Parental imprinting: potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II (*Igf2*) gene

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The mouse insulin-like growth factor II (*Igf2*) gene, which is located on distal chromosome 7 (Chr7), has been shown previously to undergo tissue-specific parental imprinting. This imprinting results in expression of *Igf2* from the paternally inherited chromosome and repression of the maternally inherited allele in most tissues of the developing embryo. We are using embryos with the maternal duplication and paternal deficiency of distal Chr7 to characterize the mechanism that underlies repression of the maternal allele. We show that the chromatin of the 5' region of the repressed *Igf2* allele is potentially active for transcription rather than heterochromatic. In particular, a CpG island that comprises one of the two strong promoters is unmethylated at both parental alleles, and DNase I hypersensitive sites in and around the strong promoters are consistently present on both parental chromosomes. In agreement with the chromatin state, primary transcripts from the maternal *Igf2* allele have been detected at low but significant levels. These findings differ from observations in other instances of imprinting, namely, X-chromosome inactivation and transgene imprinting in mice. Although no parent-specific differences were detected in either DNA methylation or sensitivity to nucleases at these promoters, we have observed parental methylation differences in a region several kilobases upstream of the first exon. The differential activity of the parental *Igf2* alleles could be achieved through epigenetic modifications situated outside the promoters or by subtle and yet unidentified modifications at the promoters.

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The concept of genomic or parental imprinting has become increasingly important in many fields of biology. In mammalian embryology, it has been well established that both a maternal and a paternal genome are vital for normal mouse development (Surani 1986; Surani et al. 1990; Solter 1988). This notion has been further supported by genetic complementation studies with uniparental disomies, which have identified several mouse autosomal regions whose activities are affected by their parental origins (Searle et al. 1989; Beechey et al. 1990; Cattanaach and Beechey 1990). Furthermore, some genetic traits in mice (Johnson 1974, 1975) and a variety of genetic disorders in man (Reik 1989; Clarke 1990; Hall 1990) show different phenotypes, depending on whether the affected allele is inherited from the father or the

mother. Genesis of some types of tumor also appears to be influenced by the parental origin of the relevant genetic loci (Ferguson-Smith et al. 1990; Sapienza 1991).

Despite its relevance to many biological phenomena, little is known about the molecular basis of imprinting. The primary imprint must be established during gametogenesis, and the germ line-specific epigenetic imprint must persist through fertilization and subsequent rounds of DNA replication and cell division during development. However, reprogramming should be possible upon a subsequent passage through the germ line of the opposite sex. Thus, heritability, reversibility, and the ability to affect gene expression are the requirements for the mechanism of imprinting (Surani 1986; Sapienza et al. 1987; Monk 1988). Both DNA methylation and condensed chromatin, such as heterochromatin, fulfill these criteria, and their possible role in imprinting is suggested by findings associated with X chromosome inactivation and the imprinting of transgenes (see below).

In somatic cells of female eutherian mammals, com-

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pensation for the sex differences in dosage of X-linked genes is achieved by a random inactivation of the maternal or paternal X chromosomes. However, a preferential inactivation of the paternal X chromosome occurs in all cells of female marsupials (Cooper et al. 1971; Richardson et al. 1971; Sharman 1971) and in the extraembryonic tissues of developing female mice (Takagi and Sasaki 1975; West et al. 1977). Therefore, X chromosome inactivation can be affected by parental imprinting. Cytologically, the inactive state is associated with late replication and heterochromatinization. A redistribution of DNA methylation is observed in eutherian mammals at the molecular level and this is almost certainly involved in maintaining the inactive state (Monk 1986; Grant and Chapman 1988). Thus, the CpG islands, generally free of methylation (Bird 1986), are methylated on the inactive X while the remaining portions of these genes are often less methylated (e.g., Toniolo et al. 1984; Wolf et al. 1984; Yen et al. 1984). This inactive X-specific island methylation is seen primarily in the embryo proper, but recent reports suggest that it may also occur in the extraembryonic lineages (Lock et al. 1987; Singer-Sam et al. 1990), where inactivation is nonrandom. DNA methylation is often accompanied by decreased sensitivity of the chromatin to nucleases. Loss of DNase I hypersensitive sites has been reported for the *HPRT* and *PGK* genes (Riley et al. 1984, 1986; Yang and Caskey 1987; Lin and Chinnault 1988), and accessibility to the endonuclease *MspI* has also been used to differentiate between active and inactive X chromatin (Wolf and Migeon 1985; Hansen et al. 1988; Antequera et al. 1989).

Another model system used to study mechanisms of imprinting has been provided by imprinted transgenes with methylation levels altered by parental origin (Hadchouel et al. 1987; Reik et al. 1987, 1990; Sapienza et al. 1987; Swain et al. 1987; Chaillet et al. 1991; Sasaki et al. 1991). In some cases, transgene expression is affected along with methylation (Hadchouel et al. 1987; Swain et al. 1987). Although the relationship between transgene imprinting and endogenous imprinting is not clear, this model system has attracted much attention and has been studied in detail. One outcome is the characterization of genetic background effects due to modifier genes (McGowan et al. 1989; Sapienza et al. 1989; Allen et al. 1990; Reik et al. 1990; Surani et al. 1990; Engler et al. 1991); and based on their similarities to the phenomenon of position-effect variegation in *Drosophila*, the possible involvement of heterochromatinization in imprinting has been suggested (Allen et al. 1990; Sapienza 1990; Tartof and Bremer 1990; Engler et al. 1991; Singh et al. 1991). Significantly, in one reported case of an imprinted transgene, the maternal inhibition of tissue-specific expression has been shown to be accompanied by loss of a DNase I hypersensitive site (Pourcel et al. 1990).

