Maternal-Specific Methylation of the Human IGF2R Gene Is Not Accompanied by Allele-Specific Transcription


* Department of Human Genetics, University Hospital Nijmegen, Nijmegen, The Netherlands; † Department of Pediatrics, University Children's Hospital, Cincinnati, Ohio 45229; § Tissue Typing Laboratory, University Hospital Maastricht, Maastricht, The Netherlands; and § Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, D-14195 Berlin (Dahlem), Germany

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The human insulin-like growth factor type 2 receptor gene (IGF2R) is biallelically expressed in a variety of fetal and adult tissues. In contrast, the imprint mouse Igf2r gene is expressed exclusively from the maternally inherited chromosome. The mouse gene contains two CpG islands that are methylated in a parent-specific manner. Methylation of the CpG island in the promoter region occurs on the repressed paternal gene copy. Methylation of the CpG island in intron 2 is specific for the active maternal allele and may represent the primary imprint. Here, we have analyzed the human IGF2R gene to investigate whether these motifs and their parent-of-origin-specific epigenetic modifications have been conserved. As in the mouse, the human IGF2R gene was found to contain two CpG islands, one encompassing the transcription start site (CpG 1) and the other in the second intron (CpG 2). CpG 2 is hypermethylated on the maternal IGF2R allele. In contrast to the situation in the mouse, however, the human CpG 1 is completely unmethylated on both parental chromosomes. The human and mouse intronic CpG islands lack significant sequence homology, which suggests that DNA conformation plays a role in allele-specific methylation. © 1996 Academic Press, Inc.

INTRODUCTION

In mammals, certain genes show parent-specific expression in the embryo and in the adult because paternal and maternal genomes are differentially modified, or imprinted, during male and female gametogenesis. Aberrant imprinting of genes or the deviation of both homologous chromosomes from one parent (uniparental disomy) can be the cause of genetic disorders (Hall, 1990a,b; Clarke, 1990) and may also play a role in carcinogenesis (Wilkins, 1988; Sapienza, 1991, 1992; Reik, 1992; Feinberg, 1993; Mariman et al., 1995). At present, the nature of the primary imprint and the molecular basis by which genes are recognized as paternally or maternally derived are still unknown. First evidence that methylation plays an important role in genomic imprinting came from the observation that many transgenes become hypomethylated after passage through the male germline and hypermethylated after passage through the female germline (Surani et al., 1988). Analysis of four imprinted genes in the mouse, H19 (Bartolomei et al., 1993; Ferguson-Smith et al., 1993; Brandeis et al., 1993; Feil et al., 1994), insulin-like growth factor II (Igf2) (Sasaki et al., 1992; Brandeis et al., 1993; Feil et al., 1994), Igf2 receptor (Igf2r) (Stöger et al., 1993), and U2af1-rs1 (SP2) (Hatada et al., 1995), has revealed that regions within the gene or adjacent to it are methylated in a parent-specific manner. The mouse Igf2r gene is expressed exclusively from the maternally inherited chromosome in fetal and adult tissues (Barlow et al., 1991), with the exception of the head and the brain, where biallelic expression has been observed (Villar and Pedersen, 1994). During preimplantation development, both maternal and paternal Igf2r alleles are expressed (Latham et al., 1994; Szabó and Mann, 1995), indicating that silencing of the paternal allele is a secondary event. Characterization of the mouse Igf2r gene revealed the existence of two distinct CpG islands that show parental-origin-specific methylation differences. The first CpG island that includes the promoter (region 1) is methylated on the repressed paternal allele, whereas the intronic CpG island 27 kb downstream of the promoter (region 2) is methylated on the expressed maternal allele. The paternal-specific methylation pattern of region 1 is acquired after fertilization and is unchanged in the adult. Methylation of region 2 is inherited through the female gamete and is maintained during preimplantation development (Stöger et al., 1993). Therefore, it is thought to represent the primary imprinting signal. On the unmethylated paternal allele, this region may act as a...
powerful “gene silencer” by binding into an inhibitory factor (Stöger et al., 1993; Barlow, 1994). This model is supported by the recent finding that in the absence of DNA methylation, the otherwise active maternal Igf2r gene is repressed (Li et al., 1993).

