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Promoter-restricted histone code, not the differentially methylated DNA regions or antisense transcripts, marks the imprinting status of *IGF2R* in human and mouse

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Imprinting of the mouse *Igf2r* depends upon an intronic differentially methylated DNA region (DMR) and the presence of the *Air* antisense transcript. However, biallelic expression of mouse *Igf2r* in brain occurs despite the presence of *Air*, and biallelic expression of human *IGF2R* in peripheral tissues occurs despite the presence of an intronic DMR. We examined histone modifications throughout the mouse and human *Igf2r*/*IGF2R* using chromatin immuno-precipitation (ChIP) assays in combination with quantitative real time PCR. Methylation of Lys4 and Lys9 of histone H3 in the promoter regions marks the active and silenced alleles, respectively. We measured di- and tri-methyl Lys4 and Lys9 across the *Igf2r* and *Air* promoters. While both di- and tri-methyl Lys4 marked the active *Igf2r* and the active *Air* allele, tri-methyl Lys9, but not di-methyl Lys9, marked the suppressed *Air* allele. We show here that enrichment of parental allele-specific histone modifications in the promoter region, rather than the presence of DNA methylation or antisense transcription, correctly identifies the tissue- and species- specific imprinting status of *Igf2r*/*IGF2R*. We discuss these findings in light of recent progress in identifying specific components of the epigenetic marks in imprinted genes.

INTRODUCTION

Genomic imprinting is a parent-of-origin epigenetic mechanism whereby one of the two parental alleles is preferentially suppressed, while the other parental allele is normally transcribed. In the mouse genome, 38 maternally imprinted (paternally expressed genes, PEG) and 35 paternally imprinted (maternally expressed genes, MEG) genes have been identified to date (http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html). In imprinted genes, the epigenetic information that is transmitted independently of DNA sequence is conveyed through alterations in nucleosome structure, resulting from covalent modifications of DNA (methylation) and of histones (e.g. acetylation and methylation). Recent studies of the epigenetic marks associated with imprinted genes have revealed that these epigenetic modifications occur with a differential preponderance on the expressed and silenced alleles (1–12).

Imprinted genes often cluster on large chromosome regions, forming imprinted domains that are regulated by *cis*-acting imprinting control regions (reviewed in 13,14). One of the well-characterized imprinted domains contains the gene that encodes the insulin-like growth factor-II (IGF-II) receptor/mannose-6-phosphate receptor (*IGF2R*). The gene is imprinted (maternally expressed) in rodents, marsupials and artiodactyls, but it is biallelically expressed in primates, including humans (15–17). The IGF-II receptor regulates IGF-II, a potent mitogen, by binding it, internalizing it and then transporting it to lysosome for degradation. Loss of function of the IGF-II receptor by mutation in the coding regions and by loss of heterozygosity of *IGF2R* has been observed in numerous malignancies (18,19). It has been suggested therefore that *IGF2R* is a tumor suppressor gene (20).

The mouse *Igf2r* gene encodes two reciprocally imprinted transcripts, each of which is associated with a differentially

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methylated DNA region (DMR) (21). The first DMR (DMR1) includes the promoter for the sense *Igf2r* transcript, whereas DMR2, which is located within the second intron of the gene, includes the promoter for an antisense transcript, *Air*. The paternally expressed *Air* RNA suppresses the expression of the sense *Igf2r* as well as *Slc22a2* and *Slc22a3* on the paternal chromosome (22). Deletion or premature termination of *Air* leads to loss of *Igf2r* locus imprinting (22,23). However, *Igf2r* expression in the CNS does not appear to be regulated by *Air*. Although *Air* is paternally expressed in the CNS as well as in peripheral tissues, *Igf2r* sense transcripts are biallelically expressed in brain (24,25).

The human *IGF2R* gene contains a single DMR in intron 2 (26), but no antisense transcripts have been detected and the gene is biallelically expressed in all tissues including Wilms' tumors (27). Thus, it appears that although the DNA is 'marked' for imprinting, the putative imprint is never read. To understand this lack of epigenetic readout, we used a chromatin immuno-precipitation (ChIP) assay in combination with quantitative real time (Q)-PCR (Q-PCR) to scan for enrichment of various histone modifications throughout the mouse and human *Igf2r*/*IGF2R*. We have found that the human DMR lacks enrichment of acetylated and methylated histones. The absence of differentially modified histones (DMH) in the human DMR may account for the epigenetic readout failure. We show here that enrichment of parental allele-specific histone modifications in the promoter region, rather than the presence of DNA methylation or antisense transcription, correctly identifies the tissue- and species-specific imprinting status of *Igf2r*/*IGF2R*.

RESULTS

Histone acetylation and Lys4 methylation are enriched in the human *IGF2R* promoter exon and are absent in the DMR

We ran triplicate Q-PCR assays (PCR primers shown in Table 1) on ChIP preparations of human embryonic fibroblast cells that maintain the normal imprinting of all tested imprinted genes (28 and unpublished data). We used a panel of antibodies against acetyl lysines (H3 and H4) and methyl lysines (Lys4 and Lys9), as reported previously (11,12). Relative enrichment compared to input chromatin DNA was calibrated with *GAPD* (measured 0.8 kb downstream of the *GAPD* transcription site; Fig. 1C, right panel), calculated as previously described (11) and plotted on the same graph (under appropriate scales for comparison) in Figure 1A. We observed a specific and symmetric distribution of acetylated histones (H4-Ac and H3-Ac) across the 3 kb region of the *IGF2R* promoter exon (Fig. 1A, green lines). The enrichment of acetylated histones near the *IGF2R* transcription site was often $>$10-fold when compared with the enrichment of acetylated histones at 1.5 kb upstream or at 2 kb downstream of the transcription site. Low levels of acetylated histones were found in the intronic DMR or in other exons. Methyl Lys4 of histone 3 (H3 K4-Me) was also enriched in the promoter exon and was essentially absent in the DMR (Fig. 1A, blue line), whereas methyl Lys9 (H3 K9-Me) was depleted near the *IGF2R* transcription site (Fig. 1A, red line). Histone acetylation and H3-Lys4 methylation have been found in transcriptionally active genes (29,30); the absence of these activating histone modifications in the human DMR correlates with the absence of a potential human '*AIR*' antisense transcript (27).