The recent identification of endogenous imprinted mouse genes has now enabled us to examine the mechanism of parental imprinting. Two lines of evidence indicate that the mouse insulin-like growth factor II (*Igf2*) gene, located on the distal region of chromosome 7 (Chr7), undergoes parental imprinting. First, targeted

mutagenesis experiments have shown that in heterozygous mice bearing one disrupted allele, *Igf2* transcripts are detectable only when the remaining intact allele is derived from the father (DeChiara et al. 1991). Second, in mouse embryos with a maternal duplication and paternal deficiency of distal Chr7 (MatDi7), both maternal copies of *Igf2* are repressed (Ferguson-Smith et al. 1991). Thus, it is assumed that only the paternal allele is expressed and the maternal allele is repressed in normal cells. *Igf2* encodes an embryonal mitogen widely expressed in embryonic and extraembryonic tissues in the mouse (Lee et al. 1990). Expression of *Igf2* is driven by three promoters (P1, P2, and P3), which generate alternate 5' exons (E1, E2, and E3), each spliced onto the shared downstream exons E4–E6 (Rotwein and Hall 1990; Ikejiri et al. 1991). Assuming that the *Igf2* imprinting is achieved at the level of transcription, transcription from the two stronger promoters P2 and P3 is apparently affected because the degree of repression is at least 10-fold (DeChiara et al. 1991; Ferguson-Smith et al. 1991). In the gene-targeting experiment, the *neo* gene driven by the endogenous P2, which is a typical CpG island, also exhibited the differential expression pattern on the disrupted chromosome (DeChiara et al. 1991). Interestingly, *Igf2* imprinting is tissue specific: The maternal allele is not repressed in the choroid plexus and leptomeninges of the brain during late embryonic development (DeChiara et al. 1991).

Our experimental system for characterizing the molecular mechanism of parental imprinting utilizes MatDi7 embryos containing two repressed maternal copies of *Igf2*, which can be analyzed and compared with normal control embryos. Here, we report our initial attempts to identify epigenetic changes responsible for the maternal repression of *Igf2*. In light of the two possible mechanisms discussed above, we have carried out DNA methylation analysis and nuclease sensitivity studies of the maternal copy of *Igf2*, especially of the P2 and P3 regions. Our results clearly exclude extensive CpG island methylation as the mechanism being used for maternal repression. We also show evidence that the maternal *Igf2* chromatin is potentially active for transcription rather than heterochromatic. In a region several kilobases upstream of E1, however, we have detected several sites differentially methylated, depending on the parental origin. On the basis of these findings, the possible molecular mechanisms that result in the parental imprinting of *Igf2* are discussed.

Results

Experimental system

Previous studies have shown that the differential expression of the parental alleles of *Igf2* is seen at least from embryonic day 9.5 (e9.5) onward (DeChiara et al. 1991; Ferguson-Smith et al. 1991). Because MatDi7 embryos die around e16 (Searle and Beechey 1990), nucleic acids and nuclei were prepared from e13 to e15 embryos. In contrast to MatDi7 embryos, the reciprocal paternal disomies die at an unidentified earlier stage and cannot be

obtained. Therefore, normal littermates of the MatDi7 embryos were used as controls throughout this study. Thus, comparative analyses were carried out between MatDi7 embryos harboring two repressed *Igf2* alleles and control embryos carrying one active and one repressed allele. This allows us any epigenetic differences identified between the two embryo types to be attributed to the active paternal allele. Normal embryos at e13–e15 actively express the paternal copy of *Igf2* in a variety of tissues, and the transcripts are readily detectable by Northern blot analysis in total RNA prepared from whole embryos (Ferguson-Smith et al. 1991). In the choroid plexus and leptomeninges of the brain, however, both parental copies are active during late gestation (DeChiara et al. 1991). Therefore, we carefully removed the head region before sample preparation for very sensitive experiments such as reverse transcriptase–polymerase chain reaction (RT–PCR).

DNA methylation analysis of the promoters and the peptide-coding region

To examine whether DNA methylation is involved in repressing the maternal *Igf2* allele, the methylation status of the two *Bam*HI fragments of 5.6 and 2.4 kb shown in Figure 1A was analyzed. The 5.6-kb fragment contains the two stronger promoters P2 and P3 (hereafter called the P2–P3 region), and the 2.4-kb fragment contains the polypeptide-coding exons. P2 is included in a CpG island, which also comprises the 5' half of E2. DNAs extracted from MatDi7 and control embryos at e15 and normal epididymal sperm were double digested with *Bam*HI plus methylation-sensitive enzymes *Hpa*II, *Sma*I, *Xho*I, or *Sal*I.