We (Kalscheuer et al., 1993) and others (Ogawa et al., 1993) have recently shown that in contrast to the murine gene, the human Igf2r gene is expressed from both parental alleles in first and second trimester fetuses, although in a minority of individuals monoallelic expression of the maternal allele has been reported (Xu et al., 1993). This raises the question whether differential imprinting in humans and mice is due to methylation differences. As reported here, we have isolated the human Igf2r gene, identified two intragenic CpG islands, and found that one of these, but not the other, shows parent-specific methylation differences.

MATERIALS AND METHODS

YAC characterization and cosmid mapping. Screening of the ICI human YAC library was performed by PCR with primer pairs generated from the 5' and 3' ends of the human Igf2r cDNA sequence (Morgan et al., 1987) (5' UTR: 16-1 AGTGGAGGCGCTCCTACCT, I6-2 CTGCAAGCCTGGGAAACG; 3' UTR: I6-3 AATCAGGGCCAGGCTTCC, I6-4 GTTGAATCAAACGAAGCCG). One clone was positive both for sets of primers and was further analyzed. Yeast cultures were grown in selective synthetic medium lacking tryptophan and uracil. High-molecular-weight yeast DNA was prepared from agarose plugs using standard protocols (Mueller and Wold, 1989). For long-range restriction mapping, single and double digestions were performed on the plugs with enzymes cutting infrequently in human DNA and analyzed by pulsed-field gel electrophoresis. DNA fragments were separated in 1.5% agarose with running conditions at 150 V, 16°C for 38 h and transferred overnight by capillary blotting to GeneScreen Plus membranes. To visualize the specific YAC fragments, the filter was hybridized sequentially with probes generated from YAC right and left insert ends, 5' and 3' PCR fragments, and total human DNA. Chimerism of the clone was excluded by FISH analysis on human metaphase chromosomes exactly as described (Suijkerbuijk et al., 1993). The probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989). These probes, as well as the 5' and 3' PCR fragments of cosmid C12 were isolated by ligation-mediated PCR according to Mueller and Wold (1989).

Methylation analysis with restriction enzymes. Twenty micrograms of genomic DNA derived from blood, liver, and placenta was digested with 100 U HindIII (Gibco BRL) according to the manufacturer’s recommendations, extracted once with phenol/chloroform and once with chloroform, and precipitated with ethanol. The DNAs were divided into two portions and digested for 3 h with 50 U of either HpaII or MspI (Gibco BRL), followed by a digestion with another 50 U of the respective enzyme. The DNAs were then phenol extracted as described above and redigested with 50 U of HpaII or MspI for 3 h. For comparison of genomic and cosmid DNA digestion patterns, 10 μg genomic DNA and 1 μg cosmid DNA were digested with 50 or 5 U HindIII, respectively. After phenol extraction the DNAs were digested with methylation-sensitive enzymes (BstIII, NolI, NruI, PvuI, SmaI, and XhoI; Gibco BRL or New England Biolabs) as described above. Finally, the DNAs were ethanol precipitated and electrophoresed on a 1 to 1.5% agarose gel, transferred on a GeneScreen membrane, and hybridized with a 4.1-kb HindIII fragment comprising Cpg 1 and a 3-kb HindIII fragment specific for Cpg 2. Hybridization was performed at 65°C with random-prime labeled probes (Feinberg and Vogelstein, 1984). Filters were washed at 66°C with 2x SSC/0.1% SDS (15 min), 1x SSC/0.1% SDS (15 min), and 0.1x SSC/0.1% SDS (two times, each 15 min) successively. Autoradiograms were exposed for 2 h to 2 weeks.