Histone H3 Lys4 and Lys9 methylation are enriched in mouse *Igf2r* DMR1 and DMR2

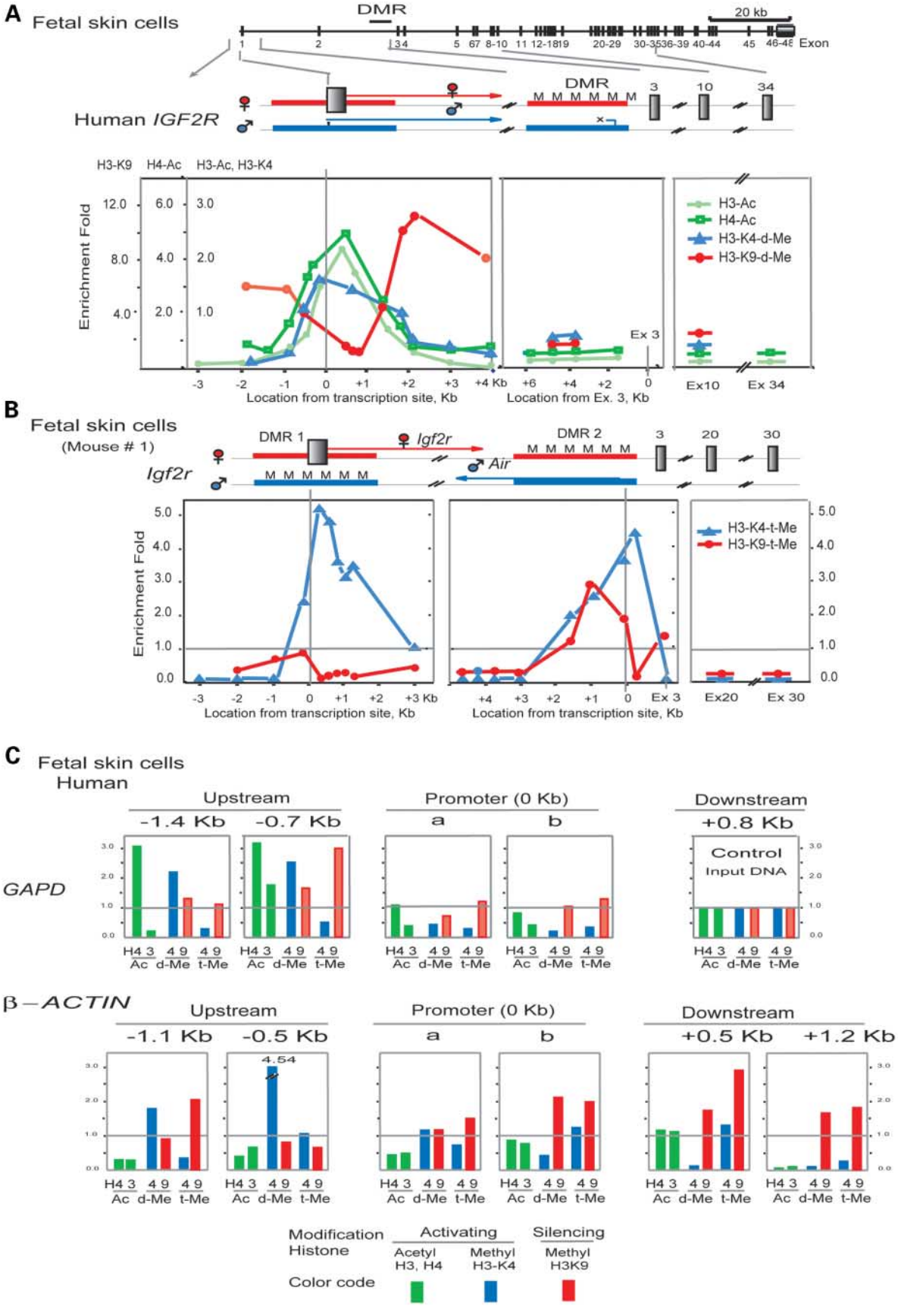
We scanned the distribution of methyl Lys4 and methyl Lys9 of histone H3 across the mouse *Igf2r* in cultured fetal skin cells where *Air* antisense is actively transcribed from the paternal allele (23). Allele-specific histone modifications in the promoter region of *Igf2r* and *Air* have been reported previously (10,11) but the distribution of modified histones across the *Igf2r* promoter region was not clarified. As shown in Figure 1B (blue line), tri-methyl Lys4 demonstrated peak enrichments within $\sim$3 kb region of both DMRs, confirming the promoter-specific enrichment of activating modified histone in transcriptionally active regions. The peak enrichments occurred near the transcription site of *Igf2r* and *Air* despite the fact that our *Igf2r*/*Air* scanning results reflect the sum from both transcriptionally active and inactive parental alleles in each region. Examining the enrichment of di- and tri-methyl Lys4 on each parental allele in the *Igf2r* and *Air* promoter regions suggests a similar pattern of di-methylation and tri-methylation of H3-Lys4 in various tissues and in fetal cells (Fig. 3). This result suggests that H3-Lys4 methylation marks the active promoter region of mouse and human *Igf2r*/*IGF2R* gene and that the absence of histone acetylation and Lys4 methylation marks in the human DMR is associated with lack of active gene transcription. Tri-methyl Lys9 of histone 3 was more abundant than di-methyl Lys9 in fetal skin cells (Fig. 3C), and it was more enriched in the *Igf2r* DMR2 than in DMR1 (Fig. 1B, red line).

Absence of promoter-restricted enrichment of histone modifications in human *GAPD* and *β-ACTIN*

We examined various histone modifications across two housekeeping, autosomal non-imprinted genes, *GAPD* and *β-ACTIN* (Fig. 1C). *GAPD* and *β-ACTIN* are relatively small genes, and they are embedded in gene-rich regions on chromosomes 12p13 and 7p22, respectively. *GAPD* spans a short DNA region of 3.85 kb located 2.87 kb downstream of *CNAP1* whereas *β-ACTIN* spans 3.44 kb, 1.92 kb upstream of the putative transcript (LOC 402247). Because of the presence of other nearby transcripts, we examined two locations upstream of the *GAPD* and *β-ACTIN* promoters ($\sim$1 kb), two locations downstream (*β-ACTIN*, $\sim$1 kb) and two adjacent locations near the *GAPD* and *β-ACTIN* transcription sites (promoter *a* and *b*, Fig. 1C; for PCR primer, see Table 1). The patterns of various histone modifications observed at the two adjacent locations near the transcription site were similar, confirming our reliable ChIP–Q-PCR assays and revealing low levels of histone modifications in the promoter region, when compared with those in the upstream or downstream regions. This result is in sharp contrast to the enrichment of acetylated histones and H3-methyl Lys4 near

