Parental Chromosome-specific Chromatin Conformation in the Imprinted U2af1-rs1 Gene in the Mouse*

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The imprinted U2af1-rs1 gene on mouse chromosome 11 is expressed exclusively from the paternal allele. We found that U2af1-rs1 resides in a chromosomal domain that displays marked differences in chromatin conformation and DNA methylation between the parental chromosomes. Chromatin conformation was assayed in brain and liver, in fetuses, and in embryonic stem cells by sensitivity to nucleases in nuclei. In all these tissues, the unmethylated paternal chromosome is sensitive to DNase-I and MspI and has two DNase-I hypersensitive sites in the 5′-untranslated region. In brain and in differentiated stem cells, which display high levels of U2af1-rs1 expression, a paternal DNase-I hypersensitive site is also readily apparent in the promoter region. On the maternal chromosome, in contrast, the entire U2af1-rs1 gene and its promoter are highly resistant to DNase-I and MspI in all tissues analyzed and are fully methylated. No differential MspI sensitivity was detected in this imprinted domain. The parental chromosome-specific DNA methylation and chromatin conformation were also present in parthenogenetic and androgenetic cells and in tissues from animals maternally or paternally disomic for chromosome 11. This demonstrates that these parental chromosome-specific epigenotypes are independently established and maintained and provides no evidence for interallelic trans-sensing and counting mechanisms in U2af1-rs1.

Parental imprinting is an epigenetic mechanism in mammals that gives rise to differential expression of the maternally and paternally inherited alleles of certain genes. The identification of the epigenetic modifications (imprints) responsible for parental allele-specific expression has gained considerable impetus since the discovery of the first imprinted genes, and it is generally assumed that primary modifications are established in the germline or prior to synangy after fertilization (1, 2). CpG methylation is an epigenetic modification of DNA that has been found to correlate with the allelic expression of imprinted genes (3). In several imprinted genes, such as H19, SNRPN, and U2af1-rs1 (4–8), DNA sequences involved in expression have shown that DNA methylation is clearly required for at least the somatic maintenance of monoallelic expression (11, 12). In the imprinted genes analyzed so far, with the possible exception of a region upstream of H19 (13) and “region 2” of Igf2 (10), the allelic methylation patterns seem, however, to arise after fertilization, during early development (14–16). They would therefore not necessarily constitute primary imprints and may reflect other, pre-existing epigenetic features of chromatin.

It has been found that autosomal imprinted gene regions replicate earlier on the paternal than on the maternal chromosome during S phase (17, 18). Earlier studies had established asynchrony of replication of genes on the differently compacted, active, and inactive X chromosome in female mammals (19). The asynchronous replication of imprinted and X-linked genes suggests that differential chromatin compaction may be another characteristic of mono-allelically expressed genes. For the imprinted Igf2/H19 domain on mouse distal chromosome 7, this has been studied more directly by our laboratory and by others in assays that analyze allelic sensitivity to nucleases in nuclei. No parental chromosome-specific chromatin configuration was thus detected in the Igf2 gene, including its promoters (20, 21), and in the body of the H19 gene. Both regions were equally sensitive to DNase-I on the maternal and the paternal chromosome, and the same was found for the 90-kb region between the two genes (22). However, the H19 promoter was found to be more sensitive to nucleases on the expressed maternal than on the repressed paternal chromosome (4). This finding suggests chromosome-specific chromatin compaction in the regulatory sequences of H19, which could be associated with the allelic expression of this imprinted gene.

To gain more insight into the role of chromatin in the regulation of imprinting, we analyzed allelic chromatin conformation in the imprinted mouse U2af1-rs1 gene. U2af1-rs1 (named SP2 (6) or U2afbp-rs (7) in some previous studies) is a small, intronless gene that is paternally expressed in embryonic and adult tissues. It codes for a protein with homology to the splicing factor U2 small nuclear ribonucleoprotein auxiliary factor (6, 7). It is the only imprinted gene identified on mouse proximal chromosome 11, the paternal disomy of which gives fetal overgrowth, whereas maternal disomy leads to fetal growth retardation (23). It has been shown previously that specific