Figure 2A shows such digests hybridized with probe C. The 5.6-kb *Bam*HI fragment was cut into very small pieces by *Hpa*II, and only one band of 0.4 kb was observed in all samples. This band was also the only one yielded by *Msp*I digestion. Therefore, most of the *Hpa*II sites that are detected by this probe appeared to be unmethylated. Similarly, four *Sma*I sites, Sm1–Sm4, and an *Xho*I site, X1, were cut to completion in all samples. The faint bands seen in the 3- to 4-kb region of the *Sma*I and *Xho*I lanes arose from a cross-hybridization of the probe (also visible in the *Bam*HI lane) because their sizes were inconsistent with those expected for partial digestions. Hybridization with probe D confirmed the unmethylated state of this region (Fig. 2B). *Hpa*II digestion yielded small fragments of 0.8 and 0.6 kb identical to the *Msp*I digestion (except for the background smear due to a cross-hybridization). Enzymes *Sal*I, *Sma*I, and *Xho*I demonstrated that sites Sa2, Sm5, and X1, respectively, were completely unmethylated. The unmethylated states of Sm5 and X1 were further evidenced by hybridization with probe F (Fig. 2C). This probe also showed that the *Hpa*II sites around E3 were only slightly methylated. Therefore, the P2–P3 region, including the CpG island, was strikingly unmethylated irrespective of the source of DNA. This clearly excludes the possibility of extensive CpG island methylation, such as that seen on the inac-

Figure 1. Overall structure and restriction map of the mouse *Igf2* gene (A) and summary of methylation analyses (B). (A) The positions of six exons (E1–E6; solid boxes), three promoters (P1–P3), and transcription initiation sites (arrows) are indicated. ATG, TGA, and pA denote the initiation codon, termination codon, and major polyadenylation site, respectively. The *Bam*HI sites (B) and the distances (in kb) between these sites are shown. Solid lines indicate the probes used for the methylation assays: (C) *Bam*HI–*Bgl*II, 1.5 kb; (D) *Sal*I–*Bst*EII, 1.7 kb; (F) *Bst*EII, 1.0 kb; (H) *Bam*HI, 2.4 kb. The sites for methylation-sensitive enzymes are *Sma*I (Sm1–Sm6), *Xho*I (X1), *Sal*I (Sa1, Sa2), and *Hpa*II–*Msp*I (H/M). The positions of these sites are derived from the genomic sequence (Rotwein and Hall 1990), cDNA sequence (Stempien et al. 1986), and the restriction mapping experiments that we performed. The gapped portions were not determined for *Hpa*II–*Msp*I. (B) The methylation-sensitive enzyme sites are shown by open circles if unmethylated or a solid one if methylated. Other circles denote intermediate levels of methylation. The methylation status of sites without circles was not determined.

tive X chromosome, being involved in the repression of the maternal allele.

The downstream *Bam*HI 2.4-kb region showed a somewhat different picture (Fig. 2D). Upon *Hpa*II digestion, both MatDi7 and control DNA gave a partially methylated pattern, whereas sperm DNA gave a totally methylated pattern. Similarly, site Sm6 was partially cut in MatDi7 and control embryos but remained uncut in sperm. Site X2 was partially methylated in all cases but clearly more methylated in the sperm DNA. Thus, the methylation level of this region was higher in epididymal sperm than in e15 embryos. However, again, no difference was found between MatDi7 and normal embryos. The results obtained from all of these experiments are summarized in Figure 1B.

Figure 2. Autoradiographs showing the methylation status of the *Igf2* gene in MatDi7 and control embryos at e15 and sperm from adult epididymis. DNA was digested with *Bam*HI only or double digested with *Bam*HI plus either *Hpa*II (H), *Msp*I (M), *Sma*I (Sm), *Sal*I (Sa), or *Xho*I (X). The digests were hybridized to probe C (A), D (B), F (C), or H (D). Fragment sizes are in kb.

*Msp*I sensitivity of the promoter regions in nuclei

Elsewhere we show by RNA analysis that the *Igf2* imprint is maintained in cultured cells derived from MatDi7 embryos (P.A. Jones et al., in prep.). In both MatDi7 and control cells, the CpG island region described above was found to remain unmethylated. It was

also found that the same region was significantly methylated in the established C3H 10T1/2 Cl 8 (10T1/2) and its transformed derivative MCA Cl 15 Cl (MCA) cell line (Reznikoff et al. 1973a,b). Various sites within this region were partially methylated in 10T1/2 cells and heavily methylated in MCA cells. Using these cells, we then addressed the question of whether the chromatin struc-

ture of the repressed allele differed from that of the active allele. Nuclei isolated from the cultured cells were digested with various concentrations of *MspI*, and DNA was then purified and restricted with *BamHI*. The samples were analyzed by Southern blotting using probe C.

As shown in Figure 3, the P2–P3 region was highly sensitive to *MspI* in both MatDi7 and control chromatin because the *BamHI* 5.6-kb band disappeared even at the lowest concentration of the enzyme. There was little difference between the two digestion profiles. In contrast, this region was highly resistant to *MspI* cleavage in MCA nuclei. The nuclei from 10T1/2 cells gave a pattern intermediate between the embryo-derived cells and MCA cells. Accordingly, the accessibility to *MspI* showed a good correlation with the observed level of methylation. Because the reaction conditions employed here were unable to distinguish MatDi7 and control chromatin, these nuclei were also digested with reduced amounts of *MspI* (final concentration, 5, 10, or 50 U/ml). In these experiments we observed a gradual disappearance of the *BamHI* 5.6-kb band; but again, no difference in sensitivity was detected (not shown). The same results were obtained with nuclei isolated from e15 embryos (not shown).

DNase I hypersensitivity of the promoter regions

To further examine the chromatin structure of the P2–P3 region, we then identified and compared DNase I hy-

persensitive sites in MatDi7 and control embryos. Nuclei were treated with increasing concentrations of DNase I, and subsequently the purified DNA was digested with *BamHI* and analyzed by Southern blotting. Probes C and G, located at the 5' and 3' ends of the *BamHI* 5.6-kb fragment, respectively, were used to map the hypersensitive sites by "indirect end-labeling" (Fig. 4A).

