature.

Allele-specific expression analysis of individual GRB10 splice variants

RT–PCR amplifications were performed on total RNA from multiple tissues of three heterozygous fetuses for exon 3 polymorphism using primer sets 388–579 or 756, 755–756, 732–579 and 733–579. Primers used were: 388: 5′-CTCT-TCGCTTTGTGATT-3′; 579: 5′-CTGACAGCGAGAT- GTGC-3′; 755: 5′-CACAGCCACGTGATACC-3′; 756: 5′- CGGCGAGATGAGGTTC-3′; 732: 5′-GAAGGAAC- CCAATGACCTTA-3′; 733: 5′-CCCGGAACAAACA- CATCC-3′. First strand cDNA synthesis and RT–PCRs were performed as above, except for 3 mM MgCl2 included in the RT–PCR. Amplifications consisted of a total of 40 cycles at 95°C for 1 min, 59°C/59°C/61°C/60°C (for 388–579 or 756, 755–756, 732–579 and 733–579, respectively) for 1 min, 72°C for 1.5 min. For each isoform, at least two independent RT–PCR products were sequenced directly on an ABI automated sequencer using the corresponding primers. For strand-specific RT–PCRs, 2 μg of RNase-free DNase I (Gibco BRL)-treated total RNA was mixed with 0.6 pmol of primer 756 which anneals to the sense transcript of GRB10. Denaturation was at 70°C for 10 min and annealing at 61°C for 15 min. First-strand cDNA was synthesized at 42°C for 60 min in the presence of 200 U SuperScript II Reverse Transcriptase (Gibco BRL). The reaction was inactivated at 70°C for 15 min. 500 ng of cDNA was amplified with primer set 388–579.

Methylation analysis

For methylation analysis, genomic DNAs from tissues, blood and cell lines were digested with the appropriate restriction enzymes exactly as described previously (42). Fragments were separated by agarose gel electrophoresis, transferred to nylon membranes and hybridized under standard conditions.

Mutation screening by SSCP

Mutation screening included coding exons from all GRB10 isoforms and all exon–intron boundaries. PCR products were denatured in 95% formamide, 10 mM EDTA and resolved in 8% polyacrylamide gels (29:1 or 49:1, acrylamide/bisacryl- mide) containing 2% glyceraldehyde. Gels were run in 1x TBE at 15°C or 20°C for 3–5 h at 500 V or 16 h at 150 V. Part of the exons was analysed on 10% polyacrylamide gel (49:1) containing 5% glyceraldehyde. Gels were run at 60 V for 16 h or 180 V for 3 h at room temperature and at 70V for 16 h or 200 V for 3 h at 4°C. Silver staining was performed according to standard.

In order to assess allele sharing among SRS patients, we typed the recently published polymorphic microsatellite marker GRB10-(CA)n in intron 14 of the GRB10 gene (27). PCR was performed as described by Angrist et al. (27). Following electrophoresis on denaturing sequencing gels, the alleles were visualised by silver staining.

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