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1 The abbreviations used are: kb, kilobase(s); Ag, androgenetic; ES, embryonic stem; HSS, hypervarsensitive site(s); MNase, micrococcal nuclease; Matdi-11, maternal disomy 11; Patdi-11, paternal disomy 11; Pg, parthenogenetic; RFLP, restriction fragment length polymorphism; UTR, untranslated region(s).
CpG dinucleotides in the promoter and in the 5'-untranslated region (UTR) of U2af1-rs1 are methylated on the repressed maternal allele (6, 7, 24). In this study we show that, in fact, a larger domain comprising the entire U2af1-rs1 gene becomes completely methylated on the maternal chromosomes during early development but remains unmethylated on paternal chromosomes. Most interestingly and in contrast to the previous studies on the imprinted Igf2 and H19 genes, the parental chromosomes also showed a pronounced difference in general-ized sensitivity to nucleases in the entire U2af1-rs1 domain of differential methylation. We also characterized paternal DNase-I hypersensitive sites (HSS) in the 5'-UTR and the promoter of the gene. These sites have been independently identified by Shibata and colleagues (25) but only in a single tissue. Our study, however, specifically addresses the nature and the developmental regulation of differential chromatin conformation in the U2af1-rs1 domain. It shows that the par-ental chromosome-specific chromatin conformation and the maternal DNA methylation in the U2af1-rs1 domain are intrin-sically linked and become established before implantation.

It has been proposed that intergenicomic and trans-allelic interactions play a role in the mechanism of genomic imprinting (16, 26–29). Analysis of allelic expression and methylation interactions play a role in the mechanism of genomic im-printing before (21), and the unit definition was as defined by the manufacturer (Boehringer Mannheim). Micrococcal nuclease (MNase) digestions were performed on purified nuclei for 5 min at 37 °C in 15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 0.34 M sucrose, 10 mM NaH₂SO₄, 1 mM CaCl₂. MspI digestions on nuclei were performed at 37 °C in 50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol.

After incubation with DNase-I, MNase, or MspI, the 200-bp reactions were terminated by the addition of an equal volume of 20 mM EDTA, pH 8.0, 1% SDS. Proteinase-K was added to 200 µg/ml, and proteinase-K digestion was carried out overnight at 37 °C, after which genomic DNA was extracted.

**EXPERIMENTAL PROCEDURES**

**Mice and ES Cell Lines—**Hybrid F1 mice were produced by crossing C57BL/6 females to Mus spretus males, and the reciprocal F2 mice were produced by backcrossing F1 females to C57BL/6 males. Mice maternally (Mat-11) or paternally (Pat-11) disomic for proximal chromosome 11 were produced by intercrossing mice heterozygous for the Robertsonian translocation, Rb(11.13)4Bnr (23). Pg embryos and Pg and Ag ES cells were derived as described before (32). Biparental, hybrid ES cells were derived as follows: mature (C57BL/6 × CA/Ca)F1 eggs were in vitro fertilized with M. spretus sperm, embryos were cultured in vitro to the blastocyst stage, and ES cell lines were derived subsequently as described before (32). ES cell lines were main-tained on feeder cells, but for the chromatin and methylation assays, culture was performed in the absence of feeder cells, and only semicon-fident early passage (-passage 6) cells were used, which showed <10% of morphologically apparent differentiation. For in vitro differentiation, Ag and normal ES cells were seeded at low density on tissue-culture dishes and grown in ES medium without LIF. After 5 days, semi-confluent early differentiating cells (>90%) were obtained that appeared mostly parietal endoderm in morphology (32).

**Genomic Cloning of the U2af1-rs1 Region—**Phage AmU2D1 was isolated from a genomic library of 129/Sv DNA (33) using a Nor1-EcoRV fragment from the human pSP2 (6). Mapping of endonuclease restriction sites in this 15-kb phage (see Fig. 1) was performed by partial digestion and labeling with left arm- and right arm-specific oligonucleotide probes.