Hybridization with probe C revealed that both MatDi7 and control embryo nuclei at e15 contain a number of hypersensitive sites (Fig. 4B). The site giving rise to the most prominent sub-band was located within the P2 region of the CpG island (long arrow). When the same membrane was rehybridized with probe G, four major hypersensitive sites were clearly detected (Fig. 4C, long and medium arrows). Among these, the one in the P2 region gave the strongest signal (the long arrow), confirming the above result obtained with probe C. The P3 region also contained one prominent hypersensitive site. The other two major sites mapped outside the promoters, but the one in the middle of E2 had also been reported in the rat gene (Evans et al. 1988). The location of these major hypersensitive sites, together with those of minor ones (short arrows), are summarized in Figure 4A.

These studies therefore showed no significant differences between the MatDi7 and control embryos. All hypersensitive sites found in normal embryos were also present in MatDi7 embryos. This result was reproducibly observed in two other experiments, one of which

Figure 3. Accessibility of the *BamHI* 5.6-kb (P2–P3) region of *Igf2* to *MspI* in nuclei from various cells. Nuclei were treated with increasing concentrations of *MspI* and analyzed by probe C. Markers are in kb.

Figure 4. DNase I hypersensitivity assay of the *Bam*HI 5.6-kb (P2–P3) region of *Igf2*. (A) Map of the *Igf2* gene (see Fig. 1 legend) and summary of the hypersensitivity analysis. Thick solid lines (bottom) indicate the positions of the probes used for indirect end-labeling: (C) *Bam*HI-*Bgl*II, 1.5 kb; (G) *Bst*EII-*Bam*HI, 0.6 kb. The positions of hypersensitive sites are shown by long (the most prominent site), medium (other major sites), or short (minor ones) upward arrows. (B) DNase I hypersensitive sites analyzed by probe C. Nuclei isolated from tissues and cells were digested with increasing amounts of DNase I. Sub-bands representing the hypersensitive sites are marked by long, medium or short arrows (see above). Markers are in kb. (C) Hypersensitive sites assayed by probe G (see above).

was done with cultured cells derived from normal and MatDi7 embryos. Slight differences in the general intensities of sub-bands were sometimes observed (e.g., compare the middle lanes in Fig. 4C), but these changes were not consistent in repeated experiments. Therefore, despite the fact that the maternal copy of *Igf2* is significantly repressed at the mRNA level (DeChiara et al. 1991; Ferguson-Smith et al. 1991), it does retain DNase I hypersensitive sites.

In contrast to the above results, MCA cells, in which *Igf2* is heavily methylated and not expressed, did not exhibit any hypersensitive sub-bands (Fig. 4B). This is in concordance with the observation of high resistance of MCA chromatin to *Msp*I. Similarly, no hypersensitive sites were detected in nuclei from adult spleen (Fig. 4B), where the CpG island remains unmethylated but *Igf2* transcripts cannot be detected (H. Sasaki et al., unpubl.). These observations indicate that under certain conditions, either in established cells or adult tissues, the P2–P3 region is no longer hypersensitive to DNase I and suggest that the *Igf2* gene can exist in several chromatin states.

Low-level expression of the maternal *Igf2* allele

Our findings suggest that in embryos, the chromatin of the repressed maternal allele of *Igf2* may be potentially

active for transcription. It is not known exactly to what degree the maternal allele is repressed. We designed a sensitive RT–PCR protocol to specifically quantitate primary transcripts initiated from all of the three promoters using sequence-specific oligonucleotide primers. The primary transcripts were assayed because large differences between the levels of primary transcripts and spliced mature mRNAs have been reported in some cases (Chang et al. 1991). Figure 5A shows the locations of the primers and probes used in this experiment. To detect primary transcripts specifically, the 5' primers (OP1 and OP5) were designed to hybridize to exon sequences, and 3' primers (OP2 and OP6) to intron sequences. PCR products were identified by hybridization with probe F or H to double-check the specificity. Throughout this study, care was taken to avoid contamination with chromosomal DNA: All samples were treated with DNase I before RT reaction; and to control for successful removal, samples were split and incubated with and without RT before PCR. No amplification was observed in RT– samples, thus ruling out any contamination with chromosomal DNA (see RT– lanes, Fig. 5C).

Pilot experiments with samples from normal e13 embryos were performed to determine the conditions adequate to measure relative abundance of *Igf2* transcripts.

Figure 5. RT-PCR assay of the primary transcripts for *Igf2*. (A) Map of the *Igf2* gene region corresponding to the PCR-amplified portions. The positions of the oligonucleotide primers (OP1, OP2, OP5, and OP6) are shown. Thick solid lines indicate the positions and lengths of the hybridization probes: (F) *Bst*II, 1.0 kb; (H) *Bam*HI, 2.4 kb. (B) Amplification curves obtained by primers OP1 and OP2. Different amounts of nuclear RNA from normal e13 embryos were used as templates. The amount of products after 40 cycles of RT-PCR initiated with 500 ng of the template RNA is shown as 100%. The proportionality of the RT-PCR products was maintained linearly up to 25 cycles. Very similar results were obtained with the other primers. (C) Autoradiographs of the Southern blots representing the

results. Reverse transcription (or cDNA synthesis) was carried out with (RT+) or without (RT-) reverse transcriptase. RT- lanes show the absence of DNA contamination. Transcripts for β -actin were detected by primers OP9 and OP10 and a cDNA probe. Quantitation of the PCR products was carried out by slot-blotting and densitometric scanning (see text).