Table 1. PCR primers for ChIP–PCR of human *IGF2R*, *GAPD* and *β-ACTIN*

Primer set	Location to transcription site (kb)		Laboratory primer no.	Sequence (5′–3′)	PCR (bp)	Laboratory ID no.
1	*IGF2R*	−3.0	1316	CCGGTGCCAAA CTfCCATGTGTATGA	83	TV1316HIIRI-3K83 + 1326, BstNl 59/83
			1317	GAAAATCTCTGG AGATGGGCACC		TV1317HIIRI-3K83 − 1224
2		−2.0	1366	GACCAATT TTCCATCTAT AGACAACCT	81	TV1366HIIR-2K81 − 927
			1367	GGA AAATCCAAGG AGAATCCAAC A		TV1367HIIR-2K81 + 1024
3		−1.0	1370	TG CAAAACGGAT GCAATAAAGG CA	94	TV1370HIIR-1K94 + 1024
			1371	GT ACGAAGTACT GAAGTGTTCG GA		TV1371 HIIR-1 K94 − 1024
4		−0.6	1318	CGTTCTACTTTCATTGTGGCTAGCTCA	95	TV1318HIIRI-.5K95 + 1225
			1319	GACAAAAGTGTCGCTGTCAAGCAAGT		TV1319HIIRI-.5K95 − 1224
5		−0.5	796	C TTGACAGCGA CACTTTTGTC CACT	110	TV796-HIIR + 340 12/25
			797	CTCTCAGAC CTTCTGTGGG AATGT		TV797-HIIR-449 12/24
6		−0.3	1382	ACC TCCCTGGCAC TTfGATCCAA GT	86	TV1382HIIR + .3K86 + 1325
			1383	GTG CAACCCAGGTTAGAGCAACT GT		TV1383HIIR + ,3K86 − 1325
7		+0.4	1390	AGGAGC TCCTGGGGTC TCCAAGT	82	TV1390HIIR + 04K82 + 1423
			1391	ACACCA CGCAGGCAAC TTTCCCT		TV1391 HIIR + 04K82 − 1323
8		+0.5	1392	CACTTGTGGC TGTCGCTGAG T	111	TV1392HIIR + 05K111 + 1221
			1393	C TGCCTCCAAC TTTCCCGGAC		TV1393HIIR + 05K111 − 1321
9		+0.6	1322	GTCCGGGAAAGTTGGAGGCAG	72	TV132211RI + .5K72 + 1321
			1323	TGGCG AAACTCTGAT GCAAACGCGCA		TV132311RI + .5K72 − 1323
10		+1.2	1376	CTGA CCATCCT1Tr CTGGACTGT	88	TV1376HIIR+1 K88 + 1123
			1377	CATC CTGCTTCCTT TTTACCAAAG T		TV1377HIIR+1 K88 − 1025
11		+1.6	1324	GAGAAATGGTGGCAGCTTAAACGA	84	TV132411RI + 2K84 + 1124
			1325	TTG GTCACAGAAG CCATTATTCC A		TV132511RI + 2K84 − 1325
12		+2.1	1378	AAG GTGGCTTCAA GGGCAGACTG A	84	TV1378HIIR + 2K84 + 1324
			1379	C AAGACACTGC CATGACTAAC CT		TV1379HIIR + 2K84 − 1123
13		+3.1	1380	CCGTAAATG TAGCATGTAG TTGAGGA	97	TV1380HIIR + 3K97 + 1126
			1381	CACGTCAC ACCTTGGTGA CACGA		TV1381 HIIR + 3K97 − 1323
14		+4.0	1326	GTAGAGCAA GAAGAGAGGG CATTCA	97	TV1326HIIRI + 4K87 + 1225
			1327	GCTGAGAT CACTTTAGAC ACTGTTGT		TV1327HIIRI + 4K87 − 1126
15	Upstream exon 3	+5.6	1042	GATGATACCTGGTTGAGTTTTGGATAC	135	TV1042-HIIRIT1 + 135F11/27
			1043	CTTCTATCCGTTTGAGTCACAAAAGA		TV1043-HIIRIT1 − 135R10/26
16		+4.6	1044	TGCCT GTGAAGTCAG GATCACCAG	120	TV1044-HIIRIT2 + 120F13/24
			1045	AGTCA GGCCACCAAG TCGCATTCT		TV1045-HIIRIT2 − 120R13/24
17		+3.9	1046	ATCTGATTGG ATCCTGGATC TTGCCA	110	TV1046-HIIRIT3 + 110F12/26
			1047	GCACAGATGAACCAAGCTTG CAACA		TV1047-HIIRIT3 − 110R12/25
18		+1.6	1052	GTTGTTGAACCAGTTAACATTTCACTAGC	105	TV1052-HIIRIT6 + 105F12/29
			1053	CCATTTACAACCATCCCTCTGGCAA		TV1053-HIIRIT6 − 105R12/25
19	Intron–exon 10/11		1036	GGATT GCAGAATGTC ACTGGGTGC	98	TV1036-HIIRNG2 + ggF14/23
			1037	TTTGATATGCCAGTG CCTTGGTGAC T		TV1037-HIIRNG2 − 98R12/26
20	Exon 34		701	AGTGTGATCAGTTTCGTGTGCAGGC	153	TL701-HIIR/4969F- SNP -MspI
			702	GCTTCCATTCCTCACGGAACATTCG		TL702-HIIR/5121R
21	*GAPD*	−1.4	1570	ACACCAACCATGCCAGTGGCAGCCAGA	89	TV1570HGAP-1391 + 1525
			1571	TCCCT GACCC TGCCT TTCTG GGAT		TV1571 HGAP-1391 − 1425
22	Upstream	−0.7	1572	GGATTGTCTGCCCTAATTA TCAGGT	72	TV1572HGAP-708 + 1125
			1573	AGGTT TCTGC ACGGA AGGTC ACGAT		TV1573HGAP-708 − 1325
23	Promoter *a*	−0.1	1574	TAGGGCCCGGCTACTAGCGGTTTTA	71	TV1574HGAP-85 + 1425
			1575	AGCCA GTCCC AGCCC AAGGT CTTGA		TV1575HGAP-85 − 1525
24	Promoter *b*	0	1576	CGGTTTCTATAAATTGAGCCCGCAG	73	TV1576HGAP + 36 + 1225
			1577	AGATG CGGCT GACTG TCGAA CAGGA		TV1577HGAP + 36 − 1425
25	*β-ACTIN*	−1.1	1556	AGG GTCTTCCCAG GCTGGCTTTG A	73	TV1556H873-1101 + 1424
			1557	CAAGACTCCATGTGCCACAGAGGAT		TV1557H873-1101 − 1325
26	Upstream	−0.5	1558	CACCC AGCAC ATTTA GCTAG CTGA	75	TV1558HB75-501 + 1224
			1559	TTCAGAGCAACTGCCCTGAAAGCA		TV1559HB75-501 − 1224
27	Promoter *a*	−0.03	1560	AAATGCTGC ACTGTGCGGC GAAG	88	TV1560HB88-32 + 1323
			1561	CGAGCCATAAAAGGCAACTTTCGGA		TV1561HB88-32 − 1225
28	Promoter *b*	−0.01	1566	CCTATAAAACCCAGCGGCGCGA	82	TV1566H + 15ACT82 + 1323
			1567	ATCGGCAAAGGCGAGGCTCTGTGCT		TV1567H + 15ACT82 − 1525
29	Downstream	+0.5	1568	TGGT CCTTTGGGCG CTAACTGCGT	56	TV1568H + 454ACT56 + 1424
			2569	GCACGCGCAATT AGCGCCAATT C		TV1569H + 454ACT56-1323
30		+1.2	1564	GCATGGGTC AGAAGGATTC CTATGT	83	TV1564HB83 + 1230 + 1225
			1565	GTGCTCGATGGGGTACTTCAGGGT		TV1565HB83 + 1230 − 1424
31	Control *GAPD* (0.8 kb downstream)		1084	CGG TGA CTA ACC CTG CGC TCC TG	77	YY1084HGAPD1967F1523
			1085	AGC TAG CCT CGC TCC ACC TGA CTT		YY1085HGAPD2043R1424



the human and mouse *IGF2R*/*Igf2r* transcription site in Figure 1A and B.

Absence of allele-specific histone modifications in human *IGF2R*

To evaluate histone modifications on each of the two parental alleles of *IGF2R*, we genotyped five fetal subjects for potential SNPs across the promoter and the DMR (we selected SNPs of >10% heterozygous frequency from the NCBI database). We identified two subjects (HFB #1 and HFB #5) who were informative for allelic analysis in the DMR and the *IGF2R* promoter. We performed ChIP assays using a panel of six antibodies, and we ran triplicate RFLP–PCR (with duplicate samples) on the two informative fetal skin cell lines. To enhance detection sensitivity we used [^{32}P] dCTP radioisotopes, and we quantified by PhosphoImager the relative enrichment in each parental allele with reference to the parental allele ratio observed in 'input DNA'. Figure 2 shows representative RFLP–PCR gels with relative allelic ratios from duplicate samples. Although the parental origins are unknown, it is clear that all histone modifications including acetylation (H3 and H4) and methylation (di- and tri-methylation of both H3-Lys4 and H3-Lys9) were enriched equally in both parental chromosomes at the locations near the promoter region (+1.9 kb) and near the intronic DMR (0.3 kb upstream of exon 3). In contrast to the allele-specific histone modifications in the DMRs of the mouse *Igf2r*/*Air* (10,11), the absence of allele-specific histone modifications in the human *IGF2R* promoter is consistent with the biallelic expression of the human *IGF2R.*

Surprisingly, despite the presence of allele-specific DNA methylation in the intronic DMR (26), both parental chromosomes harbor equally low levels of histone modifications. The absence of these DMHs near the intronic DMR highlights the discordance between histone modifications (both acetylation and methylation) and DNA methylation in the 'non-functional' DMR. Furthermore, the observation of very low levels of histone modifications in the intronic DMR in both parental chromosomes correctly identifies the absence of antisense '*AIR*' transcription from both parental alleles.

Allele-specific histone acetylation and Lys4 methylation in the mouse DMR2

To examine histone modifications on each of the two parental chromosomes across DMR1 and DMR2 of *Igf2r*, we performed ChIP–Q-PCR from various tissues (kidney, liver and CNS) and embryonic cells (skin and CNS) from interspecific F1 mice (C57/BL/6J female × *Mus spretus* male). To this end, we designed PCR primers to amplify both parental alleles and three sets of parental allele-specific primers encompassing the three polymorphic sites (11) to amplify each parental allele (Table 2). In Figure 3, activating modifications are shown as green (acetyl lysines of H3 and H4) and blue columns (H3-methyl Lys4), whereas the silencing modification (H3-methyl Lys9) is shown in red color. The dark, light and medium intensities of the three colors (green, blue and red) represent paternal, maternal and (pat + mat) alleles, respectively (see color code in Fig. 3B and D).