**Nuclease Sensitivity Assays—**Nuclei were isolated from tissues and ES cells as described previously (21). DNase-I assays were described before (21), and the unit definition was as defined by the manufacturer.
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(Fig. 3), and completely digests the unmethylated paternal allele, DNase-I sensitivity was thus assayed specifically on the maternal chromosomes, which did not show any HSS sites in the U2af1-rs1 domain (Fig. 2B). The paternal chromosome-specific promoter HSS was not as pronounced in liver (Fig. 2A) or embryonic stem cells (see Fig. 5A), where U2af1-rs1 expression levels are lower than in brain (6). However, in (C57BL/6 × M. spretus) F1 day 15 fetuses we detected paternal hyper-sensitivity in the promoter region, albeit not as strong as in adult brain, possibly due to the proportional contribution of brain (or other high expressing tissues) in these fetuses (not shown).

To establish whether the two HSS in the 5′-UTR were solely responsible for the preferential paternal DNase-I sensitivity or whether a generalized sensitivity difference is established over a larger domain, we analyzed DNase-I sensitivity in the 3′ part of the gene (in SacI+BgII digests). No HSS were detected in this part of the gene in brain and liver, but in both tissues, the paternal chromosome was again more sensitive to DNase-I than the maternal chromosome (Fig. 2C). Hence, preferential paternal sensitivity appears to be established in the entire U2af1-rs1 gene. In both liver and brain, we also analyzed the parental chromosomes for their accessibility to the methylation-insensitive restriction endonuclease MspI, which has multiple recognition sites throughout the gene and its promoter (Fig. 1B). Like in the DNase-I assays, a pronounced difference in sensitivity between the parental chromosomes was detected, with paternal chromosomes displaying much higher sensitivity to MspI than maternal chromosomes, both in the polymorphic BglII fragment covering the entire gene (not shown) and in the SacI-BgII fragment covering the 3′ part of the gene (Fig. 2C).

To investigate further the nature of the differential chromatin conformation, we analyzed the U2af1-rs1 domain for its sensitivity to MNase, an enzyme that digests preferentially the linker DNA between nucleosomes. Hence, nuclei from (C57BL/6 × M. spretus) F1 livers were incubated with increasing concentrations of MNase, and the BglII RFLP polymorphism was used to differentiate between the parental chromosomes. We found that both in the BglII fragment covering the entire gene (not shown) and in the SacI-BglII fragment covering the 3′ part of the gene (Fig. 2C), the domain of maternal and paternal chromosomes were equally sensitive to MNase. In particular, the degree of MNase digestion of the paternal chromosome and the maternal SacI-BglII fragments related to their respective lengths, with the 2.6-kb maternal fragment being about twice as sensitive as the 1.3-kb paternal fragment (Fig. 2C).

The Entire U2af1-rs1 Gene Is Maternally Methylated—Because preferential paternal DNase-I sensitivity was detected in all the tissues analyzed (liver, brain, fetus, and stem cells), it was of interest to determine whether maternal chromosome-specific methylation was equally widespread. Indeed, the NotI restriction site in the 5′-UTR was maternally methylated and paternally unmethylated in all embryonic and extraembryonic tissues analyzed, including brain, choroid plexus, liver, spleen, kidney, heart, skeletal muscle, and placenta (not shown). The paternal chromosome is not required for the establishment and maintenance of maternal methylation in U2af1-rs1, because the NotI restriction site was fully methylated in fetuses that were MatDi-11. Conversely, the maternal chromosome is maintained unmethylated during development, even in the absence of the maternal chromosome, because in PatDi-11 fetuses, the NotI restriction site was completely unmethylated (data not shown, but see Fig. 3). To determine the extent of maternal methylation in more detail, we analyzed the allelic methylation status of all the HpaII restriction sites in three adjacent HindIII fragments (together spanning 10.8 kb of DNA; see Fig. 1) by comparing the MatDi-11 and PatDi-11 fetuses and tissues (Fig. 3). All HpaII restriction sites in the gene and in the promoter were methylated on the maternal allele and appeared fully digested by HpaII on the paternal allele (probes 1 and 13). However, a HpaII restriction site at 1 kb upstream of the gene was partially demethylated on the maternal allele in liver and brain (probe 13), and two HpaII sites at ~4 kb downstream of the gene were methylated on both parental chromosomes in the fetus and in liver (probe 4). Also in brain the latter two HpaII sites were fully methylated on the paternal allele, but one of these two sites was partially demethylated on the maternal chromosome. It follows that the domain that is methylated on the maternal allele and unmethylated on the paternal allele comprises at least the entire U2af1-rs1 gene and its promoter.