The amplification reaction started with 0.5 μ g of the nuclear RNA proceeded in an exponential manner until 25–30 cycles of PCR and then reached the plateau phase. Therefore, we decided to use 25 cycles for quantitation. Further experiments with a series of dilutions of the same RNA sample showed that it was possible to determine the relative abundance of the *Igf2* transcripts fairly precisely under the conditions that we established (Fig. 5B).

When nuclear RNAs prepared from MatDi7 or control embryos (excluding the head region) at e13 were analyzed, both primer sets detected a low but significant expression of the maternal allele (Fig. 5C). To determine the transcript levels more precisely, the PCR products were slot blotted and the hybridization signals were measured by densitometry. The results (normalized by the β -actin levels) indicated that the level of primary transcripts in MatDi7 embryos is 3.5% (OP1 + OP2) or 7.4% (OP5 + OP6) of that in controls. We then examined the *Igf2* expression at an earlier e10 stage using parthenogenetic embryos instead of MatDi7 embryos. The parthenogenones collected for RNA preparation had variable morphology, but the majority of the embryos contained >10 somites. The relative level of primary transcripts in e10 parthenogenones compared with con-

trols was 4.7% (OP1 + OP2) or 7.5% (OP5 + OP6) (see also Fig. 5C). Thus, the two maternal copies of *Igf2* are repressed to very similar levels in MatDi7 and parthenogenetic embryos. In contrast, we could not detect any *Igf2* transcripts in MCA cells using either of the two primer sets, although the control primers readily detected transcripts for β -actin (Fig. 5C). Therefore, it appears that the degree of repression in MCA cells is greater than the degree of repression of the maternal alleles in MatDi7 and parthenogenetic embryos. Because the relative primary transcript levels described above are for two maternal copies, the estimated activity of the maternal allele is ~2–4% of that of the paternal allele.

The observed levels of transcript in MatDi7 and parthenogenones can be due to either a high level of expression in a small proportion of cells or a rather uniform low level of expression in almost all *Igf2*-expressing cells. The in situ hybridization data on mice with a disrupted *Igf2* gene support the latter idea because the maternal allele of the intact *Igf2* was active only in the choroid plexus and leptomeninges of the brain (which was excluded in our RT-PCR) (DeChiara et al. 1991). To address this question further, we have carried out in situ hybridization analysis on MatDi7 and parthenogenetic embryos. Figure 6 shows the *Igf2* expression patterns in

Figure 6. In situ hybridization analysis of control (A,B) and MatDi7 (C,D) embryos at e15. A and C are bright-field illumination; B and D are corresponding dark-field illumination. The control embryo (B) shows widespread expression of *Igf2* in many tissues. In the experimental MatDi7 embryo (D), expression was undetectable except that high-power magnification revealed clear expression in the choroid plexus and leptomeninges (not shown).

MatDi7 and control embryos at e15. There was a marked difference in the levels of expression between these embryos, with greatly reduced expression in the MatDi7 embryos. Careful microscopic examination under high magnification of individual tissues did not reveal tissues with high levels of expression in MatDi7 embryos except for the leptomeninges and choroid plexus. Computer analysis of in situ grain counts for these expressing tissues, when compared with the background in nonexpressing tissues, gave values of 328 and 94 per unit area, respectively. We also examined e10 parthenogenones, but no significant *Igf2* expression was detected in any cell type (not shown).

Methylation differences upstream of the gene

Because no parent-specific differences were found in ei-

ther DNA methylation or sensitivity to nucleases within the P2–P3 region, we have set out to determine whether such changes exist in other parts of this genomic region. This analysis was initially carried out in interspecific hybrids between *Mus domesticus* and *Mus spretus*, in which the parental alleles are distinguishable. A double digest with *DraI* and *HpaII* of adult liver DNA hybridized with probe B, located 3 kb upstream of E1 (Fig. 7A), reveals a 5-kb band in *M. domesticus* (C57BL/6J strain, B6), which is absent in *M. spretus* (Fig. 7B). This is because of a *HpaII*–*MspI* site polymorphism between the two species. Crosses were therefore performed to obtain reciprocal combinations of the two types of chromosomes in hybrid offspring. In offspring of the cross B6 (female) $\times$ *spretus* (male), the 5-kb band is almost completely undetectable; whereas in the reciprocal cross [actually a (B6 $\times$ *spretus*) $\times$ B6 backcross in which the *spretus* Chr7 was maternally inherited], this band is prominent. This observation suggests that *HpaII* sites within this fragment are more methylated on the paternal chromosome.

To confirm this finding in the MatDi7 system and to get a better idea of where these sites might be located, we performed *EcoRI* and *HpaII*, and *XbaI* and *HpaII* double digests on DNA from MatDi7 and control embryos. Hybridization with probe A (Fig. 7A) showed that the 2.2-kb band in the *EcoRI* digest and the 1.45-kb band in the *XbaI* digest are much stronger in normal embryos than in MatDi7 embryos (Fig. 7C). Additional bands are stronger in normal embryos, for example, at 1.7 kb (*EcoRI*) and at 0.9 kb (*XbaI*). Hybridization of the same blot with probe C (Fig. 1) controls for the completeness of digestion (Fig. 7C). These results therefore indicate that multiple *HpaII* sites in this region are more methylated in normal embryos than in MatDi7 embryos, suggesting that the paternal chromosome, which has the active *Igf2* allele, is more methylated. Although our preliminary experiment on DNase I sensitivity did not show signs of hypersensitive sites in this region, more detailed studies of both DNA methylation and chromatin structure are now under way. Attempts to identify epigenetic changes in other regions around the locus are also being made.