Bisulfite modification, PCR amplification, and sequencing. Genomic DNA (10 μg) and cloned DNA (2 μg) were first digested with HindIII and then alkaline denatured in 0.3 N NaOH for 15 min at 37°C. Treatment with sodium bisulfite was essentially as described by Clark et al. (1994). Modified DNAs were desalted and concentrated using GeneClean (Bio 101), ethanol precipitated, and resuspended in 110 μl 100 mM Tris–HCl (pH 8.0), 1 mM EDTA. An aliquot of bisulfite-modified DNA was amplified by PCR under the following conditions: initial denaturation for 5 min at 95°C, 1 min at 94°C, 2 min at 63°C, and 3 min at 72°C. Amplification was performed for 35 cycles and then 6 min at 72°C. An aliquot was then reamplified using one additional internal primer. Cpg 1: 16-34 CCCAATCRAACCRCRCTCACCTCRAACTCC-CRT (944-977), 16-35 TTTGTATGTTGGGAAAGGYGGGTGGT-TGGTTT (1237-1204), 16-36 CRCRATCTAAACCCCRCTTAC (902-935), 16-37 TTTTATTTGATTGGATTTTGGATTTTGTTATGTG (447-480); 16-38 GAGTTTTTTTGGGTTTTTTGTATT-GTTTGG (447-480); 16-39 TATTTTAGTTTTTTAGTTTAGY-GTTTAG (447-480); 16-40 GAGTTTTTTTGGGTTTTTTGTATT-GTTTGG (447-480). Oligonucleotides (exon 1, ATGCGACTGC; exon 2, TGAGGAAGCATGATCTCAATCC; 3' UTR, GCCGCGCTCACCT; exon 2, CATGGGAAGCTGTTGATACC; exon 3, AATCAGGGCCAGGCTTCC; exon 4, ACTTCTGAGGACGGCAAGAGT; exon 5, TGAGGAAGCTGTTGATACC; exon 6, AATCAGGGCCAGGCTTCC) were phosphorylated using T4 poly­nucleotide kinase (Gibco BRL) and (γ-32P)PATP and purified using Sephadex G50 (Pharmacia). Hybridization was for 3 h at 50°C under standard conditions (Sambrook et al., 1989). Filters were washed once with 2x SSC and twice with 5x SSPE/0.3% SDS at 50°C.

DNA sequence analysis. EcoR1 fragments of cosmid C12 were subcloned into plasmid Bluescript or plasmid pT7T3. From these, clones containing Cpg 1 and Cpg 2 were further subcloned and sequenced using T7 and T3 primers and several Cpg 1- or Cpg 2-specific primers. Sequencing was performed using the T7 sequencing kit (Pharmacia), the T7 DyeDeoxy terminator cycle sequencing kit (Applied Biosystems), and the T7 track sequencing kit (Promega) in combination with terminal transferase as described by DeShazer et al. (1994). Homology analyses were performed using the program FASTA, and sequences were aligned using the programs BESTFIT and GAP. Cpg observed/expected ratios and C + G richness were calculated according to Gardiner-Garden and Froummer (1987), using the program CpGplot.
RESULTS

Isolation of a YAC Clone Containing the Human IGF2R Gene

To isolate a YAC clone containing the complete human IGF2R gene, we first generated STS primers using the published cDNA sequence of Morgan et al. (1987). These primers, amplifying 290 bp of the 5'-untranslated region (UTR) and 324 bp of the 3' end of the IGF2R gene, were used to screen the human ICI YAC library by PCR. Three clones were isolated, one of which turned out to contain a human insert of 215 kb comprising the complete IGF2R gene. As expected, FISH analysis on human metaphase chromosomes showed a hybridization signal at the telomeric region of the long arm of chromosome 6 (6q26) (not shown).

Single and double digestions of YAC DNA with restriction enzymes that cut infrequently in human DNA (BssHII, NotI, SalI, MluI, and SfiI), followed by pulsed-field gel electrophoresis and Southern hybridization with human DNA, yielded the long-range restriction map shown in Fig. 1A. In a second step, a cosmid library was established, and a complete cosmid contig was constructed. Oligonucleotide hybridizations of exon 1 and the 3' UTR to Southern blots of EcoRI-digested C12 and B12 cosmids enabled us to estimate the length of the gene as 140 kb and to determine its position within the YAC (Fig. 1A).

Two CpG Islands Are Present in the Human IGF2R Gene

An 8.5-kb EcoRI fragment containing the 5' part of the IGF2R gene was subcloned and partially sequenced. Sequence analysis comprised a 1.6-kb fragment centered around exon 1 and revealed the existence of a CpG island (Figs. 1B and 2A). This CpG island, termed CpG 1, spans the putative promoter region and extends into intron 1. The size of the promoter-associated CpG islands of the human and mouse IGF2R genes is conserved. Three E-box-like sequences and several Sp1 boxes but no TATA or CAAT boxes were found in the region of 700 bp immediately upstream to exon 1 (not shown). Sequence comparison of the 5' end of the human and mouse IGF2R genes revealed 71% homology between positions -700 and +455 bp of the human gene and positions -656 and +346 bp of the mouse gene (Accession Nos. X91875 and L06445). A similar degree of homology was found when the promoter region, exon 1, and intron 1 were analyzed separately.