A PstI polymorphism between C57BL/6 (5.5-kb fragment) and M. spretus (7-kb fragment) was used to analyze DNAse-I sensitivity allele-specifically in the biparentally methylated region downstream of U2af1-rs1. For the chromatin analysis of this region we used fragment 4, which is 4 kb downstream of the gene, to probe Southern blots. The maternal and paternal chromosomes appeared equally accessible to DNase-I in this region, both in liver (Fig. 4A) and in brain (not shown).

In addition, two closely linked, strong HSS were detected in liver (Fig. 4A) but not in brain (not shown), and these two sites are likely to be on both parental chromosomes (Fig. 4A). To map the two HSS more precisely, we digested the DNase-I treated liver nuclei DNAs with the enzyme EcoRV and hybridized the electrophoresed DNA with probe 4 (not shown). This indicated that the two HSS are 5.5 kb downstream of the gene (Fig. 1). This location was verified because no strong HSS were detected
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Fig. 2. Parental chromosome-specific nuclease sensitivity in the U2af1-rs1 gene. A, paternal DNase-I sensitivity and hypersensitivity sites in the 5′-UTR. A BglII RFLP was used to analyze adult liver nuclei, isolated from (C57BL/6 × M. spretus) F1 and (C57BL/6 × M. spretus) × C57BL/6 (F2) mice. After incubation at increasing concentrations of DNase-I (lanes 1–7) were 0, 20, 50, 100, 150, 200, and 300 units/ml, respectively. DNA was extracted from the nuclei, digested with BglII, electrophoresed, and probed with fragment 1. To the left, the BglII fragments in C57BL/6 (M) and M. spretus (S) adult livers, and the 1.8-kb BglII-NotI digestion product that derives from the unmethylated paternal chromosome in the (M × S) F1 adult liver. For the (M × S) F1 DNase-I series, the ratios of the intensities of the maternal (5.6 kb) and the paternal (4.3 kb) bands were 1.1, 1.5, 3.2, and 3.7 for lanes 1–4, respectively. Arrows indicate DNase-I digestion products corresponding to HSS. Fragment sizes are in kb. B, a promotor-specific paternal DNase-I HSS in adult brain. In the left panel, BglII-digested (M × S) F1 nuclei DNA, probed with fragment 1, showing the presence of the two HSS in the 5′-UTR, and a prominent HSS in the promoter. DNase-I concentrations (lanes 1–7) in this analysis were as in A. The ratios of the intensities of the maternal (5.6 kb) and the paternal (4.3 kb) bands are 0.9, 1.0, 1.5, 3.4, and 4.9 for lanes 1–5, respectively. In the right panel, the same DNase-I-digested nuclei DNA, were digested with BglII and HpaII. The methylation-sensitive restriction enzyme HpaII digested sites on the unmethylated paternal allele (4.3-kb fragment) exclusively and thereby allowed analysis of DNase-I sensitivity on the maternal chromosome. The weak band of 5.3 kb corresponds to a single, partially demethylated HpaII site on the maternal chromosome 1 kb upstream of U2af1-rs1 (see also Fig. 3). Fragments corresponding to DNase-I HSS are indicated with arrows. C, paternal chromosome-specific nuclease sensitivity in 3′ part of the U2af1-rs1 gene. In the left panel, DNase-I digested (M × S) F1 liver nuclei DNA (the DNase-I concentrations in 1–8 were 0, 20, 50, 100, 200, 300, 500, and 800 units/ml, respectively) were digested with BglII and SacI, electrophoresed, and probed with fragment 1. The parental 1.3-kb fragment is more sensitive to DNase-I digestion product that derives from the unmethylated paternal chromosome in the (M × S) F1 adult liver. For the (M × S) F1 DNase-I series, the ratios of the intensities of the maternal (5.6 kb) and the paternal (1.3 kb) bands are 1.4, 1.6, 4.0, and 4.4 for lanes 4–7, respectively. In the middle panel, the hybrid liver nuclei were incubated for increasing periods of time (0, 1, 2, 5, 7.5, 10, 15, and 30 min, in lanes 1–8, respectively) with 30 U/ml of MNase. DNA was extracted and digested with BglII for allelic DNase-I sensitivity and DNA methylation using the BglII polymorphism between the two mouse species (Fig. 1). The hybrid cell lines were obtained by in vitro fertilization of M. m. domesticus eggs with M. spretus sperm, followed by in vitro culture of the hybrid embryos to blastocysts, from which ES cells were then derived. In total, five early passage hybrid lines were analyzed. U2af1-rs1 was found to be maternally methylated in the 2.8-kb HindIII fragment downstream of U2af1-rs1 (Fig. 4B). However, a less prominent HSS was present in liver and brain in this HindIII fragment (Fig. 4B). The relative weakness of this HSS, however, did not allow us to determine (using the PstI polymorphism) whether it was also present on both parental chromosomes. Finally, with probe 6 we analyzed DNase-I sensitivity in a 4.5-kb BamHI fragment located upstream of the U2af1-rs1 gene (Fig. 1A), but no strong HSS were apparent in this upstream region (not shown).