Discussion

We have analyzed the methylation status and chromatin structure of the maternal allele of *Igf2*, which is repressed by parental imprinting, and have found that the situation is unique in many respects when compared with the current models of imprinting. First, we have shown that methylation of the entire CpG island, such as that seen with genes on the inactive X chromosome of female mice, is not involved in repressing the maternal allele. Moreover, a number of methylatable sites located within the gene but outside the island did not display any signs of differential methylation. Thus, the methylation behavior of *Igf2* also seems to differ from the imprinting of transgenes, where parental origin-dependent methylation differences are readily detectable at multiple sites distributed over considerable distances (several

Parental imprinting and chromatin of the *Igf2* gene

Figure 7. Differential methylation in the upstream region of the *Igf2* gene. (A) Map of the upstream region. Positions of E1 and two pseudogenes ($\Psi 1$ and $\Psi 2$) (Rotwein and Hall 1990) are shown. Restriction sites are: *EcoRI* (E); *XbaI* (Xb); *DraI* (D); and *HpaII*–*MspI* (H/M). Positions of enzyme sites are based on restriction mapping and sequencing by R. Feil et al. (unpubl.). Probe A is a 1.5-kb *EcoRI*–*HindIII* fragment; probe B is a 0.5-kb *MspI* fragment. (B) Adult liver DNA from C57BL/6J, *M. spretus*, the F₁ hybrid (B6 $\times$ *spretus*), and the backcross progeny [(B6 $\times$ *spretus*) $\times$ B6], in which the presence of the maternal *spretus* Chr7 was verified by using the polymorphism] was double digested with *DraI* and *HpaII* and hybridized with probe B. (C) DNA from MatDi7 and control fetuses was digested with *HpaII* plus either *XbaI* or *EcoRI* and hybridized with probe A. Control hybridization with probe C shows the identical digestion pattern of the two samples. Sizes are in kb.

kb) (Hadchouel et al. 1987; Reik et al. 1987, 1990; Sapienza et al. 1987; Swain et al. 1987; Sasaki et al. 1991). Because the methylation level at most sites within the polypeptide-coding region was almost 100% in sperm but considerably less than 50% in embryos, the paternal chromosome underwent demethylation at these sites during early development. Second, the P2–P3 region of *Igf2* was equally and highly accessible to *MspI* in maternal and paternal chromatin. The increased resistance, which we observed in the established cell lines, can be the result of the binding of methylated CpG-binding proteins caused by a higher density of methylated CpGs (Antequera et al. 1989). This is supported by the good correlation between the degree of resistance and the level of methylation in these cells. Third, both parental chromosomes exhibited a number of identical DNase I hypersensitive sites in the P2–P3 region. This is again in

contrast to the cases reported for X chromosome inactivation (Riley et al. 1984, 1986; Yang and Caskey 1987; Lin and Chinault 1988) and the imprinting of transgenes (Pourcel et al. 1990). Because it is well established that DNase I hypersensitive sites represent nucleosome-free regions generated by the displacement of histones by activator or other DNA-binding proteins (Elgin 1988; Gross and Garrard 1988), our observations indicate that the P2–P3 region of the maternal *Igf2* allele is accessible to nuclear factors and capable of forming protein-DNA complexes. Because the basis of heterochromatin is thought to be the 30-nm fiber formed by the coiling of the uninterrupted array of nucleosomes, it is unlikely that extensive heterochromatinization involving the promoters is responsible for the repression of the maternal allele.

Altogether, our results suggest that the chromatin of

the maternal allele of *Igf2* is in a potentially expressible or transcriptionally competent state at its promoters. We have observed a low but measurable level of expression from the maternal chromosome. The observed level (2–4% of the paternal activity) seems significant as the transcripts were not detectable when the heavily methylated and nuclease-resistant *Igf2* alleles in MCA cells were examined. By comparison, a recent RT–PCR study on a human X-linked gene, *TIMP*, showed that the activity of this gene is at least 100-fold less on the inactive X chromosome (Brown et al. 1990). Because high-level expression of the maternal *Igf2* allele is restricted to the choroid plexus and leptomeninges (which were excluded in our RT–PCR), as shown by in situ hybridization, it is very likely that the observed expression represents the level of activity of the maternal allele in most *Igf2*-expressing cells.

Although we did not observe any global difference in either DNA methylation or nuclease sensitivity at the promoters, we cannot rule out the possibility that subtle changes, present within the examined region, were overlooked. For example, we have only examined the methylation of CpG sites that are testable by restriction enzymes. Also, some nuclear proteins or protein complexes that may constitute the primary imprint could be associated with either of the parental alleles in a way that does not affect nuclease sensitivity. Another possibility, which we sought in this study, is that the epigenetic modifications associated with imprinting could be present somewhere outside the promoters. We found that multiple *HpaII* sites located 3–4 kb upstream of E1 are more methylated on the paternal chromosome, from which the gene is expressed. This was found both by comparing MatDi7 and normal embryos, as well as in adult liver when comparing offspring from reciprocal interspecific crosses. It is interesting to note that the Prader–Willi/Angelman region in the human shows methylation imprinting, with some sites being more methylated on the paternal chromosome and others on the maternal chromosome (Nicholls et al. 1992). We cannot therefore exclude the possibility that there are sites elsewhere in the locus that are more methylated on the maternal chromosome. At present, we do not know whether the observed differential methylation is in any way involved in establishing or maintaining the imprint of the *Igf2* gene. It will also be interesting to see whether the changes are already present in the gametes or whether they arise after fertilization.

