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Conservation of a maternal-specific methylation signal at the human IGF2R locus

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The human IGF2R gene has been reported to be either biallelically or very rarely monoallelically expressed, in contrast to the maternally expressed mouse counterpart. We describe here an analysis of the 5′ portion of the human IGF2R gene and show that it contains a maternally methylated CpG island in the second intron. A similar maternally methylated intronic element has been proposed to be the imprinting box for the mouse gene and although the relevance of this element has yet to be directly demonstrated, methylation has been reported to be essential to maintain allele-specific expression of imprinted genes. Allelic expression analysis of human IGF2R in 70 lymphoblastoid cell lines identified only one line showing monoallelic expression. Thus, in this tissue monoparental methylation of the IGF2R gene does not correlate with allele-specific expression. We also confirm here that the human IGF2R gene is located in an asynchronously replicating chromosomal region, as are all other imprinted genes so far analyzed. The mouse and human IGF2R intronic CpG islands both contain numerous large direct repeats that are methylated following maternal, but not paternal, transmittance. Thus features that attract maternal-specific methylation are conserved between the mouse and human genes. Since these intronic CpG islands share organizational rather than sequence homology, this suggests that secondary DNA structure may play a role in attracting a maternal methylation imprint.

INTRODUCTION

Gametic imprinting is a multi-step process that can render the maternal and paternal alleles of a gene functionally non-equivalent in diploid cells (1,2). Thirteen imprinted genes have been identified in mammals, nine of these show exclusive paternal expression, three are maternally expressed and the orientation of one is not yet known (3–6). Monoallelic expression at imprinted loci is not always permanently established in the early embryo, instead it can be dynamically regulated. Imprinted genes can be either biallelically or monoallelically expressed, depending on cell-type and developmental stage (7,8) or pathological state (9). While the molecular mechanism of imprinting is not yet understood, it is predicted that imprinted genes will inherit an 'imprinting signal' from one parent that generates an inequality between parental alleles which is thought to result in monoallelic expression. The identification of monoparental methylation as a unique marker of all the imprinted genes so far examined, has suggested DNA methylation could be the imprinting signal (10). This suggestion is supported by the demonstration that imprinted gene expression becomes de-regulated, such that allele-specificity is lost and genes are either silenced or expressed from both homologues, in mutant mice deficient for DNA methyltransferase (11). However, while this experiment supports a role for methylation in maintaining allele-specific expression, it does not directly identify monoparental methylation as the initiating imprinting signal. Nor does it identify methylation as the key distinguishing mark of an imprinted gene, since non-imprinted genes have not yet been rigorously examined for this type of modification.

In the mouse, the imprinted Igf2/M6pr (insulin-like growth factor type 2/mannose 6-phosphate receptor) gene is maternally expressed and inherits from the female gamete, a maternal-specific methylation mark that persists throughout development and has been proposed as the 'imprinting signal' for this gene. This methylation-imprinting signal is carried by an intronic CpG island that has thus been proposed to be an imprinting box for this gene (12). The relevance of maternal-specific methylation of the putative intronic imprinting box has not yet been experimentally tested. As part of our investigations into the significance of the putative mouse imprinting box, we have asked if this sequence is conserved and similarly modified in the human IGF2R locus (that also encodes the insulin-like growth factor type 2/mannose 6-phosphate receptor). The imprinting status of the human IGF2R gene has yet to be fully resolved. Biallelic expression, suggesting an absence of imprinting, has been demonstrated in studies of embryonic and adult tissues (13,14), but one study identified sporadic monoallelic expression (15) and raised the possibility that this locus was subject to polymorphic imprinting.

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We report here the isolation of 45 kb from the 5′ portion of the human IGF2R locus and the identification and characterization of the human homologue of the mouse Igf2/M6pr putative imprinting box. The human IGF2R homologous element is conserved in its intron location, sequence organization and similarly carries a maternal-specific methylation mark. The strong organizational similarity between the mouse and human maternally methylated elements coupled with the lack of obvious direct sequence homology, raises the possibility that DNA conformation rather than a sequence-specific motif may play a role in attracting maternal methylation in the gamete. The maternal-specific methylation mark of the human IGF2R gene is not translated into paternal repression in a large sample of lymphoblastoid cell lines. However, we confirm that the human IGF2R locus replicates asynchronously in the cell cycle, a feature shown by all imprinted or random-monoallelically expressed gene regions tested so far (16,17).

RESULTS

Genomic organization of the 5′ portion of human IGF2R

An *Eco*RI/*Bam*HI restriction map of the human IGF2R 5′ portion was constructed by enzyme digestion and Southern blot analysis of cosmids SBV and C06 and of P1 clone P1–17 (Fig. 1a). Exon containing fragments were identified using oligonucleotides designed from the human cDNA sequence (18,19) and were based on homology to mouse exon/intron junctions (20). The genomic organization of the first 45 kb of the human IGF2R gene is similar to the mouse locus, in that the first three exons (149, 140 and 125 bp respectively), are separated by large introns (20 and 21 kb respectively) with conserved splice donor and acceptor sites (GenBank accession nos X83699–X83702). However, the first three exons span 42 kb in the human, in contrast to 27 kb in the mouse locus. The promoters of both the human and the mouse gene are located