To simplify the allelic analysis of various histone modifications across the three polymorphic sites, we have marked on top of each panel in Figure 3 the predominant allele, 'M' (maternal) or 'P' (paternal) when the ratio of the predominant versus the less abundant allele was greater than 3.0. When both alleles were enriched (enrichment >1.0-fold), the panel was marked as 'Bi' for 'bi-allelic enrichment' if the allelic ratio was within 1.0–2.0; or marked as '(Bi)'—in parentheses—if the allelic ratio was 2.0–3.0. Low levels of modified histones on both parental alleles (enrichment <1.0-fold) were marked as 'x'.

In kidney, liver and fetal skin cells, activating modifications (green and blue columns) were found exclusively in the maternal allele in the *Igf2r* promoter (Fig. 3A and C, −0.2 kb panel). These activating modifications switched exclusively to the paternal allele in the *Air* promoter region (Fig. 3A and C, +1 and +0.1 kb panels). In contrast, in CNS and in cultured brain cells (predominantly fetal astrocytes), despite the paternal allele-specific modifications (H3-acetylation, H3-Lys4 di- and tri-methylation) observed at *Air* (Fig. 3B and D, +1 and +0.1 kb panels), the activating modifications marked both paternal and maternal chromosomes in the *Igf2r* promoter (−0.2 kb panel). Although there was likely a case of maternal bias in the tri-methyl Lys4 in brain cells (Fig. 3D, −0.2 kb panel), this case might be considered as a marginal '(Bi)' since both parental alleles were enriched (enrichment >1.0-fold).

Tri-methyl Lys9 methylation marks the suppressed allele in the mouse *Igf2r* DMR2

The silencing modification, methyl Lys9 of H3, occurred primarily in DMR2 of the maternal chromosome (*Air* promoter) but was much lower in DMR1 (*Igf2r* promoter) (Fig. 3, red columns). This was more obvious in fetal skin and brain cells (Fig. 3C and D) than in kidney and liver (Fig. 3A and B), and was observed exclusively in tri-methyl Lys9 but not in di-methyl Lys9 (Fig. 3C and D, panels +1 kb and +0.1 kb, compare d-Me and t-Me). As DMR2 is a DNA

Figure 1. Distribution of modified histones across the promoter regions of human and mouse *IGF2R*/*Igf2r.* Top panel shows the *IGF2R* map and details of the promoter and the DMR. *IGF2R* is transcribed from both parental alleles with no detectable antisense transcript from DMR. (**A**) ChIP–Q-PCR assay across human *IGF2R* showing enrichment of activating histone modifications (acetylation and H3-Lys4 methylation) near the *IGF2R* transcription site and low levels of modifications in the intronic DMR, exon 10 and exon 34 regions. H3-Lys9 methylation was absent near the transcription site. (**B**) ChIP–Q-PCR assay across mouse *Igf2r* showing enrichment of H3-Lys4 methylation in both promoter regions of *Igf2r* and *Air* (DMR1 and DMR2). (**C**) ChIP–Q-PCR analysis of the promoter, upstream and downstream regions of human *GAPD* and *β-ACTIN* showing a broad distribution of histone modifications in the housekeeping genes. The promoter regions were examined by two sets of adjacent PCR primers *a* and *b* that were separated by less than 50 b. The histone enrichment in the *GAPD* (+0.8 kb downstream) served as a calibration control.

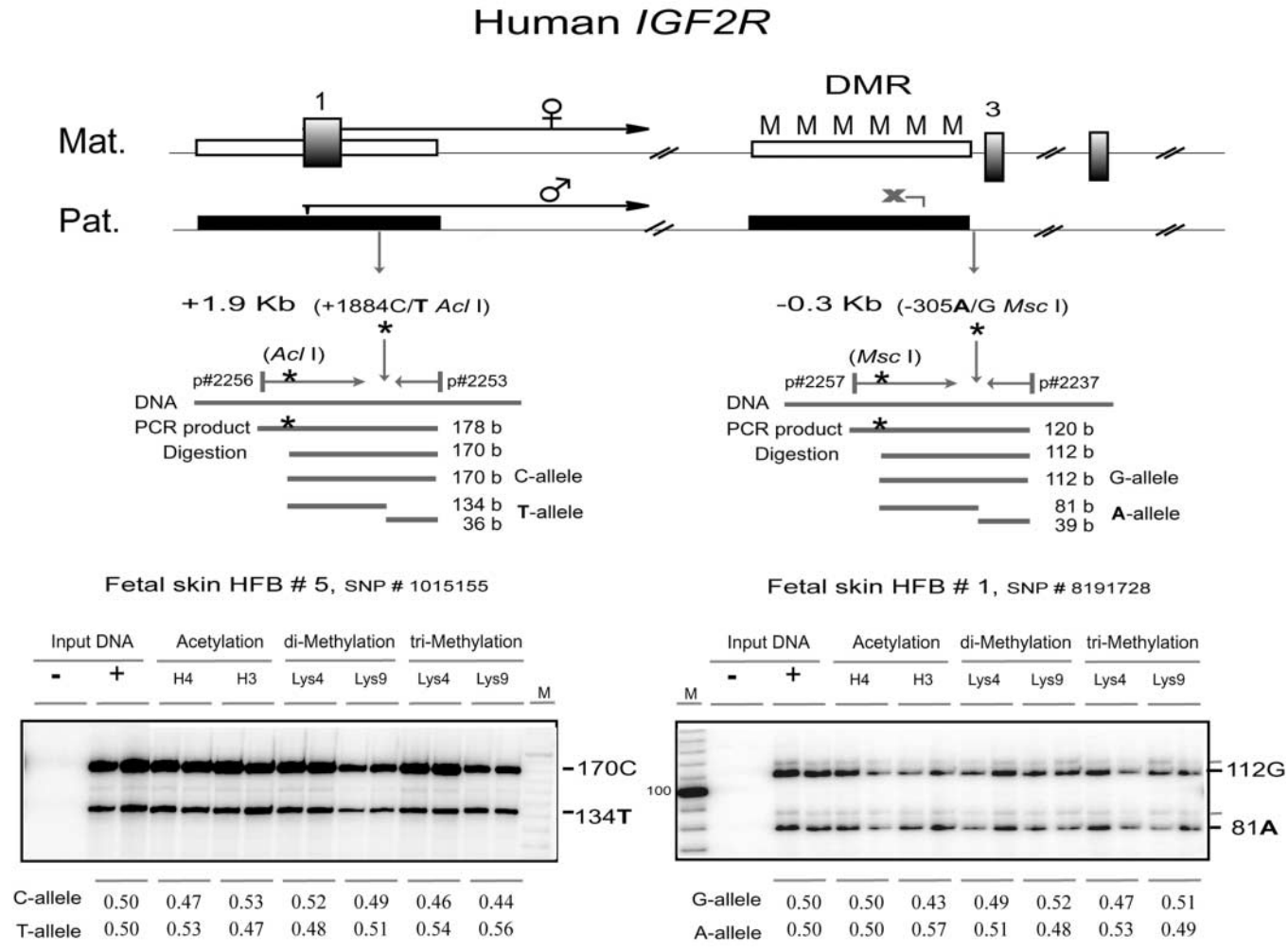


Figure 2. Allelic distribution of histone modifications at sites near the *IGF2R* promoter and the DMR by ChIP RFLP–PCR. ChIP DNAs from human fetal skin HFB # 5 were PCR amplified across a polymorphic +1884 *Acl*I site (SNP no. 1015155). Digestion with *Acl*I revealed an undigested 170C allele and a digested 134**T** allele. ChIP samples from HFB #1 were amplified and digested with *Msc* I (SNP no. 8191728) to reveal an undigested 112G and a digested 81**A** alleles. PCR with (plus) and without (minus) input DNA served as positive and negative controls, respectively. An internal restriction site was integrated to the PCR primers (asterisk), which served as an internal digestion control. Traces of incomplete digestion products were marked (minor bands) along with the major digested products (major bands). Results of quantification by PhosphoImager from duplicate samples, after calibration with control input DNA (equal parental alleles), are shown at the bottom of each panel.

methylation imprint that is inherited from the female gamete, whereas DMR1 CpG methylation is not completed until postnatal day 4 (31), DMR2 has been referred to as the primary DNA-gametic imprint. Our ChIP–Q-PCR results indicate that tri-methylation of Lys9 of histone H3 accompanies the primary gametic imprint of the *Igf2r.* In the human *IGF2R* gene, the absence of such a marker in the intronic DMR (Fig. 1A) corresponds to the absence of a potential imprinted antisense '*AIR*' and the presence of biallelic expression of *IGF2R* in all tissues.