Long-range restriction mapping revealed the presence of recognition sites for NotI and BssHII in a small region downstream from exon 2. A 6-kb EcoRI fragment of cosmid C12 (Fig. 1B) was subcloned, and sequence analysis of 3 kb surrounding both restriction sites con-
FIG. 2. Plots showing the G + C density (broken line) and CpG content (solid line) per 100 bp for a region of 1.8 kb encompassing CpG 1 (A) and a region of 3 kb encompassing CpG 2 (B). The data are expressed as the observed number of CpGs per 100 bp over the expected number of CpGs per 100 bp. The diagrams display that CpG 1 and CpG 2 contain core sequences of approximately 1 and 1.3 kb, respectively, that are more than 70% G + C. N, NotI, B, BssHII.
firmed the presence of a second 1.3-kb-long CpG island, termed CpG 2 (Fig. 2B). Following successive hybridizations of oligonucleotides specific for exons 1–4 to Southern blots of EcoRI-digested C12 DNA, we were able to localize CpG 2 within intron 2. Comparison of CpG 2 and region 2 of the mouse Igf2r gene with the program BESTFIT displayed sequence homology of 71% for a small segment of 38 bp starting at position 1457 in the sequenced DNA fragment, including CpG 2 and 1002 in region 2 (Accession Nos. X91880 and LO6446). Analysis for repetitive elements within CpG 2 showed four different tandem, direct repeats of 36, 38, 22, and 17 bp, respectively, with an overall homology of >70% (Fig. 5C), as well as a number of inverted repeats (not shown).

Despite the slightly different distances between CpG 1 and CpG 2 (i.e., 27 kb in the mouse versus 40 kb in humans), the positions of these CpG islands within the gene have been conserved.

Methylation Analysis of CpG 1 and 2

In the mouse Igf2r gene, partial methylation of CpG sites in regions 1 and 2 has been found by Southern blot analyses of genomic DNA digested with methylation-sensitive restriction enzymes (Stöger et al., 1993). To investigate methylation of the human gene, a 4.1-kb HindIII fragment comprising CpG 1 (probe 1, Fig. 3A) and a 3.0-kb HindIII fragment specific for CpG 2 (probe 2, Fig. 4A) were used for Southern analyses of MspI- or HpaII-digested human DNA isolated from peripheral blood. Following predigestion with HindIII, hybridization of MspI-digested control DNA with probe 1 revealed fragments of 1.8 and/or 1.7 kb (due to an MspI polymorphism), 0.8, and 0.4 kb (Fig. 3B, lane 1). Because of their small size, several other fragments could not be detected.

In HpaII-digested control DNA, the same probe showed one prominently hybridizing fragment of 2.6 kb (Fig. 3B, lane 2), suggesting that the two MspI/HpaII sites in intron 1, marked by M in Fig. 3A, are methylated, while other sites in this region are not. Fragments of 0.4 kb could be visualized only after longer exposure time (not shown).

The same Southern blot was used for methylation analysis of CpG 2. MspI digestion yielded bands at 2.1 and 0.4 kb (Fig. 4, lane 1) as well as several smaller fragments (not shown). HpaII-digested control DNA resulted in a similar banding pattern, but in addition, one prominent fragment of 3.0 kb was present (Fig. 4, lane 2), indicating that all MspI restriction sites of this fragment that carry CpG 2 are resistant to digestion. Therefore, some of the DNA appears to be completely methylated at all MspI recognition sites in CpG 2. Comparison of the methylation pattern of DNA isolated from peripheral blood, liver, and placenta all gave the same results (not shown). As an extension of this approach, other methylation-sensitive restriction en-

![FIG. 3. Methylation analysis of CpG 1. (A) The 4.1-kb HindIII (H) hybridization probe (probe 1) is shown, including CpG 1 and surrounding MspI and/or HpaII (M) sites; the asterisks refer to two HpaII sites. CpG 1 contains 22 MspI sites that are not depicted here. The order of 0.8, 1.8, and 1.7 + 0.1 kb fragments is not exactly determined. (B) Southern blot analysis of control DNA, paternal, and maternal UPD DNA, digested with HindIII and MspI or HpaII, after hybridization with the 4.1-kb HindIII fragment (probe 1) encompassing CpG 1. Weak signals at 3.0 and 2.1 kb are due to incomplete removal of the CpG 2 probe, which was applied first (Fig. 4). Zymolyases (BssHII, NotI, NruI, PvuI, SalI, Smal, and XhoI) were employed to compare restriction patterns in (potentially methylated) chromosomal DNA and (unmethylated) DNA from cosmids spanning CpG 1 and CpG 2, respectively. For CpG 1, banding patterns were identical in native and cloned DNA, which indicates that the respective CpGs are unmethylated in the genome. In contrast, a BssHII and a NotI restriction site in CpG2 were found to be partially methylated (not shown).