Developmental Regulation of Parental Chromosome-specific Chromatin Conformation and DNA Methylation—To determine whether the differential chromatin structure and the maternal chromosome-specific DNA methylation in the U2af1-rs1 domain are present before implantation and whether both parental genomes are required for the establishment and maintenance of the paternal chromosome structure without DNA methylation. Conversely, for the establishment of the “closed” maternal chromatin conformation with full DNA methylation, it is only the maternal genome that is required. In the five early passage Pg cell lines we analyzed, the NotI restriction site in the U2af1-rs1 gene was fully methylated, and no DNase-I HSS were detected in the 5′-UTR (Fig. 5A). We obtained the same result by analysis of DNA methylation in Pg (day 9.5) embryos, in which the U2af1-rs1 gene was also methylated (not shown). The U2af1-rs1 methylation in the Pg ES cell lines, however, was found not to be stably maintained on prolonged culture, and in several of the lines, loss of methylation was observed (not shown). Our analysis of early passage monoparental ES cell lines demonstrates that already at the blastocyst stage, at least in the inner cell mass, the parental chromosome-specific chromatin organization and DNA methylation are fully established in the U2af1-rs1 domain.

To investigate the situation in biparental ES cells, in a parental chromosome-specific manner, we derived new (M. m. domesticus × M. spretus) F1 stem cell lines and analyzed them for allelic DNase-I sensitivity and DNA methylation using the BglII polymorphism between the two mouse species (Fig. 1). The hybrid cell lines were obtained by in vitro fertilization of M. m. domesticus eggs with M. spretus sperm, followed by in vitro culture of the hybrid embryos to blastocysts, from which ES cells were then derived. In total, five early passage hybrid lines were analyzed. U2af1-rs1 was found to be maternally methylated.
Maternal chromosome-specific DNA methylation in the U2af1-rs1 domain. Matdi-11 and Patdi-11 DNAs were digested with HindIII and HpaII, electrophoresed, and hybridized with probes 13, 1, and 4. Lane 1, Matdi-11 day 16 fetus; lane 2, Matdi-11 newborn liver; lane 3, Matdi-11 newborn brain; lane 4, Patdi-11 d16 fetus; lane 5, Patdi-11 newborn liver; lane 6, Patdi-11 newborn brain. Fragment sizes are in kb. The HpaII restriction sites in the three HindIII fragments are indicated in the map shown below. One of the HpaII sites in the 5' part of the gene corresponds to the NotI restriction site analyzed in other studies (6, 7). Summarized for liver and brain are the levels of methylation of these HpaII restriction sites determined on the maternal (Mat.) and paternal (Pat.) chromosome. Filled circles, >90% methylation; open circles, no methylation; partially filled circles, blackened proportional to the level of partial methylation. We hybridized with probe 6 to determine the status of the paternal methylation of the HpaII site 1 kb upstream of the gene and found it to be partially methylated similar to the maternal allele (not shown).

To determine whether the absence of methylation on the maternal allele in some of the biparental hybrid lines was due to the genetic background of these ES cells, we have also analyzed U2af1-rs1 methylation levels in 17 homozygous ES cell lines of the 129/Sv and C57BL/6 genotypes, which were all of higher passage (>passage 10). Although nine of these lines displayed the expected methylation level of ~50% at the NotI restriction site, eight others were fully unmethylated at this site (data not shown).