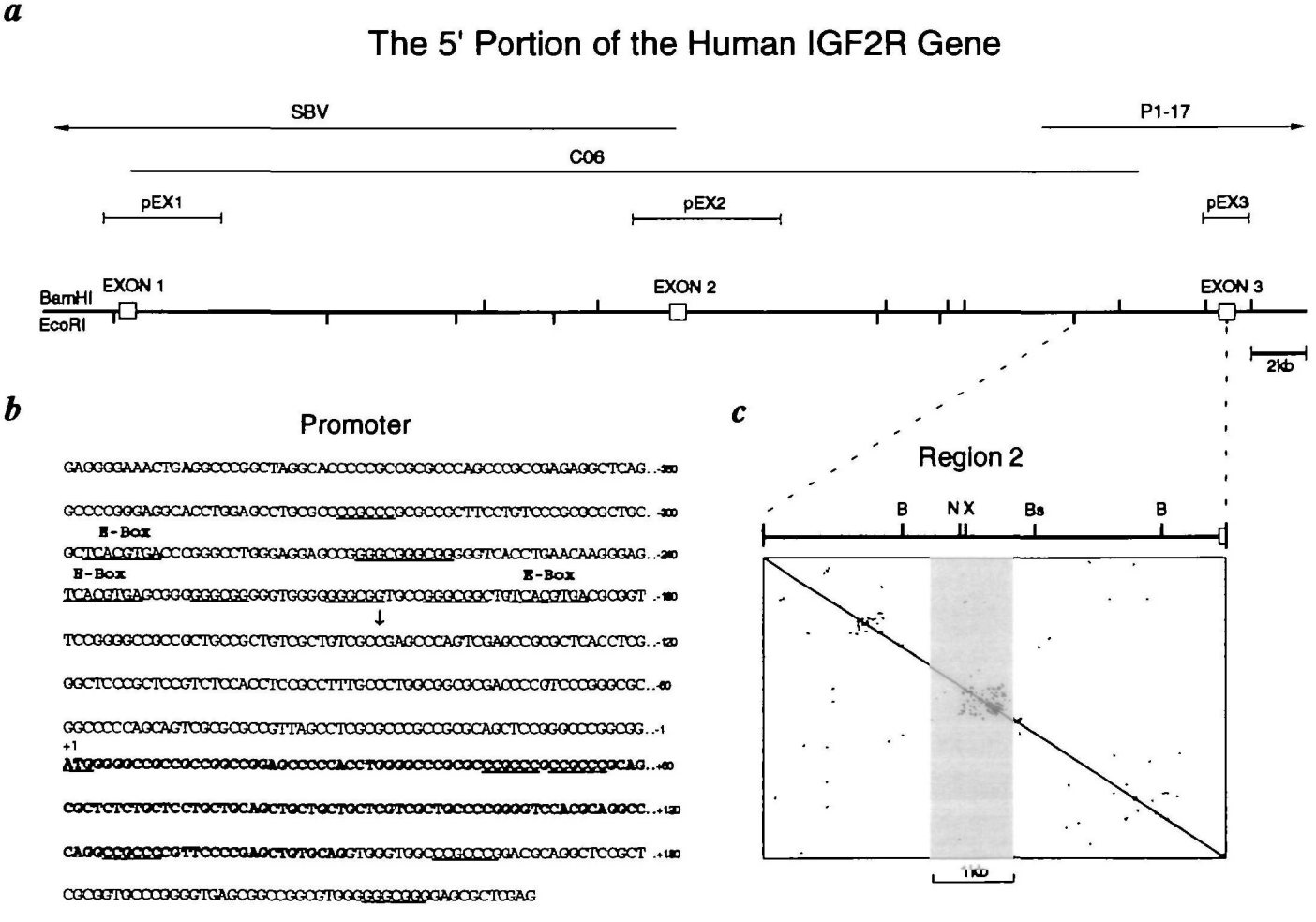

Figure 1. (a) Organization of the first 45 kb of the human IGF2R locus. The map indicates the positions of exons 1–3 and of *Bam*HI and *Eco*RI restriction sites (dashes above and below the line respectively). The location of cosmids SBV and C06, P1 clone P1–17 and subclones pEX1–3 (spanning exons 1–3 respectively), are shown. (b) Sequence of part of the promoter region and exon 1 (bold letters). The transcription start (18,19) is marked by an arrow, the start codon ATG and consensus sites for GC boxes and E-box elements are underlined. (c) Restriction map of 5.7 kb of clone pE3UP. The box below shows a self/self alignment (see Methodology). The shaded area marks the nucleotides where the moving average %C+G is >50 and the CG:GC ratio is >0.6, indicating a CG island (22). Aligning the sequence to itself shows a large number of direct repeats B, *Bam*HI; N, *Not*I; X, *Xho*I; Bs, *Bss*HII.

within CpG islands that lack TATA or CAAT boxes. Furthermore, they show 70% DNA sequence homology over 700 bp and conservation of putative regulatory elements, including three E-boxes and several GC boxes (underlined in Fig. 1b).