The mechanism by which parental-specific epigenetic modifications regulate *Igf2* expression continues to be a subject of speculation. However, whatever the imprint is, and wherever it exists, it should control the level of transcripts initiated from the multiple promoters. Our preliminary results from nuclear run-on experiments with MatDi7 nuclei suggest that the maternal allele is repressed at the level of transcription (H. Sasaki et al., unpubl.). A simple explanation for the control of the multiple transcripts was initially considered to be heterochromatinization of the entire locus, but this is now unlikely, as shown in this study. Other mechanisms that

can act on a domain basis, such as late replication and alteration of superhelical density by topoisomerase II, could be involved, but only if they are separable from heterochromatinization. There are a number of other possibilities, but the models described below have some attractive features and are therefore worth mentioning.

First, if transcriptional attenuation or premature termination occurs downstream of the three promoters, it will offer an efficient way of blocking all transcription. There is growing evidence that this type of regulation is important for many eukaryotic genes (for review, see Proudfoot 1989; Spencer and Groudine 1990; Kerppola and Kane 1991). In this connection, it is intriguing that antisense transcription of *Igf2* has been identified in chick embryos (Taylor et al. 1991), because it could interfere with the sense transcription. The second possibility, promoter occlusion, is another type of transcriptional interference, which down-regulates transcription at the initiation step (Proudfoot 1986; Corbin and Maniatis 1989). The effect is caused by transcription from an upstream promoter and could affect multiple downstream promoters. It is known that the insulin-2 (*Ins-2*) gene is ~17 kb upstream of *Igf2* (Rotwein and Hall 1990), but a systematic search for intergenic transcription has not been carried out in the mouse. The attenuation model and the promoter occlusion model are testable by detailed run-on transcription experiments. The third model assumes the presence of enhancers or silencers associated with *Igf2*. Epigenetic modifications might down-regulate transcription by controlling some of these distantly situated elements without affecting the basic activities of the promoters. Although none of these elements has been identified for *Igf2*, this model becomes particularly attractive if we consider the existence of the *H19* gene in close proximity to *Igf2*, as it has an enhancer in its 3'-flanking region (Yoo-Warren et al. 1988). The temporal and spatial distribution of *H19* expression during mouse development is similar to that of *Igf2* (Poirier et al. 1991). Moreover, this gene is imprinted in the opposite direction to *Igf2*—the maternal allele being active and the paternal allele repressed (Bartolomei et al. 1991). The *H19* enhancer consists of at least two separable enhancer elements (Yoo-Warren et al. 1988) and thus has the potential to interact with multiple promoters. Therefore, it is tempting to speculate that the *H19* enhancer interacts specifically with the *H19* promoter on the maternal chromosome and with the *Igf2* promoters on the paternal chromosome. The original model of promoter competition for an enhancer has been proposed for the developmental switching of the β -globin genes (Choi and Engel 1988). Parental origin-specific interaction between the common enhancer and the two genes could be achieved through the action of negative regulatory elements that block specific enhancer–promoter interactions. Sequence elements that inhibit enhancer activity have been identified in several mammalian genes and are called repressors (Vacher and Tilghman 1990), silencers (Nakabayashi et al. 1991), or sequestering elements (Stauffer and Ciejek-Baez 1992). The parental methylation differences that we have observed in the upstream

region could play a role in these models, especially in those involving remote regulatory signals.

Materials and methods

Animals and cells

Mice used to produce MatDi7 embryos were kindly supplied by B. M. Cattanach and C. V. Beechey (MRC Radiobiology Unit, Oxford, UK). The MatDi7 embryos were obtained from the T9Hd/+c (female) × T9H/+ + (male) intercrosses at the expected frequency of ~17% (Searle and Beechey 1990). A lack of eye pigment owing to the two maternal *c* alleles allowed us to distinguish the MatDi7 embryos from littermates. Parthenogenetic embryos were produced from unfertilized (C57BL/6J × CBA/Ca)F₁ eggs by the method described previously (Surani et al. 1988). After cytochalasin-B treatment, the parthenogenetic eggs containing two pronuclei were cultured for an additional 2 days and then transferred to the oviducts of day-1 pseudopregnant F₁ recipients. The derivation and characterization of cells from the MatDi7 embryos and their littermates will be described elsewhere (P.A. Jones et al., in prep.).

Isolation of nuclei

Whole embryos, portions of embryo and adult spleen were homogenized manually in 5 ml of nuclear buffer [60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] (Wu 1989) containing 0.3 M sucrose (nuclear buffer/0.3 M sucrose). In some experiments, 0.2 mM spermine and 0.5 mM spermidine were added. The homogenate was filtered through a pad of cheesecloth, layered over a 4-ml cushion of nuclear buffer/1.2 M sucrose, and centrifuged at 8000 rpm for 20 min. Pelletted nuclei were resuspended in a small volume of nuclear buffer/0.3 M sucrose/5% glycerol and stored at -80°C. To obtain nuclei from cultured cells, cells were first washed with phosphate-buffered saline in 100-mm dishes and then allowed to lyse in 1 ml/dish of a cold solution composed of 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.5 mM DTT, 0.1 mM PMSF, 0.32 M sucrose. Crude nuclei were collected by centrifugation at 4000 rpm for 20 min. Samples were resuspended and stored as described above.