The levels of U2af1-rs1 expression were relatively low in the undifferentiated Ag and normal, hybrid, ES cell lines (Fig. 5C). They may therefore not be very significant relative to the overall methylation of the chromatin status of the parental chromosomes in these lines. However, the allele-specificity of expression correlated absolutely with parental chromosome-specific DNA methylation and chromatin configuration. No expression was detected on Northern blots in Pg ES cells, and in the hybrid lines with differential chromatin structure and methylation (SF1-1 and SF1-11), exclusive paternal expression was detected by allele-specific reverse transcription-polymerase chain reaction analysis. In the hybrid lines with a nucleo-
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accessible to transcription factors and therefore permissive to expression. The levels of expression may depend on the tissue-specific availability of such trans-acting factors. In addition to this consistent property, the chromatin organization at the presumed regulatory sequences of the gene (24) varies according to the levels of U2af1-rs1 expression. Thus, an additional, paternal HSS was readily present in the promoter in brain and in differentiated ES cells but was not detected in liver and in undifferentiated ES cells and may therefore be directly associated with paternal gene expression.

We found the U2af1-rs1 gene to be maternally methylated and paternally unmethylated in all embryonic and extraembryonic tissues analyzed. These included adult kidney, heart, skeletal muscle, and liver, tissues that have either very low or nondetectable levels of U2af1-rs1 expression (6). This shows that the maternal methylation, like the parental chromosome-specific chromatin conformation, may be permissive for allelic expression of U2af1-rs1 and does not reflect levels of paternal expression.

Differences in nuclease sensitivity have been found previously at the promoter of the imprinted H19 gene (4). In addition, Shibata et al. (25) have independently identified the paternal chromosome-specific HSS in the U2af1-rs1 gene and examined allele-specific methylation but only in adult liver. Our more comprehensive studies examined a range of tissues and developmental stages, using monoparental in addition to biparental material and employing different nuclease probes. In particular, we document generalized paternal chromosome-specific DNase-I and MspI sensitivity over a larger region comprising the entire U2af1-rs1 gene.

By comparing HpaII digestion profiles in DNAs from Matdi-11 and Patdi-11 mice, we estimate the domain of allele-specific methylation at least to encompass the entire gene. This appears to coincide well with the region of generalized differential nuclease sensitivity. Outside this region, biallelic methylation was detected, and DNase-I HSS were present on both chromosomes ~4 kb downstream of the gene. It is likely that these downstream HSS are not associated with U2af1-rs1 expression. The U2af1-rs1 gene appears to be a recent retrotransposon, located within an intron of a larger gene that is not imprinted (Murr-1; Ref. 34), so it is possible that the downstream HSS are involved in the expression of this new gene.

The relative contributions made by methylation and chromatin structure to the maintenance of monoallelic expression of U2af1-rs1 are not easy to quantitate; the two were invariably linked in all the material we examined. Methylation can repress transcription directly by inhibiting transcription factor binding (35, 36), although some factors are indifferent to the methylation of CpGs in their recognition sites (37). Demethylation by 5-azacytidine is sufficient to induce transcription factor binding, HSS formation and derepression of silent genes (38, 39), but not invariably (40). There is also evidence for a less direct mechanism in transcriptional repression. Methylated transfection constructs appear to require organization into chromatin before expression becomes repressed (41, 42). Methyl-CpG-binding proteins, whose binding to DNA is determined by the density of methylation rather than sequence, are thought to be at least partially involved in this indirect repression of transcription (37, 43-45). It may be significant that we found the methylated maternal U2af1-rs1 domain to be highly resistant to MspI in nuclei, but to which extent this can be accounted for by methyl-CpG-binding proteins bound to these CpG-containing restriction sites remains to be determined.

At first glance, it may seem surprising that we detected no difference in the rate of digestion by MNase of the parental alleles, despite marked differences in sensitivity to DNase-I and MspI in the same nuclei. This would appear to indicate that both alleles are similarly packaged into nucleosomes and that if the closed chromatin conformation of the maternal allele represents a more compact or higher ordered structure, this has not altered accessibility of linker DNA to MNase. We cannot, however, rule out the possibility of more subtle differences in nucleosomal organization. Several studies suggest that the composition of nucleosomes, including the distribution of linker histone H1 (46-48) and acetylation of core histones (49-51), may differ on active and inactive genes, and similar differences may exist between the active and repressed alleles of imprinted genes.