Conservation of a maternally methylated element in the human IGF2R gene

In the mouse Igf2/M6pr gene a putative imprinting box, here named M-region 2 to distinguish it from the human element, has been identified in the second intron. This element is CG rich, shares features of CpG islands, is methylated only on the maternally derived allele and contains a series of single copy, direct repeats (12,21). Restriction enzymes with CG rich recognition sites (*Sac*II, *Not*I, *Xho*I and *Bss*HII) were used to scan the first two introns of the human gene for evidence of an element homologous to the M-region 2. A 5.7 kb *Eco*RI fragment within the second intron (named E3UP, derived from clone P1–17) was found to contain a cluster of sites recognized by these enzymes. This region was mapped 3 kb upstream of exon 3 (Fig. 1c) and named H-region 2 in analogy with the mouse sequence that is also located in the second intron. The 5.7 kb *Eco*RI fragment from clone pE3UP was sequenced (EMBL accession no. X83701). The CpG island spans nucleotides 2100–3100 bp of this sequence and most of the repeat rich region is contained within these nucleotides. No significant sequence homology to M-region 2 was found, although short stretches, not exceeding 20 bp could be found using a reduced stringency of <80%. However, three common features of the sequence were found. First, a consensus MDBP binding sequence, that has been associated with transcription repression (22), could be identified at 944 bp. Secondly, 4 Sp1 consensus binding sites, that have been associated with methylation free CpG islands[23] were found at 2448, 2699, 3038 and 3069 bp. Finally, an 8 bp consensus motif (CCCTHGNG) was found to occur four times in the H-region 2 island and 10 times within the M-region 2 island.

In contrast to the lack of direct sequence homology, clone pE3UP showed a high level of organizational similarity to M-region 2. H-region 2 contains a central core with a CG:GC ratio >0.6 and a moving average value %C+G >50, spanning 1.0 kb (shaded area in Fig.1c). These values are similar to those found for M-region 2 and are typical of CpG islands in general (12,24). In addition, alignment of the pE3UP sequence to itself (Fig. 1c) revealed a striking density of direct repeats within the CG rich region, similar to those identified in M-region 2 (21). The repeats identified varied slightly according to the search parameters chosen using the COMPARE program (38). However, repeats are consistently found and do not reflect a bias due to high GC composition of islands, since a search of 13 randomly chosen CpG islands from the database (25), plus the IGF2R promoter CpG island, did not identify similar repeats (data not shown). The H-region 2 repeats lie mostly within the CpG island, and are related to each other, but bear no homology to the highly repeated DNA families of Mus musculus, for example B1 sequence (26). The organization and sequence of the H-region 2 repeats, that are up to 45 bp in length (allowing for one mismatch per 16 bp), are shown in Figure 2.

Human region 2 carries a maternal-specific methylation signal

As previously shown, the mouse Igf2/M6pr M-region 2 carries a maternal-specific methylation signal (12). To verify the extent of conservation between the mouse and the human sequences, H-region 2 was subjected to methylation analysis using Southern blotting. Genomic DNA prepared from peripheral blood leukocytes (five individuals) or EBV transformed lymphoblastoid cell lines (six individuals), was digested with *Bam*HI or *Eco*RI in combination with *Hpa*II or a variety of methylation-sensitive restriction enzymes (peripheral blood analysis from five individuals is shown in Fig. 3b). Sequences spanning 900 bp (from 2100 to 3000 bp in clone pE3UP) were found to be digested to ~50% by methylation-sensitive enzymes suggesting the possibility of monoparental methylation. Restriction sites flanking these 900 bp were totally resistant to digestion by the same enzymes indicating complete methylation of both parental alleles (Fig. 3b,d). Analysis of the promoter CpG island (Fig. 3a and legend) showed that six methyl-sensitive enzyme sites were completely digested, indicating absence of methylation at the promoter.

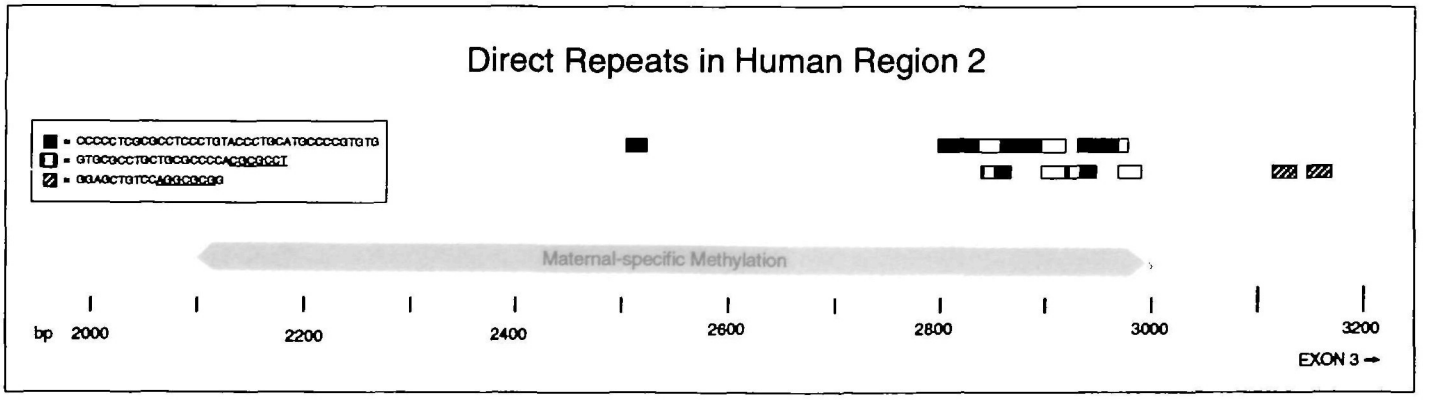

Figure 2. H-region 2 contains direct repeats which are clustered in two areas, the larger is between bp 2800 and 3000 and the smaller between bp 3100 and 3200 of clone pE3UP. Only repeats >16 bp, allowing one mismatch each 16 bp, are displayed, sequence relationships within the repeats are represented by filled, open or hatched boxes respectively. Only the larger repeat group is located within the nucleotides carrying the maternal-specific methylation imprint (shaded bar). The nucleotide sequences of the repeats are displayed and aligned to show related repeat motifs. The underlined sequence motif (CGCGCCT) contained in the larger repeat group appears in reverse orientation in the smaller repeat group (AGGCGCG).