DISCUSSION

The epigenetic code of imprinted genes is likely to consist of both DNA methylation (DMR) and histone modifications. The DNA methylation code appears to be clear: CpG methylation near the promoter region correlates with suppression of transcription. In contrast, a variety of histone modifications including acetylation and methylation (mono-, di- and tri-) at various lysine residues and other modifications constitutes a complex histone code (29,32). In this report, we examined histone acetylation and di- and tri-methylation throughout the mouse and human *Igf2r*/*IGF2R* by ChIP–PCR in order to clarify the epigenetic marks governing tissue- and species-specific imprinting of the *Igf2r*/*IGF2R.*

We have shown that histone modifications were enriched in the promoter region of *Igf2r*/*IGF2R* and that the promoter-specific enrichment was independent of the tissue- or species-specific imprinting status of the gene. The enrichment of histone acetylation and Lys4 methylation near the transcription site within a 3 kb promoter region of human *IGF2R*

Table 2. Allele-specific primers and biallelic PCR primers for ChIP–PCR assay of mouse *Igf2r*

Primer set	Location to transcription site (kb)		Laboratory primer no.	Sequence (5′–3′)	PCR (bp)	Laboratory ID no.	Notes
1	*Igf2r*	−3.2	964	ATCA GGTCTCATAT AGTGCAGGCT	107	TV964-M2R + 134 − 11/24	GenBank accession no. U26348(134-239)
			166	TGGGCATAGTAA TTCCAG CTCTGGAAC			
2		−2.0	1502	CAAT TCTGTACCTG TGAGGTGGCT	84	TV1502M2R − 2K84 − + 1224	
			1503	CATTATCTCT TCTTG AAGTG ATGCCA		TV1503M2R − 2K84 − 1026	
3		−1.0	1504	CTTCAT CAGGAACAGG CCATCCTCT	126	TV1504M2R1K126 + 1325	
			1505	TGGCTGTGAC ACCAGGATTG GTCAA		TV1505M2R1K126 − 1525	
4		−0.5	1506	CTCCAG TATAGCCTGA GCTACATAGT	117	TV1506M2R.5 + 117 + 1226	
			1507	G TGACTGGTGG ACTAATATGC AGTGT		TV1507M2R.5 − 117 − 1226	
5		−0.2	6458	GGTGCTGGACGGGGAAACTGAGGTC	105/89		16-base deletion in *M. spretus*
			8-04	GGCACGAGCGCCAGGTACCTACTCGA		8-04 M2R − 907	GenBank accession no. L06445(NT # 803-908)
6		−0.2	1508	GACGGGGAA ACTGAGGTCT **CCCT**[a]	82	TV1508M2R.2SP82 − 1423	Paternal spretus-specific primer
			8-04	GGCACGAGCGCCAGGTACCTACTCGA		8-04 M2R − 907	
7		−0.2	1509	GGGGAA ACTGAGGTCT **GGCTCTGA**[a]	95	TV1509M2R.2C95 − 1424	Maternal C57-specific primer
			8-04	GGCACGAGCGCCAGGTACCTACTCGA		8-04 M2R − 907	
8		+0.2	756	AGGCC GTCGA CTTGG ACGCC CTGT	88	TV756-M2R + 236 16/24	
			757	CGGG GCGGA GGTCT GCGTG TCCT		TV757-M2R-56 17/23	
9		+0.5	1510	GCTGCA CGTTTCCATT GAAGTTCCA	74	TV1510M2R.5 + 74 + 1225	
			148	ACTCCCTG GAGACTGCAC ACTAGA			
10		+0.7	150	GTTG AGAGGTGGTC ATCTCTGTAG GCT	101		
			149	CTCTGGA GACAATGACA TCATGTGAGC A			
11		+1.0	965	G CTCTTTAGGG TGAAGGGAAA CCA	148	TV965-M2R + 8830 − 12/24	GenBank accession no. L06445
			152	GAAACATCAAGTTTG GCTCAGCC			(NT#2052-2191)
12		+1.3	163	CTTCATGATATGGAGGAAG GGTGC	114		
			1511	CTTGGTCAC AGCAGCCCTA AATCCA		TV1511M2R1K + 98 + 1325	
13		+3.0	1514	CAGCTCCT TATGTGGTCA GGACT	111	TV1514M2R3K + 111 + 1324	
			1515	CAC CAAACCACAC CACATCCATC T		TV1515M2R3K − 111 − 1224	
14	*Air*	+6.0	1517	GTTG TGAGCTCTTT CTCCTGTTGA	89	TV1517M2RE2 + 89 + 1124	+1.3 kb downstream of exon 2

Continued

Table 2. Continued

Primer set	Location to transcription site (kb)		Laboratory primer no.	Sequence (5′–3′)	PCR (bp)	Laboratory ID no.	Notes
			769	GTAATA ACTTGGACC ACCCCACTT		TV769-M2R − 255 11/24	
15		+5.0	1518	GAC ACTGGATGTA CAGAACCAAG A	83	TV1518M2RE2 + 83 + 1124	+2.3 kb downstream of exon 2
			770	GGTCAGATGG TTTATGGAGC AGAA		TV770-M2 R − 1250 11/24	
16		+3.0	1530	GGAATAATTGGTTGGG ATCCAGGA	99	TV1530M2RAir99 + 1124	
			1531	CCA CTGCTACCCT CCTGTGACTG AA		TV1531 M2RAir99-1425	
17		+2.0	1542	CCGAGTGAT AAGAACTACTAAGGCT	110		
			1543	GGCTATTGCTA AGTGGCTACT ACA			
18		+1.5	1528	CTGAG GGTCCAACCC TTTGAACTCT	84	TV1528M2RAir84 + 1325	
			1529	TCTGCACTA TCCAGGCTGT GCTCA		TV1529M2RAir84-1324	
19		+1.0	1524	AG CACAACCAAG GATCACGGCA CA**T**[a]	126	TV1524M2RSp126 + 1324	Paternal *spretus*-specific primer
			1525	AGGC TAAGGGTGAAAAGCTGCACA		TV1525M2R126 − 1224	
20		+1.0	1526	AG CACAACCAAG GATCACGGCA CA**A**[a]	126	TV1526M2RC126 + 1324	Maternal C57-specific primer
			1525	AGGC TAAGGGTGAA AAGCTGCACA		TV1525M2R126-1224	
21		+0.1	1522C	TGGAACT CTTGGTCGGA GCCCTC**C**[a]	98	TV1522M2RSp98 + 1241	Paternal *spretus*-specific primer
			6460	TACGCGAGGTGAGGGTTCCACTGAT		JH6460M2R2010R	GenBank accession no. L06446 (NT#1913-2010)
22		+0.1	1523G	TGGAACT CTTGGTCGGA GCCCTC**G**[a]	98	TV1523M2RC98 + 1241	Maternal C57-specific primer
			6460	TACGCGAGGTGAGGGTTCCACTGAT		JH6460M2R2010R	
23		0	199	GAATCCTAC CCTCATCTGC AGAATC	86	JH6460M2R2280F	
			1521	CATCCTG GGGAACTGAG GTAAGCTA		TV1521 M2RAir86 − 1325	
24		−0.3	1520	TC CTTGC TTGTC TTGAACCCCG AGA	75	TV1520M2RAir75 + 1325	
			201	ACTGCATACTCTG CATCCAAGCA ATG		JH6460M2R2597R	
25		−1.0	225	TCACTTT TGAGAAGTTC AGCCAGAT	89	JH6460M2R3169F	Exon 3
			1519	GT CTGGATTCTG TGCTGTGAAT CTGA		TV1519M2Re3-89 − 1226	
26	Exon 20		1534	C ACTTTCAACT GGGAATGTGT GGTCA	85	TV1534M2Re20 + 85 + 1226	∼70 kb downstream of *Igf2r*
			1535	CTGG TCTGTTTCTG TGATTGTCTG GA		TV1535M2Re20-85 − 1226	
27	Exon 30		1536	AACT CCATTGACCT CTCCTCACTG T	96	TV1536M2Re30 + 96 + 1325	∼80 kb downstream of *Igf2r*
			1537	GC ATACATTGAT GAGGTAATGC TCAGT		TV1537M2Re30-96 − 1126	
28	Control L7		1290	GAAAGGCAAGGAGGAAGCTCATCT	80	TV1290mL7 + 80f1224	GenBank accession no. NM-011291
			1291	AATCTCAGTGCGGTACATCTGCCT		TV1291 mL7 − 80r1224	(NT#224-303)

[a]Allele-specific primers with allele-specific nucleotides in bold.