Developmental Control of Chromatin Conformation, DNA Methylation, and Allelic Expression—Whether methylation and/or chromatin organization constitute the primary imprinting signals of the U2af1-rs1 gene and initially determine monoallelic expression remains to be determined. The absence of methylation and the hypersensitivity in the 5′-UTR in early passage Ag stem cells (derived by pronuclear transfer) and full methylation and a highly DNase-I-resistant chromatin conformation in early passage Pg ES cells (derived by activation of eggs), however, indicate that germline imprints must exist on both paternal and maternal alleles. Recently, using a polymerase chain reaction assay to detect methylation of HhaI sites (up
to four) at the U2af1-rs1 promoter, Hatada et al. (24) reported that these sites were unmethylated in oocytes and two-cell embryos. This region is also unmethylated in sperm DNA in which, in contrast, we found the NoI restriction site in the 5′-UTR to be fully methylated. The maternal allele has previously been found to become methylated by embryonic day 11 (24), and our results from early passage ES cells indicate that distinct methylation and chromatin states exist already at the blastocyst stage. Expression of U2af1-rs1 RNA is detected at the two-cell stage, preferentially or exclusively from the paternal chromosome (24, 31), and therefore, in the apparent absence of allele-specific methylation. It is possible that a germ-line methylation imprint does indeed exist but was not included in the regions examined so far. For example, at the imprinted H19 locus, HpaII and HhaI sites close to the promoter are methylated both in sperm and oocyte DNA, whereas sites further upstream are differentially methylated in the gametes, remain so in preimplantation embryos, and could constitute an imprinting signal (13).

What could give rise to monoallelic expression, however, if there was indeed no differential methylation in the zygote? Although it is not technically feasible to assay chromatin organization in preimplantation embryos, there are grounds for believing that distinct chromatin states, sufficient to determine monoallelic expression, could become established on the parental chromosomes at such early stages. The oocyte and sperm derived chromosomes both undergo remodeling in the fertilized oocyte (52), in particular, histones must replace protamines on the sperm chromosomes. It is therefore possible that the U2af1-rs1 gene could assume a chromatin organization permissive to expression preferentially in the paternal pronucleus. In this context, it is interesting to note that U2af1-rs1 has been reported to be among the earliest known genes to be activated in the embryonic genome (24, 31). In contrast, in the absence of transcriptional activation, the maternal allele may adopt a closed conformation that is subsequently “fixed” by methylation. The direct repeats in the 5′-UTR (6, 7), a possible characteristic of imprinted genes (53), could influence local DNA structure (54) and be involved in this early regulatory decision. In three of the newly derived hybrid ES cell lines and in about half of the higher passage homozygous lines we analyzed, the U2af1-rs1 gene was biallelically unmethylated. This may indicate that on derivation and subsequent culture of stem cells, the allelic methylation and chromatin conformation (hybrid ES cells) are not faithfully maintained. Indeed, we observed loss of U2af1-rs1 methylation on prolonged culture of the Pg ES cell lines, and in other imprinted genes changes in methylation levels have also been observed on culture of stem cells (55). The consequences for gene expression and development of epigenetic changes that result from in vitro manipulation during early development are being investigated in our laboratory.

Finally, our analysis of monoparental material allows us to conclude that the distinct methylation and chromatin states of the parental chromosomes are established and maintained independently. The methylation state of U2af1-rs1 in Patdi-11 DNA appeared like two copies of the paternal allele and like two maternal alleles in Matdi-11 DNA. Therefore, we do not envisage the necessity of trans-sensing between alleles (26–29) or the operation of a “counting mechanism” (16, 26). Furthermore, the fact that we detected a maternal chromatin and methylation epigenotype in the early passage Pg ES cell lines and in Pg embryos indicates that the maternal imprint is correctly established without the input of the paternal genome. The same applies to the paternal imprint because Ag ES cell lines had the correct paternal epigenotype, characterized by the DNase-I HSS in the 5′-UTR and unmethylated DNA. This could therefore be in contrast to the situation for clusters of imprinted genes, where normal biallelic inheritance appears to be necessary for some aspects of imprinting (16, 29).

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