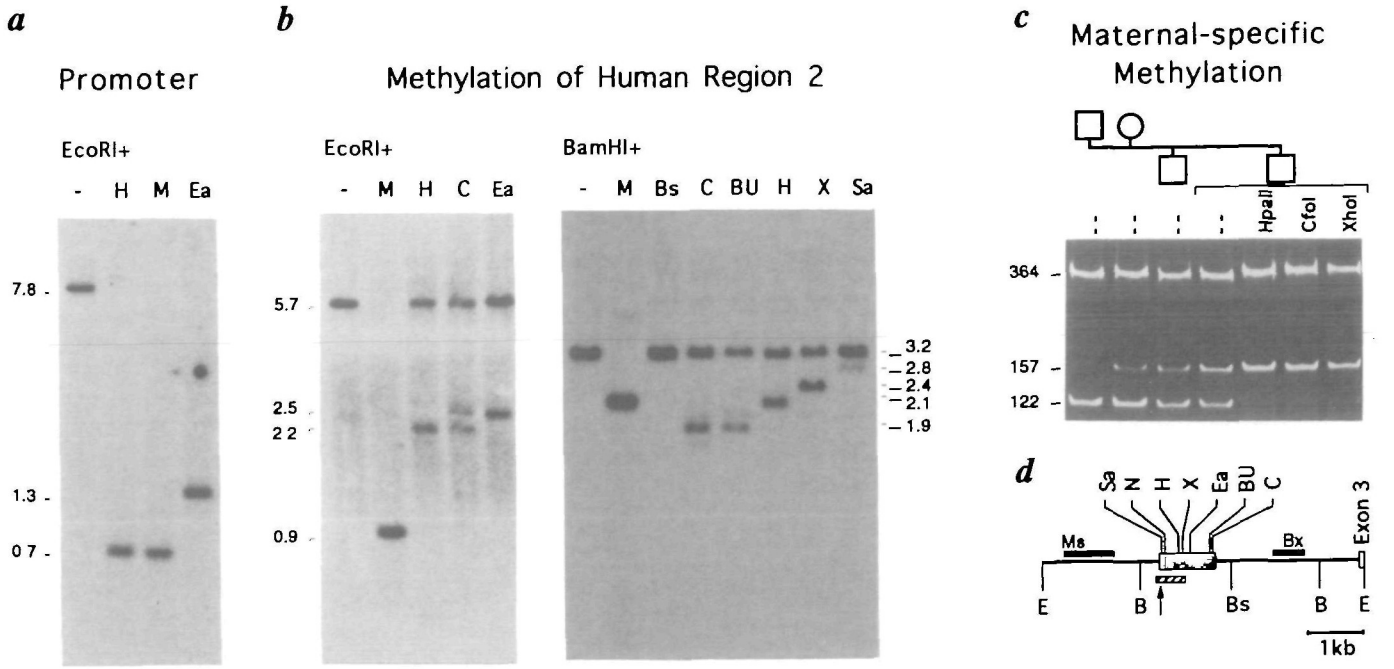

Figure 3. (a) Southern blot analysis of the promoter region using probe HB and genomic DNA from peripheral blood leukocytes from an adult male. A 7.8 kb band is seen after digestion with *Eco*RI and is reduced to 0.7 kb following digestion with either *Msp*I or its methylation-sensitive isoschizomer *Hpa*II and to 1.3 kb when digesting with *Eag*I, indicating the absence of methylation on both parental alleles at these sites Complete digestion was also seen using enzymes H, C, BU, X and Bs (data not shown). (b) Southern blot analysis of H-region 2 using a variety of methyl-sensitive enzymes and probes Ms (left panel) and Bx (right panel) whose locations are shown in d. The 5.7 kb *Eco*RI fragment (lane 1, left panel) was digested to ~50% by *Hpa*II, *Cfo*I and *Eag*I, indicating partial or monoparental methylation at these sites (lanes 3–5, left panel). Two *Cfo*I fragments are seen since the probe covers an additional *Cfo*I site that is fully methylated on both parental chromosomes site and lies at the border of the 50% methylated region (bp 2190 of pE3UP). The *Msp*I fragment is smaller than the *Hpa*II fragment because probe Ms extends beyond the partially methylated region. The 3.2 kb *Bam*HI fragment (lane 1, right panel) was not digested by *Bss*HII (lane 3) and only digested to ~10% by *Sac*II (bp 2115 of pE3UP) (lane 8) indicating the borders of the partially methylated region. The *Bam*HI fragment was digested to ~50% by *Cfo*I, *Bst*UI, *Hpa*II and *Xho*I indicating partial or monoparental methylation at these sites (lanes 4–7). The position of the analyzed sites and the extent of the partially methylated region is shown in d. (c) An AFLP pedigree showing the father, the mother and two children. Genomic DNA from peripheral blood leukocytes was amplified by PCR using primers 2015F and 2549R, to generate a 557 bp amplification product whose location is indicated by the dashed bar in d. This fragment is reduced to either 122 or 157 bp following digestion by *Msp*I, which cuts 4 times in the 557 bp amplification product, the position of the polymorphic *Msp*I site is indicated by an arrow in d. The father is homozygous for the 122 bp allele and the mother is heterozygous for the 122 and 157 bp alleles. Both children are heterozygous and have inherited the 122 bp allele from the father and the 157 bp allele from the mother (lanes 3,4). A maternal-specific methylation imprint was identified by digesting genomic DNA of both children with methylation-sensitive enzymes prior to PCR amplification. The results from one child (lane 4), analyzed with enzymes *Hpa*II, *Cfo*I and *Xho*I, are shown in lanes 5–7. In these lanes the maternal-specific 157 bp band was amplified, a faint trace of the paternal 122 bp allele could be seen on overloaded gels. (d) The extent of maternal-specific methylation within region 2 was deduced from fragment sizes in b. Partial digestion by methylation-sensitive enzymes spanned 900 bp (shaded box), which are contained within the CG island indicated in Fig. 1c. Methyl-sensitive enzymes flanking the shaded box are fully methylated. The *Cfo*I site at bp 2190 identified in b (lane 4, left panel) and the *Sac*II site at bp 2115 (lane 8, right panel) mark the upstream border of the shaded box. Probes Ms and Bx are aligned as solid bars above the map, part of exon 3 is represented by an open box. The hatched box marks the PCR product containing the polymorphic *Msp*I site (indicated by an arrow). E, *Eco*RI; B, *Bam*HI; M, *Msp*I; H, *Hpa*II; C, *Cfo*I; Ea, *Eag*I; Bs, *Bss*HII; BU, *Bst*UI; X, *Xho*I; Sa, *Sac*II.

