Enhancer Blocking Activity of the Insulator at H19-ICR Is Independent of Chromatin Barrier Establishment

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Transcriptional insulators are cis regulatory elements that organize chromatin into independently regulated domains. At the imprinted murine Igf2/H19 locus, the H19-ICR insulator prevents the activation of the Igf2 promoter on the maternal allele by enhancers that activate H19 on the same chromosome. Given the well-demonstrated role of H19-ICR as an enhancer blocker, we investigated its ability to define a chromatin barrier, as the two activities are coincident on several insulators and may act in concert to define a functional chromatin boundary between adjacent genes with distinct transcriptional profiles. Allele-specific association of posttranslationally modified histones, reflecting the presence of active or inactive chromatin, was analyzed in the region encompassing H19-ICR using chromatin immunoprecipitation. The existence of differential histone modifications upstream and downstream of H19-ICR specifically on the maternal chromosome was observed, which is suggestive of a chromatin barrier formation. However, H19-ICR deletion analysis indicated that distinct chromatin states exist despite the absence of an intervening “barrier.” Also, the enhancers can activate the Igf2 promoter despite some parts of the intervening chromatin being in the silent state. Hence, H19-ICR insulator activity is not dependent on preventing the enhancer-mediated alteration of the histone modifications in the region between the Igf2 promoter and the cognate enhancers.

Transcription is regulated by appropriate interactions of various cis- and trans-acting factors and hence is influenced by chromatin structure and organization. It is intriguing indeed that the cis regulatory elements like enhancers can exert their influence on promoters and functionally interact with them specifically, despite being separated by several kilobases of DNA. In this context, insulators, the cis-acting elements that can curtail the activity of enhancers and/or prevent the heterochromatin spread in a position-dependent manner, are believed to play a crucial role in gene regulation (11). Insulators have been identified in the genomes of several organisms, including Saccharomyces cerevisiae, Drosophila, chickens, and mammals. Genome-wide analysis has predicted the presence of nearly 14,000 sites in the human genome that could potentially act as insulators (16, 37). However, the mechanisms underlying insulator, enhancer, and silencer activities and their interactions are only partially understood.

Several mechanisms have been proposed for the enhancer-blocking activity of the insulators based on analysis of the chicken β globin locus (cH$4$) and Drosophila insulators gypsy and acs-acs. Considering the looping model of enhancer action, insulators might create chromatin loops due to protein-mediated tethering of insulator regions to specific subnuclear structures like the nucleolus (39) or the nuclear periphery, thus restricting the enhancer activity to promoters that share the same loop (11). In view of the tracking model of enhancer activity, insulators may prevent the spreading of some positive activating signals that are initiated at the enhancer and progress till the cognate promoter, leading to its activation. In support of this hypothesis, the cH$4$ insulator from the chicken β globin locus prevented the spread of histone acetylation and interfered with RNA polymerase II transfer between the enhancer and the promoter in an analysis relying on minichromosomes (40). Thus, it has been suggested that activating modifications may be blocked by enhancer blockers just as heterochromatin spread is stopped by barrier elements (33). In this context, it is interesting to note that several insulators have a combination of enhancer-blocking and chromatin barrier activities when tested in vitro (36). Importantly, the proposed mechanisms are not mutually exclusive and may be context dependent (11).

An insulator regulates imprinted monoallelic expression of the mammalian Igf2 gene. A differentially methylated region (DMR), a complex regulatory region located upstream of the H19 gene (Fig. 1), governs several aspects of the parental allele-specific expression of the Igf2 and H19 genes that are activated by the shared enhancers. The DMR acts as an imprint control region (ICR) and harbors an insulator. Murine H19-ICR has four methylation-sensitive sites that bind CTCF, a zinc finger protein important for insulator function (2, 13). The unmethylated DMR on the maternal allele binds CTCF and organizes a functional insulator responsible for preventing maternal Igf2 expression. Methylation of the DMR on the paternal chromosome abrogates CTCF binding and insulator function (9), leading to transcription of paternal Igf2. Deletion of the insulator carrying DMR (14, 30, 31) and site-specific mutagenesis of the CTCF sites (26) lead to activation of maternal Igf2, demonstrating a crucial role of enhancer blocking by the insulator for Igf2 regulation. The DMR also harbors a silencer active only in its methylated state on the paternal allele and responsible for silencing downstream H19 (30). In the paucity of CTCF binding due to methylation, the maternal allele acquires a paternal epigenotype which is hypermethyl-
relevance of chromatin barrier establishment in conjunction with enhancer blocking for defining functionally distinct chromatin domains in vivo. Our studies indicate remarkable allele-specific differences in the DMR suggestive of a chromatin barrier on the maternal chromosome that carries the functional insulator. However, the analysis of DMR deletion mutants (DMRdelG), a definitive test for the endogenous barrier function, indicated the absence of a functional chromatin barrier. Our results also suggested that the enhancers can activate promoters despite the presence of silent chromatin in the intervening region, in accordance with the looping model of enhancer action, although the position dependence of insulator action is expected to involve some form of tracking mechanism also.