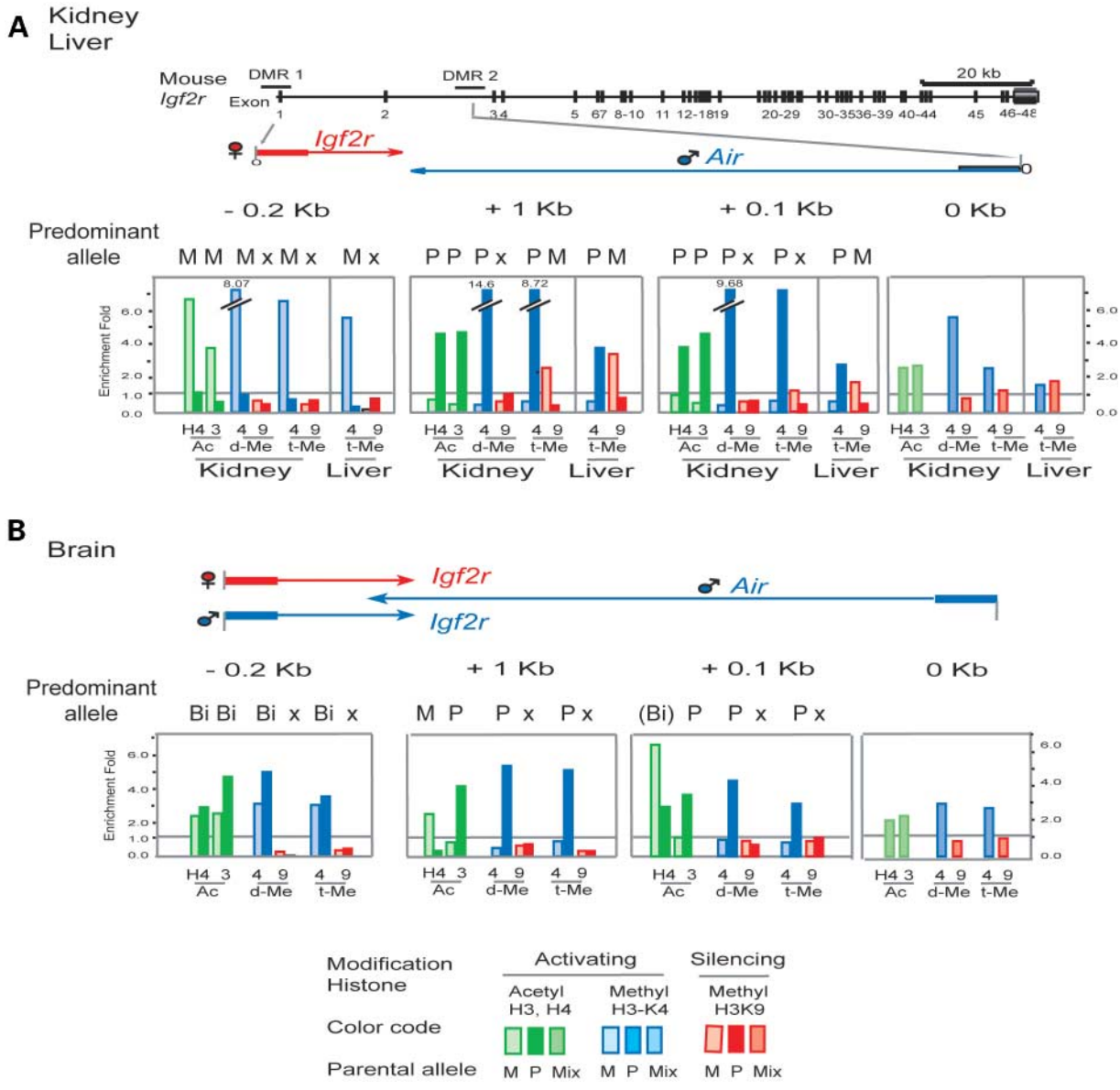


Figure 3. Allele-specific histones modifications accompany imprinting status of *Igf2r* and *Air*. Top panel shows the *Igf2r* map, promoter, DMRs and imprinted *Igf2r* and *Air* transcripts. ChIP–Q-PCR was performed on tissues from newborn C57BL/6J ♀ × *M. spretus* ♂ F1 mice (**A** and **B**) and from primary culture cells derived from skin (**C**) and brain (**D**) of the newborn F1 mice. ChIP–Q-PCR using allele-specific primers (Materials and Methods and Table 2) was performed to quantify the enrichment of the modified histones (acetylated histone H4 and H3, di-methylated H3-Lys4 and-Lys9, and tri-methylated H3-Lys4 and Lys9) on each maternal and paternal chromosomes at three locations (−0.2 kb in DMR1, and +1 and +0.01 kb in DMR2). Doublet columns at the three polymorphic locations depict the data of *maternal versus paternal* chromosomes as *lighter versus darker* colored columns. Each column represents the mean values of three PCR reactions. On top of each panel, the predominant allele—an enrichment of a modified histone in a particular parental allele—was identified as 'M' (maternal), 'P' (paternal), 'Bi' or '(Bi)' (biallelic) by criteria described in the text. Low levels of modified histones were marked as 'x'. Comparable data were obtained at nearby locations (0 and +0.1 kb, Fig. 3A and B), or by using allele-specific primers versus allele-common primers (−0.2 kb, Fig. 3C and D, compare left two panels).

was striking. We confirmed this observation by ChIP assays in fetal skin cells from three independent subjects. These activating modifications were normally found in active chromosome domains encompassing the entire gene or cluster of active genes. However, to our knowledge, the sharp increase with a 'Gaussian distribution pattern' across a gene promoter has not been demonstrated before, although sharp increases of histone acetylation were noted at a silencing boundary in the chicken β-globin (33) and in the mouse *Gnas* gene (12). The sharp increase of activating histones does not simply reflect the transcriptional activity at the promoter region since other non-imprinted, autosomal housekeeping genes such as the human *GAPD* or *β-ACTIN* demonstrated no such sharp enrichment near their promoters. Scanning of