To test whether the partial digestion observed within H-region 2 resulted from random partial or maternal-specific methylation, clone pE3UP was used to search for a polymorphism able to discriminate both parental alleles. An amplification fragment length polymorphism (AFLP) screen of 23 human DNA samples revealed an *Msp*I polymorphism within a PCR amplification product derived from H-region 2 (Fig. 3d). This polymorphism was used to determine whether the methylation was monoallelic and maternally derived. The 557 bp PCR product spans the *Sac*II, *Not*I and *Xho*I sites of H-region 2 and contains the polymorphic *Msp*I site at base pair position 122 (arrow in Fig. 3d). Amplified genomic DNA was digested with *Msp*I. Seventeen DNA samples were homozygous for the 122 bp fragment, two homozygous for the 157 bp fragment and four were heterozygous for 122/157 bp. Sequencing of both alleles revealed a single C→T transition in the 157 bp allele. Figure 3c shows a pedigree analysis in which two heterozygous children have inherited the 122 bp allele from their father whereas the 157 bp allele derives from their mother. When genomic DNA of these children was digested with methylation-sensitive enzymes *Hpa*II, *Cfo*I, *Xho*I and *Not*I prior to PCR amplification, only the maternally derived allele showed normal amplification (Fig. 3c, lanes 5–7; results for *Not*I not shown). These results show that the methylation of H-region 2 is maternal-specific.

Expression of IGF2R is normally biallelic in lymphoblastoid cell lines

In order to analyze allele-specific expression at the IGF2R locus we have used a previously defined tetranucleotide ACAA$^{-/+}$