MATERIALS AND METHODS

Experiments using mice were conducted as approved by the Institutional Animal Ethics Committee.

Analysis of Igf2 and H19 expression. Total mRNA was isolated from primary mouse embryonic fibroblasts (PMEF) derived from embryos of the required genotypes, and Igf2 and H19 were detected by Northern hybridization using standard protocols. Elongation factor (E') was used as the loading control.

Allele-specific Igf2 expression was analyzed using single-nucleotide primer extension (Snape) assays in RNA isolated from PMEF. For paternal inheritance of the DMR deletion, interspecific matings were set up such that the Igf2/H19 locus had a paternally inherited domesticus allele (wild type or DMRdelG mutant) and a maternally inherited castaneus allele (wild type). For assessing maternal inheritance of the DMR deletion, reverse crosses were set up such that the maternally inherited allele was domesticus (wild type or DMRdelG mutant) while the paternal allele was castaneus (wild type). PMEF were generated from the resulting embryos at embryonic day 13.5. Total mRNA of the PMEF was converted to cDNA, which was then subjected to Snape analysis as described previously (30) to estimate the allele-specific contribution of Igf2 to total mRNA. Control reactions with genomic DNA were used to verify the incorporation of [α-32P]dATP by castaneus DNA, of [α-32P]GTP by domesticus DNA, and of both nucleotides by DNA of F1 progeny of the interspecific cross.

ChiP. Chromatin immunoprecipitation (ChiP) grade antibodies anti-dimethyl H3K4 (catalogue number 07-074), anti-acetyl H3K9 (catalogue number 07-352), and anti-dimethyl H3K9 (catalogue number 07-441) were from Upstate Biotechnology.

For ChiP with anti-dimethyl-H3K4 and anti-acetyl-H3K9 antibodies, the Upstate Biotechnology protocol was followed with some modifications. Briefly, 107 PMEF were cross-linked in 1% formaldehyde in Dulbecco’s phosphate-buffered saline for 5 min at 25°C. Similarly, a single-cell suspension from about 100 mg of neonatal (postnatal day 4) liver was cross-linked. In either case, the reaction was quenched by glycine (0.125 M). Sonication was carried out in lysis buffer (1% sodium dodecyl sulfate [SDS], 10 mM EDTA, 50 mM Tris–Cl, pH 8.0) to shear the chromatin to an average DNA fragment size of 200 to 250 bp. Precleared chromatin was incubated overnight with the antibody. Chromatin from approximately 2 x 106 cells and 10 µl of the antibody was used for each ChiP reaction. The immune complexes were collected on protein A Sepharose beads and washed appropriately before elution. The cross-links were reversed, and the immunoprecipitated DNA was puriﬁed for analysis.

For ChiP with anti-dimethyl-histone H3K9 antibody, cells were cross-linked as above and sonicated to an average DNA fragment size of 200 to 250 bp in lysis buffer (1% SDS, 50 mM Tris–Cl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) as per the ChiP protocol by Kouzountzi and Kyrissma (http://www.epigenome.occu/research/searchprotocols.php?PROT711). Subsequent steps of a typical ChiP were followed using buffers as described in the protocol. Immune complexes were eluted, cross-links were reversed, and DNA was puriﬁed for analysis.

For each ChiP, a fraction of the “input” chromatin was also processed for DNA puriﬁcation. A mock immunoprecipitation without antibody was carried out in parallel (no antibody control). The results shown are representative of two to three independent ChiPs for each antibody.