To study parental-allele-specific methylation in CpG 2, we analyzed DNA from peripheral blood of two pre-
Maternal-specific methylation of the human IGF2R

A

Maternal-specific methylation of the human IGF2R

B

DISCUSSION

We have previously shown that in contrast to the mouse, the human IGF2R gene is equally expressed from both paternal and maternal alleles in various tissues of first and second trimester fetuses, and this has since been confirmed in several additional cases. Furthermore, both unimamaternal and unipaternal disomy

\[ \text{CpG 1 is Completely Unmethylated on Both Parental IGF2R Alleles, and CpG 2 is Methylated in a Parent-Specific Manner} \]

Since the above findings apply only to CpGs of particular restriction sites, the genomic sequencing technique developed by Frommer et al. (1992) was employed to determine the overall degree of methylation in both CpG islands. In this method, sodium bisulfite quantitatively converts unmethylated cytosine to uracil. This chemical modification is the basis for a PCR-based assay in which only methylated cytosines yield positive signals in DNA sequence ladders. After chemical modification, PCR fragments were cloned and sequenced. Analyses were performed on DNA from normal controls, including those isolated from fetal tissues with proven biallelic expression and from the patients with paternal and maternal uniparental disomy. In addition, cloned DNA was modified and used as a control to assess the efficiency and fidelity of the bisulfite reaction and PCR amplifications. In all cases, our results showed that CpG 1 is hypomethylated on both parental chromosomes. Of the 138 CpGs in the promoter-associated CpG island, not a single one was methylated (not shown). In agreement with our previous restriction enzyme analysis, CpG 2 was found to be modified by methylation in a parent-specific manner. DNA clones derived from maternal UPD DNA were methylated in most CpGs, while those derived from paternal DNA were hypomethylated. Methylation patterns of individual clones from (non-UPD) control DNA showed a clear dichotomy, i.e., either almost complete methylation or methylation of only a few cytosine residues. Examples are shown in Figs. 5A and 5B. The methylation status of three independent clones from paternal and maternal UPD DNA is given in Fig. 5C.

Thus, our results clearly demonstrate that CpG 1 of the human IGF2R gene is unmethylated on both parental alleles and that CpG 2 is modified in a parent-of-origin-specific manner, with the maternal locus being hypermethylated and the paternal locus being hypomethylated.
for the human \textit{IGF2R} gene does not give rise to specific symptoms (Welch \textit{et al}., 1990; van den Berg-Loonen \textit{et al}., submitted for publication). Biallelic expression of human \textit{IGF2R} in various tissues was also shown by Ogawa \textit{et al}.
(1993) and Xu \textit{et al}.
(1993). However, in lung and placenta of two fetuses, Xu \textit{et al}.
found that transcription was exclusively maternal, suggesting that in a small proportion of the human population the \textit{IGF2R} gene may be monoallelically expressed. The expression pattern of the human \textit{IGF2R} gene at earlier stages of development is still unknown. In the mouse, both maternal and paternal \textit{Igf2r} alleles are expressed during preimplantation development (Latham \textit{et al}., 1994; Szabó and Mann, 1995), indicating that in this species, monoallelic expression is due to secondary inactivation of one \textit{Igf2r} gene copy. Along these lines, biallelic expression of the human \textit{IGF2R} gene might result from failing repression of the paternal allele.