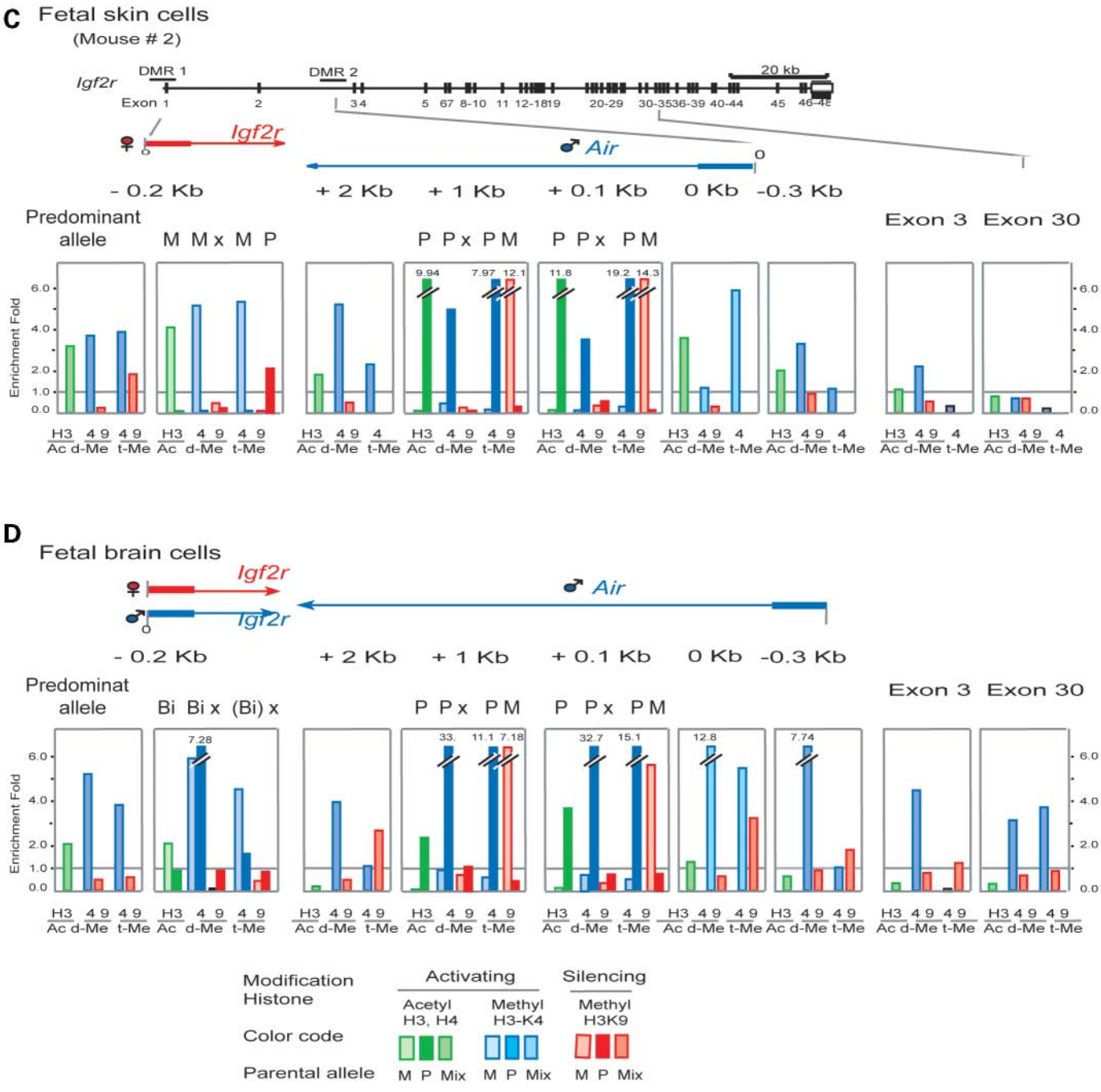


Figure 3. Continued.

histone modifications across the promoters of other imprinted genes may shed light on the role of this promoter-specific epigenetic mark in imprinted genes.

Methyl Lys9 of histone H3, which is considered to be a silencing modification (reviewed in 29,30) was enriched in a pattern reciprocal to that of the methyl Lys4 modification. This inverted pattern was more obvious in the human *IGF2R* promoter region, which showed a profound dip near the transcription site (Fig. 1A, red line). Although higher levels of methyl Lys9 were present outside of the promoter region, the human DMR and exon 10 showed low levels of methyl Lys9 modification.

The pattern of methyl Lys9 distribution in the mouse *Igf2r* appeared to be more complicated, because we presented data that resulted from the sum of opposing modifications in the active and inactive parental alleles that were measured together (Fig. 1B). Nonetheless, we observed enrichment of methyl Lys9 in DMR2 that regulates the *Air* transcript

(Fig. 1B). Differentiating the two parental alleles at three polymorphic sites using allele-specific PCR primers and antibodies against the three levels (mono-, di- and tri-) of methylation at lysine residues also confirms high levels of tri-methyl Lys9 in DMR2 versus DMR1. The allele-specific PCR demonstrated that the tri-methyl Lys9 modification was exclusively from the suppressed maternal allele. ChIP assays using antibodies against mono-methyl Lys9 (and mono-methyl Lys4) yielded weak signals (data not shown), whereas di-methyl Lys9, in contrast to di-methyl Lys4, did not mark the suppressed allele in the *Igf2r*/*Air* promoters (Fig. 3, red columns).

Promoter-restricted enrichment of DMH, rather than CpG methylation in the DMR or the presence of an antisense transcript, correctly identifies the tissue- and species-specific imprinting status of *Igf2r*/*IGF2R*. Figure 4 summarizes the tissue- and species-specific epigenetic marks, including DNA methylation (DMR) and histone modifications (DMH), in the *Igf2r/IGF2R* region and in the human intronic DMR or mouse DMR2. In the mouse, the presence of a DMR and of a DMH correlates with the imprinting status of *Igf2r*/*Air* in peripheral tissues. However, there is a dissociation of *Air* imprinting versus *Igf2r* non-imprinting in brain. Absence of *Air* imprinting and dissociation of *Air*/*Igf2r* imprinted expression also have been observed in mouse uniparental, androgenetic and parthenogenetic fetuses even in the presence of a complete DMR2 (34). The histone epigenetic marks in these uniparental fetuses, have not been investigated. In human, we have now shown that despite the presence of a DMR, the absence of activating histone modifications corresponds to the absence of a human '*AIR*' antisense, whereas biallelic presence of activating histones (i.e. absence of a DMH) in the sense promoter accompanies biallelic expression of *IGF2R*.

Rougeulle *et al.* (35) have recently suggested that the enrichment of H3-Lys4 di-methylation in the promoter versus exon regions is an epigenetic mark for monoallelic expression of X-inactivated genes and of three imprinted autosomal genes, *Igf2*, *Ube3A* and *Peg3*. They did not discuss mono-methyl or tri-methyl H3-Lys 4, however. Histone scanning of the mouse *Igf2r*/*Air* indicates that the predominance of Lys4 methylation at the promoter (versus exons 3, 20 and 30) of *Igf2r*/*Air* is not restricted to di-methylation: both di-methyl Lys4 and tri-methyl Lys4 demonstrated a similar 'epigenetic mark' pattern. Interestingly, the species-specific non-imprinted human *IGF2R* also exhibits the enrichment of Lys4 di-methylation mark in the promoter region, whereas other autosomal housekeeping genes failed to exhibit the Lys4 methylation mark. We suggest that, at least in the case of the imprinted *Igf2r* and *Air*, promoter-restricted enrichment of both di- and tri-methyl Lys4 (in the active allele) is the governing epigenetic mark. Using mouse embryonic stem cells (129 ES), we have verified the presence of the di- and tri-methyl Lys4 marks in the promoter region of X-inactivated genes (*Cdx* and *G6pd*) and in the promoter of a number of imprinted genes, including MEGs (*Cdkn1*, *Ascl2*, *Grb10* and *Meg3*) and PEGs (*Dlk*, *Nnat*, *Snrpn* and *Peg3*) (Vu *et al.*, unpublished data). Histone scanning of imprinted genes using a panel of specific antibodies against modified histones, including tri-methyl Lys9 and phosphorylated Ser10, may further define the specific components of 'epigenetic marks' in imprinted genes.

MATERIALS AND METHODS

Human tissues, interspecific mice and primary culture cells

Normal human fetal skin tissues of 6–10 weeks of gestation were obtained from the Central Laboratory for Human Embryology Tissue, University of Washington, Seattle, WA, USA. To generate F1 interspecific mice, *M. spretus* male mice were mated with *M. musculus* female mice. Housing and all procedures were performed according to protocols approved by the Institutional Care and Use Committee at the Veterans Affairs Palo Alto Health Care System. Liver, kidney and CNS tissues from newborn F1 mice were dissected, snap-frozen in liquid nitrogen and stored at −70°C. Human fetal skin cells, skin cells and brain cells from newborn F1 mice were removed, minced and cultured as described previously (11). Skin fibroblast cells at passage 3–6 were used in this study.