Real-time quantitative PCR for quantitation of immunoprecipitated DNA. Real-time PCRs were carried out on an ABI PRISM 7700 system (Applied Biosystems) using SYBR green as the reporter dye. All PCRs were done in triplicate. The crossover threshold value (Ct) was used for quantitation using a

FIG. 1. Map of the Igf2/H19 locus. (A) Schematic map of the locus showing relative positions of genes and regulatory elements. (B) Genomic regions deleted in the mouse mutants used in this study. The region deleted in mutant alleles DMRdelG and H19del13 is shown as the gray box in each case in reference to the wild-type allele (top line). Four black columns above the DMR represent the CTCF binding sites. (C) Relative positions of the ampiclons used for ChiP analysis (primer sequences in Table 1). Restriction sites EcoRI (E), BamHI (B), HindIII (H), XbaI (X), and Sall (S) are depicted. The H19 transcription start site is shown as a forward arrow above H19.

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Table 1. Sequences of the primers used for ChIP analysis

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* Amplicon positions relative to the H19 transcription start site are shown in Fig. 1.

The H19-ICR insulator, located upstream of H19 and about 80 kb downstream of Igf2 (Fig. 1), is crucial for preventing maternal Igf2 expression in several tissues, including neonatal liver, heart, and skeletal muscle. Based on deletion analysis, H19-ICR has been inferred to be a cis-acting regulatory element such that its deletion from the paternal chromosome does not alter the monoallelic expression pattern of Igf2 but its deletion from the maternal chromosome leads to activation of maternal Igf2, thus rendering the Igf2 expression biallelic (14, 30, 31). We considered PMEF to be amenable for ChIP analysis and hence ascertained the role of the H19-ICR insulator in controlling the imprint expression of Igf2 in PMEF. Similar to the expression in other tissues, Igf2 was expressed in wild-type PMEF and in PMEF derived from the mutants inheriting the H19-ICR-deleted DMRDelG allele paternally (Fig. 2A). The contributions of paternal and maternal alleles in the total Igf2 RNA of PMEF were analyzed in DMRDelG mutants; semiquantitatively, using Snupene reactions. The Igf2 expression was observed to be from the paternal allele in the wild type and in cases where the DMRDelG mutation was inherited paternally, but it was biallelic upon maternal inheritance of the DMRDelG mutation (Fig. 2C), indicating that the imprint monoallelic expression in PMEF is regulated by the H19-ICR insulator in cis as in other tissues examined previously (14, 30, 31).

Also, H19-ICR is responsible for regulating imprint expression of H19, and deletion of H19-ICR from the paternal allele leads to robust activation of normally silent paternal H19 (14, 30, 31). However, in PMEF the paternal H19 activation

## RESULTS

### Table 1. Sequences of the primers used for ChIP analysis

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The enrichment of an amplicon in the immunoprecipitated sample was calculated using the following expression: (DNA after ChIP) / (DNA in input fraction) = 2^{(-2(ΔCt-ChIP-ΔCt-ChIP_control))} = 2^{-A}.

The enrichment in the test amplicon was normalized to the enrichment obtained in the same ChIP experiment for the β actin amplicon. All the values in Table 1 are expressed as enrichment relative to the enrichment of the β actin amplicon. Primer sequences used for PCR are given in Table 1.