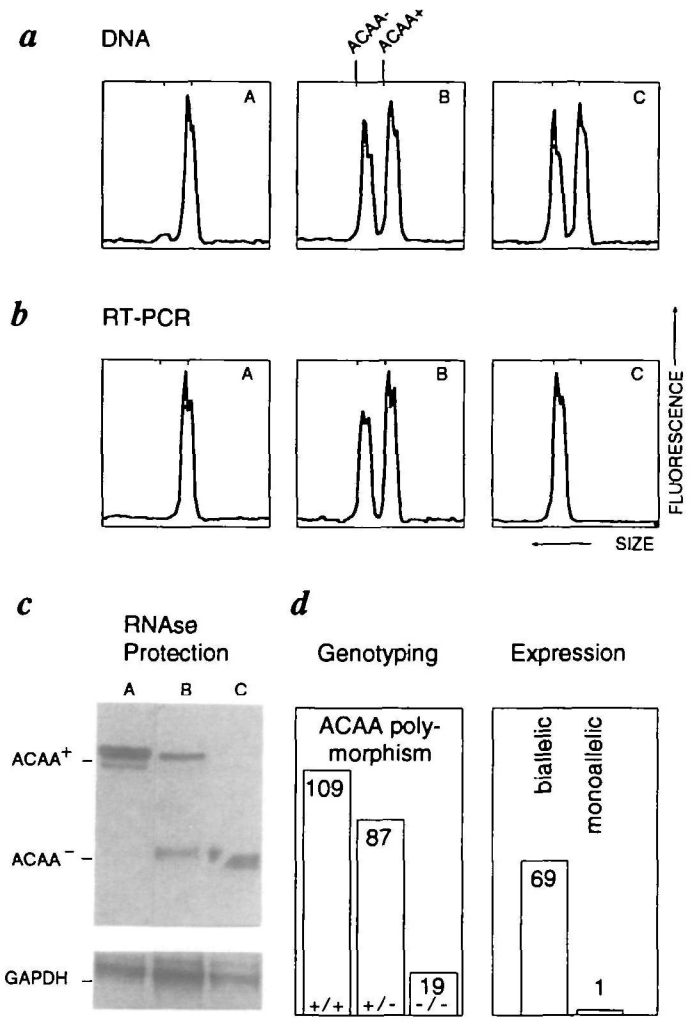

Figure 4. Amplification products from genotyping (**a**) and RT-PCR (**b**) analysis were detected using an automated sequencer. The two peaks represent both alleles of the $ACAA^{+/-}$ (insertion/deletion) polymorphism. The migration of both alleles is indicated by dashes on top of each panel. Cell line C (right panel) was the only cell line which showed monoallelic expression in early passages and later reactivation of the silent $ACAA^{+}$ allele after 2 months in culture. The remaining 69 cell lines expressed both alleles, of which cell line B is representative and shown in the middle panel. Cell line A represents an example of a non informative homozygous $ACAA^{+/+}$ sample. (**c**) RNase protection analysis confirmed RT-PCR results and showed no significant quantitative differences between the parental alleles in six biallelically expressing cell lines tested (middle lane, representative cell line B). The result for the heterozygous cell line C in early passage, was consistent with RT-PCR analysis and showed monoallelic expression of only one allele ($ACAA^{-}$, right lane). (**d**) Summary of the genotype distribution within 215 individuals analyzed for their ACAA polymorphism. IGF2R expression was analyzed in 70 informative lymphoblastoid cells between p10 and 15, of which only one (cell line C) showed initial monoallelic expression that was lost by passage 20.

(deletion/insertion) polymorphism, located at the 3′ non-translated region of the transcript (25). DNA from a total of 215 independently derived EBV transformed lymphoblastoid cell lines was screened by genomic PCR analysis. From a total number of 430 chromosomes, 305 were $ACAA^{+}$ (71%) and 125 were $ACAA^{-}$ (29%) (Fig. 4d). IGF2R expression analysis from 70 heterozygous lymphoblastoid cell lines was

Table 1. Replication timing analysis of D282 lymphoblastoid cell line at the IGF2R (16) (6q27), the paternally expressed (31) IGF2 (11p15.5) and biallelically expressed (41) PAX5 (9p13) loci

Gene		PAX5	IGF2R	IGF2
S/S		21.3	25	46.4
S/D		**11.5**	**33**	**26.8**
D/D		55.7	37	23.2

FISH signals were counted in S phase nuclei (as shown by BrdU incorporation). The percentage of analyzed nuclei showing the single/double dot hybridization pattern, indicative of replication asynchrony, are highlighted in bold type. S/S, single/single; S/D, single/double; D/D, double/double. The total number of S phase nuclei examined were 108 (Pax 5), 88 (IGF2R) and 108 (IGF2).

performed using RT-PCR. The two alleles, $ACAA^{+}$ and $ACAA^{-}$ differing by 4 bp, could be resolved and quantitated using an automated sequencer and fluorescent nucleotides (see Materials and methods and Fig. 4a,b). Sixty-nine out of 70 lymphoblastoid samples showed biallelic expression and one heterozygous cell line, C, expressed only one allele (Fig. 4a,b; sample B and C respectively). Monoallelic expression of cell line C could only be found during its initial establishment. Later RT-PCR analysis revealed reactivation of the $ACAA^{+}$ allele to ~70% for cell line C after several weeks in culture. To check for partial repression of the paternal allele in these samples and to test the reliability of the RT-PCR method, an RNase protection analysis was performed (Fig. 4c), using a 146 bp fragment spanning the $ACAA^{+/-}$ polymorphism as RNA protection probe. Results obtained by this method were consistent with those obtained from RT-PCR and showed that the biallelically expressing cell lines expressed both alleles to the same degree. RNase protection analysis also showed that expression levels were qualitatively similar between cell line C and the remaining bi-allelically expressing cells (in comparison with GAPDH, Fig. 4c). All cell lines were analyzed between passages 10 and 15. *Hpa*II digestion of genomic DNA from six biallelically expressing cell lines, including cell line C (that initially showed monoallelic expression) gave the same methylation profile at H-Region 2 as shown in Figure 3b. In addition, cell line C was not methylated at six methyl-sensitive sites, at the promoter at any stage (data not shown). Thus EBV transformed lymphoblastoid cell lines carry a monoparental methylation mark that is not normally translated into paternal repression of the IGF2R gene under the conditions examined.

Asynchronous replication timing at the human IGF2R locus

Cosmid ES, downstream from H-region 2 but within the human IGF2R locus (data not shown) was used for fluorescence *in situ* hybridization (FISH), to test if the human IGF2R locus displayed replication timing asynchrony. This had been claimed by a previous report using a cosmid probe overlapping the promoter CpG island, but data were not presented (16). Nuclei from lymphoblastoid cells were monitored for BrdU incorpora-

tion to identify cells that had undergone S phase and 33% of the BrdU positive nuclei displayed an asymmetric dot pattern for IGF2R. The results, summarized in Table 1, show that the percentage of nuclei displaying asynchrony at the human IGF2R locus exceeds that of the imprinted IGF2 locus which is monoallelically expressed in humans (28).

DISCUSSION

In this study we report the identification of an intronic element, named H-region 2, in the human IGF2R gene (Fig. 1). Several features suggest that H-region 2 is a homologue for M-region 2, the previously identified candidate imprinting box of the mouse Igf2/M6pr gene (12). These are that both H-region 2 and M-region 2 are located in the second intron of the gene, both elements are CpG islands rich in direct repeats, and both contain several copies of the consensus octamer (CCC-THCNG).

We also show that H-region 2, like its mouse counterpart, carries a methylation mark in 11 individuals and in one informative family it could be shown that this methylation is only present on the maternally inherited chromosome (Fig. 3c). In addition, we also show that human IGF2R resembles other imprinted or monoallelically expressed genes (17) in that it replicates asynchronously during S-phase (Table 1). Thus the human IGF2R gene resembles the majority of imprinted genes in some features, since all that have been examined contain a CG rich region rich in direct repeats and are subject to monoparental methylation and replication asynchrony (21). However, in contrast to other imprinted genes, including the mouse counterpart, the human IGF2R gene does not normally show monoallelic expression.