Nuclease digestion of nuclei

For *MspI* digestion, nuclei equivalent to 10 µg of DNA were resuspended in 90 µl of 10 mM KCl, 10 mM Tris-HCl (pH 7.7), 10 mM MgCl₂, and 10 mM β-mercaptoethanol. The samples were combined with 10 µl of the same solution containing 0.75, 1.5, 3, 6, or 12 U/µl of *MspI* (Stratagene) [final concentration, 75, 150, 300, 600, or 1200 U/ml, respectively] and digested at 37°C for 30 min. DNase I digestions were carried out with nuclei equivalent to 20 µg of DNA in 90 µl of nuclear buffer/0.3 M sucrose/5% glycerol. After the addition of 10 µl of the same solution containing 0.03, 0.06, 0.125, 0.25, 0.5, or 1 U/µl of DNase I (Pharmacia) [final concentration, 3, 6, 12.5, 25, 50, or 100 U/ml, respectively], the samples were digested at 25°C for 5 min. These reactions were terminated by adding an equal volume of 20 mM EDTA (pH 8.0), 1% SDS containing 0.5 mg/ml of proteinase K. Proteinase K digestion was carried out overnight at 37°C. DNA was purified by phenol-chloroform extraction, followed by ethanol precipitation, and then subjected to Southern blot analysis.

RT-PCR

Nuclear RNA was isolated from the nuclei preparations by the method of Chirgwin et al. (1979). To destroy contaminated chromosomal DNA, RNA samples (5 µg) were brought into 25 µl of 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM CaCl₂, and digested with 10 units of DNase I (RNase free, Stratagene) at 37°C for 5 min. RNA of 0.5 µg was mixed with 2 µl of 10× RT buffer [0.5 M Tris-HCl (pH 8.0), 0.75 M KCl, 30 mM MgCl₂, 0.1 M DTT], 1 µl of 3' primer (OP2, OP6, or OP10) (160 ng), and 2 µl of 5 mM dNTPs. The final volume was adjusted to 19 µl with water, and the mixture was incubated at 72°C for 2 min. The sample was then split into two halves, and 0.5 µl of Moloney murine RT (10 units, Stratagene) was added to one of them. The other half served as a negative control to confirm the absence of DNA contamination. Both tubes were incubated at 37°C for 30 min. A small portion of RT products (2 µl) was mixed with 77 µl of water, 10 µl of 10× PCR buffer (Cambio), 3 µl of 3' primer (480 ng), 3 µl of 5' primer (480 ng), 4 µl of 5 mM dNTPs, and 1 µl of *Taq* DNA polymerase (5 units, Cambio). The mixture was covered with a layer of mineral oil and amplified by PCR for 25 cycles. Each cycle consisted of 1 min at 94°C, 30 sec at 65°C, and 1 min at 72°C. A portion of the PCR product (20 µl) was analyzed by Southern blotting or by slot blotting. In each experiment, DNA contamination was excluded by the fact that no signal was obtained if no reverse transcriptase was added. The oligonucleotide primers used are as follows: OP1 (exon 3-intron 3, 5'), 5'-AGCGGCCTCCTTACCCAACTTCAG-3'; OP2 (exon 3-intron 3, 3'), 5'-TGACCTCGAAGTCCCTTCCCAAAG-3'; OP5 (exon 4-intron 4, 5'), 5'-ATCCCAGTGGGGAAGTCGATGTTG-3'; OP6 (exon 4-intron 4, 3'), 5'-AAGGGGTAGCTTAAGAGCAAGGCC-3'; OP9 (β-actin, 5'), 5'-GCTGTGCTATGTTGCTCTAGACTTC-3'; OP10 (β-actin, 3'), 5'-CTCAGTAACAGTCCGCCTAGAAGC-3'.

DNA blot analysis

Genomic DNAs isolated from embryos, sperm, and nuclease-digested nuclei were cut with appropriate restriction enzymes according to the manufacturer's recommendations. Restricted DNA fragments and RT-PCR products were electrophoresed on 0.8–1.5% agarose gels, blotted onto nylon membranes (Hybond-N⁺, Amersham), and UV-cross-linked (Stratalinker, Stratagene). A portion of RT-PCR product was also blotted by Bio-Dot SF microfiltration apparatus (Bio-Rad) for a densitometric analysis. Hybridization probes were subcloned from the cosmid clone cosIGF4 (Rotwein and Hall 1990), which was kindly provided by P. Rotwein (Washington University, St. Louis, MO), and radiolabeled by the random priming method of Feinberg and Vogelstein (1984). The membranes were hybridized with the probes in the solution described by Church and Gilbert (1984), and washing was performed in 0.1× SSC, 0.1% SDS, at 65°C. Densitometry of band intensities was done on a Chromoscan 3 (Joyce Loeb).

In situ hybridization

Preparation of embryo sections, generation of the probes, and in situ hybridization were performed as described (Ohlsson et al. 1989). The riboprobe was produced by using a plasmid clone containing a 680-bp *HinfI*–*PstI* human cDNA insert.

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