### FIG. 2. Expression analysis of Igf2 and H19 in PMEF of wild type and DMRDelG mutants.

#### A) Northern blot to detect Igf2 expression in the wild type (lanes 1 and 2, genotype +/+ and in mutants inheriting DMRDelG on the paternal allele (lanes 3 and 4, genotype +/+DMRDelG). (B) Northern blot to detect H19 expression from the wild-type paternal allele (lanes 1 and 2, genotype +/+H19del13, wild-type paternal allele (lanes 3 and 4, genotype H19del13/+), DMR-deleted maternal allele (lanes 5 and 6, genotype DMRdelG/H19del13), and DMR-deleted paternal allele (lanes 7 and 8, genotype H19del13/DMRdelG). Elongation factor (EF) was used as a loading control for the analysis, and the numbers below each lane depict the ratio of Igf2 to EF in panel A and H19 to EF in panel B. (C) Effect of DMRDelG mutation on allele-specific expression of Igf2 analyzed by Snupene analysis. The top panel depicts the control reactions with genomic DNA. Incorporation of [α-32P]dATP by human DNA (lane 1) and [α-32P]dATP by doryctous DNA (lane 2) and of both nucleotides by DNA of F1, progeny of the interspecific cross (lanes 3, 4, and 5). The bottom left panel shows the absence of any effect on the allelic contribution to Igf2 mRNA due to paternal inheritance of DMRDelG analyzed in the PMEF of embryos derived from interspecific mating such that the Igf2/H19 locus has a paternally inherited doryctous allele (wild type or DMRDelG mutant) and a maternally inherited castaneus allele (wild type). Wild-type cDNA (lane 6, genotype +/+ ) incorporated primarily [α-32P]dATP due to monoallelic expression of Igf2 from the paternal doryctous allele. In the paternal mutant cDNA (lane 7, genotype +/+DMRDelG), the allelic contribution was not altered. The bottom right panel shows the activation of maternal Igf2 upon inheritance of DMRDelG maternally analyzed in the PMEF of embryos derived from interspecific mating such that the Igf2/H19 locus has a maternally inherited doryctous allele (wild or DMRDelG mutant) and a paternally inherited castaneus allele (wild type). Wild-type cDNA (lane 8, genotype +/+ ) incorporated primarily [α-32P]dATP due to monoallelic paternal expression of Igf2 while maternal mutant cDNA (lane 9, genotype DMRdelG/H19del13) incorporated both [α-32P]dATP and [α-32P]dATP, demonstrating biallelic expression of Igf2. The genotypes of the PMEF resulting from interspecific matings for each analysis are mentioned below each lane: cas, castaneus; dom, doryctous.
due to H19-ICR deletion was hardly detectable (Fig. 2B). Surprisingly, the expression of H19 was almost entirely abrogated in the PMEF of deletion mutants when the deletion was inherited maternally (Fig. 2B). This is in contrast to observations made earlier in neonatal liver, skeletal muscle, and heart, where the H19-ICR deletion from the maternal allele does not abolish H19 expression. This suggests that some aspects of the regulation of H19 expression in PMEF are distinct from expression in other tissues.

To investigate the ability of the DMR to create a chromatin barrier, high-resolution ChIP (17) against posttranslational modifications of histones was carried out in PMEF and neonatal liver. Associations of dimethyl-H3K4 and acetyl-H3K9 were chosen to reflect the presence of active chromatin, while association of dimethyl-H3K9 was used to reflect the presence of a repressive chromatin region (4). We analyzed the chromatin structure of the region encompassing the DMR that harbors the H19-ICR insulator (Fig. 1) in an allele-specific manner, since the H19-ICR insulator is functional only on the maternally inherited chromosome. PMEF were derived from +/H19del13 embryos to analyze the maternally inherited chromosome. The 13-kb deletion of H19 and the upstream region (19) on the paternal allele ensured that the information about the chromatin structure is derived specifically from the maternal allele. Similarly, PMEF from H19del13/+ embryos were used to analyze the histone modifications from the paternal allele. The enrichment of the target sequences in the ChIP samples was analyzed by real-time PCR, and β actin and Igk loci were used as controls for the transcriptionally active and transcriptionally silent chromatin, respectively.

Chromatin structure on the maternally inherited chromosome in PMEF. We focused our attention on the maternally inherited insulator which is known to block the access of the enhancer to the Igf2 promoter. The region upstream to the DMR was practically devoid of activating histone modifications dimethyl-H3K4 and acetyl-H3K9 (Fig. 3A and B). However, the DMR and the region downstream of it, including the H19 gene, showed a significantly high enrichment in ChIP with dimethyl-H3K4. This correlates well with the active transcription of the H19 gene from the maternal chromosome. Acetyl-H3K9 presence was generally quite low through the entire region except within the actively transcribing H19 gene (Fig. 3B). The peak of activating histone modifications downstream of the fourth CTCF site suggests recruitment of activating factors by the DMR which may be important for the activation of downstream H19. Consistent with this, deletion of the DMR leads to a variable degree of loss in H19 expression (32). Alternatively, the transition in chromatin structure may suggest the inability of the enhancer-initiated histone modifications to spread past the insulator region, as observed for Ch54 in minichromosome-based assays (40).

Analysis of the maternal DMR and its flanking regions for the presence of dimethyl-H3K9 (Fig. 3C) exhibited an enrichment pattern complementary to the one observed with dimethyl-H3K4. The region upstream of the DMR was enriched in the ChIP samples, while regions encompassing the DMR and downstream of it were practically devoid of this repressive chromatin mark. Together, these results are suggestive of a putative “chromatin barrier” organized by the DMR on the maternal chromosome.