Two earlier studies (13,14) have detected biallelic expression at the human IGF2R locus in several individuals in fetal and adult tissues. However, a study by Xu *et al.* (15) detected monoallelic expression of IGF2R in three of 14 individual fetuses and proposed that monoallelic expression may be polymorphic at this locus. Our extensive analysis of 70 informative lymphoblastoid cell lines (Fig. 4) showed that these cells express IGF2R biallelically, with one exception that showed monoallelic expression only in early passage. Thus these cells do not link monoparental methylation with monoallelic expression. However, the modified assay we have devised (Fig. 4), plus the demonstration that RNase protection can be used to distinguish allelic expression at the IGF2R locus, offers the opportunity to extend this analysis to a wide range of human tissues from various developmental stages, tissue types and pathological specimens.

It is becoming increasingly apparent that imprinted genes do not always repress one parental locus. The Wilms tumor gene (WT1) (29) as well as the human IGF2R (15) gene have been reported to show polymorphic monoallelic expression and the imprinted mouse genes Igf2/M6pr, Igf2 and Ins2, have been shown to display biallelic expression in early development and in some cell types (8,30). In addition, the human IGF2 gene is monoallelic in fetal liver but biallelic in pathological states (9) and in normal adult liver, a switch that has been shown to depend on alternate promoter usage in liver development (28).

It is noteworthy that the features conserved between human and mouse-region 2, that is a CG rich element, direct repeats and monoparental methylation, are also found in elements in other imprinted endogenous genes and transgenes which do not share any direct sequence homology (21). Although, our analysis of the mouse and human IGF2R genes also identified a loosely conserved octamer sequence, their homology is only significant in terms of sequence organization. This raises the possibility that secondary DNA/chromatin structure, rather than a specific nucleotide sequence may play a role in attracting a maternal-specific methylation signal. The maintenance of information, in terms of structural similarity without specific sequence homology, has precedence in protein/DNA interactions for example Z-DNA binding proteins (32). However, the role of structural similarities versus sequence specific elements in determining maternal methylation, awaits experimental testing.

The methylation profile of H-region 2 was the same in six biallelically expressing lymphoblastoid cell lines, including cell line C that initially showed monoallelic expression (Fig. 3b and data not shown). Methylation of the promoter CpG island was never seen (Fig. 3a). Thus EBV transformed lymphoblastoid cell lines carry a monoparental methylation mark that is not normally translated into paternal repression of the IGF2R gene under the conditions examined. We have previously proposed (12) but not yet shown, that maternal methylation of M-region 2 is the imprinting signal for the mouse Igf2/M6pr gene. The analyses of human IGF2R presented here may suggest the contrary, that methylation of region 2 is not relevant for imprinting. However, alternatively, the results could support the suggestion (2) that gametic imprinting could be viewed as a multi-step process in which acquisition of the primary imprint is separated in time from the 'reading' of the imprint that results in monoallelic expression. We suggest that the first step of acquisition of a primary gametic imprint occurs in the human IGF2R gene. Whether this imprint is used to generate monoallelic expression in IGF2R is as yet untested. However, it is known that all imprinted genes so far studied are monoparentally methylated (1) and that methylation is essential for monoallelic expression of imprinted genes. Mice that lack DNA maintenance-methyltransferase (11) are unable to maintain allele-specific expression in a manner exactly as predicted by our results (12) that showed that the expressed allele carried the significant methylation. While the significance of the IGF2R 'methylation imprint' awaits further analysis, recent data describing an association between uniparental paternal disomy for human chromosome 6 (IGF2R maps to 6q24–27) and transient neonatal diabetes mellitus (33) does support the existence of an imprinted gene on this chromosome. However, it should also be noted that the Mas protooncogene, that is closely linked to Igf2/M6pr in the mouse, has been reported as imprinted and paternally expressed in mice (6) and thus could also be responsible for paternal UPD and replication asynchrony effects.

In summary, the results presented here show that the human IGF2R gene has the characteristics of monoparental methylation and replication asynchrony, that are common to all imprinted genes, but IGF2R does not show unequivocal monoallelic expression. However, the identification of organizational homology between the IGF2R mouse and human region 2 elements that carry the maternal-specific methylation signal, as well as the identification of similar elements in other imprinted genes (21), will allow the investigation of the features

that attract maternal-specific methylation. Additionally, these results should provide improved criteria to screen the mammalian genome for novel imprinted genes which may be subject to monoallelic expression in either the mouse or human.

MATERIALS AND METHODS

Genomic mapping and sequencing

Cosmid SBV was obtained by screening a human cosmid library (A.-M. Frischauf, ICRF, London) with a 465 bp *SalI/BglII* probe derived from the human IGF2R cDNA (W.Sly, St Louis, USA). Cosmid C06 was obtained by using a 0.6 kb *NotI/Eco*RI fragment from the P1 clone P1–17. Clone P1–17 was obtained by screening a human P1 library (36) with a 0.8 kb *Bam*HI/*Ssp*I fragment derived from the downstream cosmid ES (not shown). Sequencing was performed using the *Taq* Dye Deoxy Terminator Cycle Sequencing-Kit (Applied Biosystems) following the instructions given by the manufacturer. The origin and location of each sequenced clone is indicated in Figure 1a (pEX1 4.1 kb *Hin*dIII, pEX2 5.3 kb *Hin*dIII, pE3UP 5.7 kb *Eco*RI and pEX3 1.7 kb *Bam*HI). pBluescript SK$^-$II (Stratagene) was used as the cloning vector.