Antiserum against modified histones

The panel of antisera used in this report has been used in numerous studies (data from the suppliers) and in our previous studies (11,12). Antiserum specific for histone H4 acetylation (acetyl Lys5, 8, 12 and 16; cat. no. 06-866), antiserum for H3 acetylation (di-acetyl Lys9 and Lys14; cat. no. 06-599), antiserum for H3 methylation (di-methyl Lys4, cat. no. 07-030 and di-methyl Lys9, cat. no. 07-212) were obtained from Upstate Biotechnology (Waltham, MA, USA). Antiserum specific for H3-Lys9 acetylation (cat. no. 617) was from Cell Signaling Technology (Beverly, MA, USA). Antiserum specific for H3 tri-methyl Lys4 (cat. no. ab8580) and H3 tri-methyl Lys9 (cat.no. ab8898) were obtained from Abcam (Cambridge, UK).

Human *IGF2R* polymorphisms and RFLP–PCR analysis

We searched the SNPs database (GenBank, National Center for Biotechnology Information) and used RFLP–PCR to genotype five human fetal subjects for potential SNPs across ~4 kb of the *IGF2R* promoter and ~4 kb of the DMR. Only SNPs creating a restriction site and having high frequency of heterozygosity (>10%) were tested. We identified two informative subjects. The HFB #1 was a heterozygote having **A**/G alleles at 305 bases upstream of exon 3 (−305**A**/G); the two **A** and G parental alleles could be identified by the *Msc*I restriction enzyme that recognizes the TGGCC**A** sequence of the **A**-allele. Input and ChIP DNA samples were amplified using primer p 2257 (5′-TTTGA ***TGGC***C ACACT GGTGC AAGAT GGATG AG-3′) and p 2237 (5′-CGCTA AAACC ACTAC CTGCG CT-3′) to yield a 120 bp PCR product that was then digested by *Msc*I to produce 112 bp G-allele and 81 bp **A**-allele. The HFB #5 was a +1884C/**T** heterozygote having an *Acl*I polymorphic site AACG**T**T at 1884 bases downstream of *IGF2R* transcription (bold letters represent the digested allele). The DNA samples were amplified using primer p 2256 (5′-TTCAA C***A***AC***G T***TAGG CCAGC TGGGT TAATT TC-3′) and p 2253 (5′-GTCCT CCCAG TTAAG GGAGG CTGA-3′) to yield a 178 bp PCR product that was then digested by *Acl*I

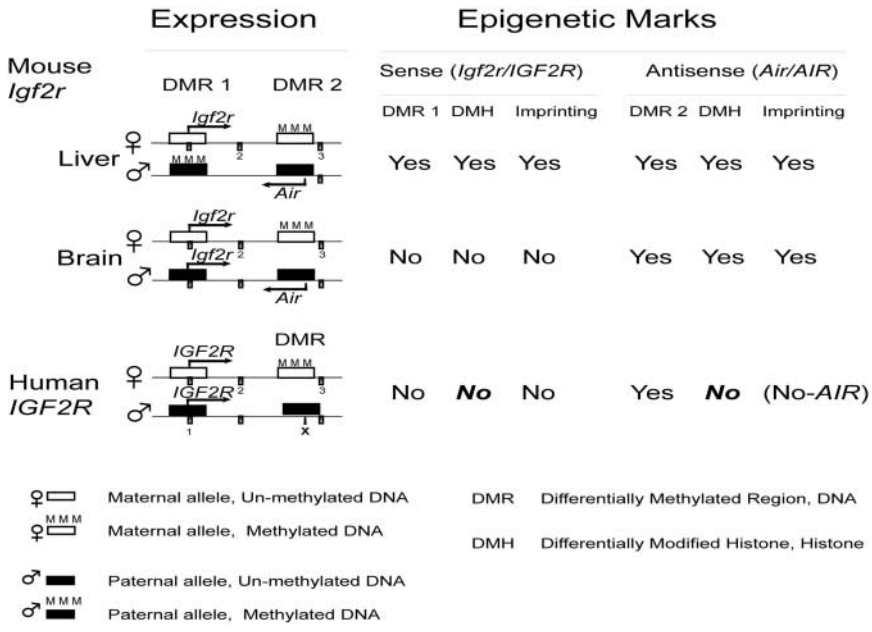


Figure 4. Schematic representing the association of epigenetic marks with tissue-specific and species-specific imprinting of *Igf2r*/*IGF2R.* In mouse liver, both *Igf2r* and *Air* are marked by DNA methylation DMR and histone DMH, and are expressed exclusively from the maternal and the patenal allele, respectively. In mouse brain, *Air* is marked by the presence of a DMR and a DMH, and is imprinted, whereas the absence of DMR and DMH correlates with biallelic expression of *Igf2r.* In human *IGF2R*, we show here that the absence of DMH, even in the presence of DMR, correctly identifies the biallelic expression of *IGF2R* and the absence of '*AIR*' antisense.

to produce 170 bp C-allele and 134 bp **T**-allele. To create an internal control for the restriction digestion, which is critical in RFLP–PCR analysis, we added an internal restriction site (underlined sequence, bold and italic nucleotides) 8 bases from the 5′ end of the forward primers. Remaining fragments of any undigested products were detected as minor bands 8 bp longer than the major bands on a polyacrylamide gel (Fig. 2).

Chromatin immuno-precipitation

ChIP assay was performed as previously described (11,12). Briefly, about 5 million cells were fixed with 1% formaldehyde, and were then sonicated for 180 s (10 s on and 5 s off) on ice by a Branson sonicator with a 2 mm microtip and setting of 40% for output control and 90% for duty cycle. The sonicated chromatin (0.6 ml) was clarified by centrifugation, aliquoted and snap-frozen in liquid nitrogen. To perform ChIP, sonicated chromatin (20 μl) was diluted 10-fold, cleared with salmon sperm DNA/protein A-agarose (80 μl) and purified with specific antiserum (2–5 μl) and protein A-agarose (60 μl). The DNA from the bound chromatin after cross-linking reversal and proteinase K treatment was further purified by MiniElute PCR purification kit (Quiagen, Valencia, CA, USA) and finally eluted in 100 μl of low-TE buffer (1 mM Tris, 0.1 mM EDTA).

ChIP–PCR and restriction enzyme digestion

Duplicate PCR reactions (5 μl under liquid wax) contained 2 μl ChIP (or input) DNA, 0.1 μM appropriate primer pairs (Table 1), 50 μM deoxynucleotide triphosphate and 0.2 units KlenTaq I (Ab Peptides, St Louis, MO, USA). Standard PCR conditions were 95°C for 60 s, followed by 30 cycles of 95°C for 10 s and 65°C annealing (and extension) temperature for 90 s and finally 72°C for 10 min. All primer sets were tested for the absence of primer–dimer products. The PCR products were digested with appropriate enzymes (New England Biolabs, MA, USA; 1 unit) in a total volume of 10 μl for 6–12 h under liquid wax. The digested products were separated on a 5% polyacrylamide-urea gel and quantified by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA).

(Q)-PCR

Enrichment of modified histones in DNA obtained from the ChIP assays was determined by Q-PCR using an ABI Prism 7900HT sequence detector following the ABI protocol. The Q-PCR assays were run in triplicate on 384-well plates. We designed allele-specific oligonucleotide primers to amplify specifically each parental allele (Table 2). All primer sets for Q-PCR were free of primer–dimer products. We used SYBR Green in our Q-PCR assays. At the end of the Q-PCR amplification we run a 'melting curve analysis' to confirm the homogeneity of all Q-PCR products. Relative enrichment of a given target sequence by a specific antibody is determined by a 'delta Ct and delta–delta Ct' calculation by an ABI protocol with reference to human *GAPD* or mouse ribosomal L7 protein gene control.

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