There is ample evidence that parental allele-specific methylation plays a crucial role in genomic imprinting. The mouse \textit{Igf2} gene is hypermethylated on the expressed paternal allele (Sasaki \textit{et al}.
, 1992; Brandeis \textit{et al}., 1993; Feil \textit{et al}., 1994), and this methylation is required for expression (Li \textit{et al}., 1993). The closely linked and reciprocally imprinted mouse \textit{H19} gene is methylated specifically on the repressed paternal chromosome (Bartolomei \textit{et al}.
, 1993; Ferguson-Smith \textit{et al}.
, 1993; Brandeis \textit{et al}., 1993; Feil \textit{et al}., 1994). In addition, tissue-specific methylation, directly proportional to the transcriptional levels, was found (Feil \textit{et al}., 1994). Analogous to the situation in the mouse, allelic methylation differences are also present in the human \textit{IGF2} and \textit{H19} genes (Reik \textit{et al}.
, 1994; Zhang \textit{et al}.
, 1993). One of the best studied imprinted genes in the mouse is the \textit{Igf2r} gene. Two CpG-rich regions of the mouse \textit{Igf2r} gene are modified by methylation in a parent-of-origin-specific manner. Region 1, which contains the promoter, is methylated specifically on the repressed paternal allele. Methylation takes place during late development, probably after transcriptional repression of the gene. In the gametes and in early embryos, this region is unmethylated. In contrast, a second CpG island within intron 2 is specifically methylated on the active maternal allele. Methylation of this region is already present in the female gamete and remains unchanged during preimplantation development and in the adult. Maternal methylation of this region was proposed to represent the primary imprinting signal for the mouse \textit{Igf2r} gene (Stöger \textit{et al}., 1993; Barlow, 1994).

Here, we have shown that the human \textit{IGF2R} gene, too, contains CpG islands that are located at comparable positions. CpG 1 extends from the promoter region into intron 1, and CpG 2 is situated in intron 2. Sequence comparison of the murine and human 5' CpG islands displayed 73 and 78% homology for the promoter region and the analyzed part of intron 1, respectively. Detailed methylation analysis of all 138 CpGs across CpG 1 re-

**FIG. 5.** Methylation analysis of CpG 2 by genomic sequencing of individual maternal and paternal chromosomes and sequence analysis for direct repeats and homology to the mouse \textit{Igf2r} gene. Following sodium-bisulfite modification of control (genomic), paternal, and maternal UPD DNA, nested PCR was performed. PCR products were cloned and sequenced. (A) An example of the segment of CpG 2 amplified with the primer set 16-37/38 followed by 16-39/38. (B) The fragment amplified with the primer set 16-40/41 followed by 16-42/41 (see Materials and Methods). As demonstrated by bands in the G-lanes, all CpGs were found to be methylated (dots and arrowheads) in genomic control and maternal UPD DNA, whereas in paternal UPD DNA most cytosine residues of CpGs are converted, indicating the presence of only a few methylated Cs (arrowheads). Aliquots of genomic control and maternal UPD DNA from the same bisulfite reaction were found to be completely converted at all cytosines of CpGs in CpG 1 (not shown). (C) Allele-specific methylation of CpG 2, sequence analysis for direct repeats, and homology to region 2 of the mouse \textit{Igf2r} gene. The methylation data were compiled from a complete analysis of three individual sodium-bisulfite-modified paternal and maternal chromosomes. CpGs unmethylated in all three clones are shown as open circles; one of three methylated as 1/3 black; two of three methylated as 2/3 black; and all three methylated as black circles. The horizontal line represents the chromosome; the paternal methylation pattern is depicted above and the maternal pattern below this line. The nucleotides correspond to the positions of these fragments in the sequenced part of intron 2 (Accession No. X91880). Dots above the circles indicate \textit{Mspl} and/or \textit{HpaII} sites. N, \textit{NotI} and B, \textit{BssHII} restriction sites. The 38-bp fragment, 71% homologous to the mouse \textit{Igf2r} gene, is shown by the black bar. Direct repeats with an overall homology of >70% are displayed by boxes, and sequence relationships within the repeats are represented by identical patterns.
Indeed, analysis of CpG 2 showed the presence of a number of direct repeats that are specific for the human IGF2R gene. Thus, the human IGF2R gene resembles other imprinted genes as far as monoparental methylation and repeats are concerned, but it is not monoallelically expressed.

It is increasingly clear that imprinted genes are clustered in the genome. In the mouse, support for an imprinted region on proximal chromosome 17 has come from the recent report that the Mas proto-oncogene, located less than 300 kb apart from the Igf2r gene, is imprinted in a developmental- and tissue-specific manner (Villar and Pedersen, 1994). MAS, the human homolog of the mouse Mas gene, is also located in the vicinity of the IGF2R locus on the distal long arm of chromosome 6 (Rabin et al., 1987). The observation that paternal (Welch et al., 1990) and maternal UPD 6 (van den Berg-Loonen et al., submitted for publication) are compatible with normal human development supports our previous finding that the IGF2R gene is not imprinted (Kalscheuer et al., 1993) and raises the possibility that this holds for the MAS gene, too. Expression and methylation studies should soon clarify this point and may shed more light on the role of gene-specific and regional factors in the control of parent-specific expression.

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