DNA samples

Genomic DNA was prepared from either human peripheral blood leukocytes or EBV transformed lymphoblastoid cells by a salting out procedure (37).

Methylation analysis

Genomic DNA was predigested with *Bam*HI or *Eco*RI, then with methylation-sensitive restriction enzymes, before DNA blot analysis. Probes: HB–0.5 kb *Hin*dIII/*Bst*EII fragment from the promoter clone pEX1. Ms–0.9 kb *Msp*I fragment. Bx–0.6 kb *Bst*XI fragment from the region 2 clone pE3UP. Completeness of digestion was checked for six sites by analyzing the same sample of digested DNA with probe HB (Fig. 3a,b). For methylation AFLPs, 0.5 µg genomic DNA was predigested with various methylation-sensitive enzymes, ethanol precipitated and amplified by PCR for 35 cycles at 94/64/72°C for 30/40/40 s. Primers for H-region 2: 2015F 5′-CGA GGC CTG GCA TGT T-3′[1 µM] and 2549R 5′-TGG GGA AGC GCG AGA GGC CTA GG-3′ [0.2 µM]. Amplification products were digested with *Msp*I, analyzed on 15% polyacrylamide or 2% agarose gels and visualized by ethidium bromide staining.

Sequence analysis

The GCG 7.0 software package (38) and the MacVector™ program (Kodak Eastman) were used for DNA computer analysis. Self/Self alignment of pE3UP in Figure 1c was performed with the COMPARE algorithm using a window size of 30 bp and a stringency of 65%. Repeat analysis in Figure 2 allowed one mismatch per 16 bp. GenBank accession nos are X83699–X83702.

Allelic expression analysis

Genotyping of the ACAA$^{+/-}$ polymorphism at the 3′ IGF2R non-translated region was performed by amplifying 0.01 µg genomic DNA with oligo 8287F 5′-AGG TTT TGG AGA GTT TGC CTG-3′ [1 µM] and the fluorescence labeled oligo 8409R 6-FAM--AAT CAA TCT TTG GGC AGG TTG-3′ [0.05 µM] with 32 cycles at 94/59/72°C for 30/20/5 s. Lymphoblastoid cell RNA was prepared by a standard guanidinium isothiocyanate/cesium chloride method and RT-PCR was performed using an Invitrogen cDNA Cycle kit. The reverse transcription was primed with oligo 8475 5′-TCA TTT TCT CTG ACA CCT CAA CTC C-3′ [1 µM]. 0.1 µl of the reaction mix was amplified using primers 8287F and 8409R-6-FAM under the same conditions as described for genotyping. Amplification products from ACAA genotyping and RT-PCR were diluted 25-fold, 1 µl was loaded on a 4.5% 8 M urea gel and analyzed by a 373A DNA Sequencer™ using 672 Genescan™ software. Alternatively, a non-denaturing 12% acrylamide minigel was used and stained with ethidium bromide. For RNase protection a 146 bp PCR fragment was amplified from genomic DNA of a homozygous ACAA$^{+/+}$ individual using primer pair 8287F/8409R and subcloned into the *Eco*RV site of pBluescript SK$^-$II. 1.5 µg of *Hin*dIII-linearized plasmid was used as template for RNA probe synthesis with T3 RNA polymerase (10 units; Promega) in a final volume of 20 µl under the following conditions: 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 30 units RNAsin (Promega) 0.5 µM each rATP, rCTP, rUTP, [α^{32}P]rGTP. RNA probe isolation, hybridization, RNase digestion was as described (39). The identity of cell line C was confirmed by DNA fingerprinting, after expression had switched from mono to biallelic, using primer sets HumTH01 and HumVWA from a Serac™ kit and HLA-DR specific primers (data not shown).

Replication timing

Cytogenetic preparations. A non-synchronized growing culture of Epstein–Barr virus-transformed human lymphoblastoid cells were incubated in the dark for 60 min with 10 mM BrdU (#B5002, Sigma-Aldrich) to label S-phase cells. Cells were pre-swollen, fixed and dropped on to microscope slides, dehydrated and stored until use in 70% ethanol at 4°C (40).
DNA probes. Positive control, IGF2 cosmid (E.Holthuizen, University of Utrecht). Negative control: Cosmid PAX5 (M.Busslinger, IMP Vienna). Cosmid IGF2R spanned exon 5 (OWS, unpublished). Probes were labeled by nick translation with bio-11-dUTP (Sigma)[40].
Hybridization, probe detection and microscopic analysis. Slides were pre-treated with RNase A (100 mg/ml) in 2×SSC for 60 min at 37°C, pepsin (50 mg/ml in 0.01 M HCl) for 10 min at 37°C and 1% acid-free formaldehyde in 1×PBS/50 mM $MgCl_2$ for 10 min at ambient temperature. Hybridization of biotinylated probes was as described (40). Immunological detection of hybridized probes was performed with FITC avidin (Vector™) for 30 min. Incorporated BrdU was detected by indirect immunofluorescence staining with mouse Anti BrdU (Becton-Dickinson) and rhodamine (TRITC) goat anti-mouse IgG (Becton-Dickinson). Cells were counterstained with 0.1 mg/ml 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Slides were analyzed with a Zeiss Axiophot epifluorescence microscope equipped with a cooled CCD camera (Photometrics, Kodak- KAF 1400 chip). Replication timing analyses were performed repeatedly, with consistent results, in interphase nuclei of EBV transformed lymphoblastoid cells and in nuclei of a fibroblast cell line (GM3876, NIGMS Camden) (data not shown).

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