Chromatin structure on the paternally inherited chromosome in PMEF. Igf2 is expressed specifically from the paternal chromosome. On the paternal allele, neither the DMR nor the regions flanking it were enriched in ChIP with dimethyl-H3K4 and acetyl-H3K9 (Fig. 3A and B) despite a high immunoprecipitation efficiency as judged by the enrichment of β actin. The silent chromatin structure of the DMR and its flanking regions on the paternal chromosome was further confirmed by ChIP against dimethyl-H3K9 (Fig. 3C). Here the region upstream of the DMR was highly enriched, while the DMR and the region downstream of it had lower but significant enrichment, consistent with the known transcriptionally inactive status of the H19 gene due to the presence of a silencer in the DMR responsible for MeCP2-dependent silencing of H19 (7). The degree of enrichment was similar to that of Igk, a gene expected to be transcriptionally silent in PMEF. The nonfunctional insulator region of the paternal allele was also not associated with a putative “chromatin barrier” and was entirely distinct from the same region on the maternal allele.

Importantly, our analysis of the paternal chromatin structure indicated that the activation of Igf2 by the downstream enhancers is brought about despite a significantly large intervening region (at least 10 kb) being in the repressed state based on histone modification status. Not only the H19 gene and the DMR but also the region examined upstream of these is in a repressed state. Since the activation of paternal Igf2 by the enhancers is possible despite a large region being associated with silent chromatin modifications, it seems that the enhancer is not responsible for altering the intervening chromatin structure in terms of histone modifications on the paternal chromosome. Therefore, the differential domains of histone modification status, as seen on the maternal chromosome, cannot be attributed to stalled or paused enhancer-mediated chromatin alterations.

Allele-specific chromatin structure analysis in neonatal liver. The absence of the activating histone modifications from the region upstream of the DMR on the paternal chromosome was interesting. It suggested the absence of a progressive spread of activating histone modifications, as might be expected if the tracking mechanism of enhancer-based activation is considered. We decided to analyze the status of these modifications in neonatal liver, a tissue which expresses both H19 and Igf2 at reasonably high levels due to promoter activation by well-characterized endodermal enhancers. As shown in Fig. 4A, dimethyl-H3K4 was associated with the paternal chromosome in the H19 gene and the region upstream of it, as in the PMEF. Also, in contrast to the PMEF, on the paternal chromosome, acetyl-H3K9 (Fig. 4B) exhibited a high degree of association with H19 and its upstream region in complete accordance with a higher level of H19 expression in liver. The region upstream of the insulator was found not to be enriched on either the paternal or the maternal chromosome upon ChIP with dimethyl-H3K4 and acetyl-H3K9, suggesting the absence of these modified histones from both the alleles. The absence of activating histone modifications from the paternal chromosome both in PMEF and liver indicates that enhancer-mediated tracking, if operational at this locus, does not involve the dimethyl-H3K4 and acetyl-H3K9 histones.

Chromatin structure in DMR-deleted chromosomes in PMEF and neonatal liver. Since the DMR on the paternal chromo-
some is in an epigenetic state entirely different from that on the maternal chromosome, in order to ascertain the relevance of the observed "chromatin barrier" with regard to spread of chromatin states, we considered it important to analyze the chromatin structure in cells that carried a germ line deletion of the functional insulator from the maternal allele. Analysis of such PMEF (DMRdelG/H19del13) using three amplicons in the region upstream of DMRdelG demonstrated that the region continued to be associated with repressive dimethyl-H3K9 and devoid of the active chromatin marks dimethyl-H3K4 and acetyl-H3K9 (Fig. 5), like the wild-type maternal chromosome (Fig. 3). Also, activating histones were associated downstream of the deletion, as in the wild type. The upstream silent chromatin clearly did not spread into the H19 region. The DMR deletion also had no pronounced effect on histone modification status analyzed by ChIP in neonatal liver (Fig. 4). Deletion of
PMEF. In the downstream region within the H19 gene, however, the DMR-deleted paternal allele acquired modifications similar to those of the wild-type maternal allele, i.e., it was associated with dimethyl-H3K4 and acetyl-H3K9 (Fig. 5), comparable to the maternal allele. There was also a reduction in the associated dimethyl-H3K9 compared to that with the wild-type allele. This correlated extremely well with the earlier inference that the DMR also harbors an epigenetically regulated silencer responsible for silencing the H19 gene on the paternal allele during early development, and its deletion leads to transcriptional activation of H19 in several tissues (30).

**DISCUSSION**

Our analysis suggested significant differences in the histone modification status of the chromatin at H19-ICR and the region upstream of it. We derived allele-specific information about the extent of association of the modified histones with the allele under investigation, relative to an unlinked β actin locus, and ensured the absence of any interference from the other parental allele. Recently, ChiP analysis was used to demonstrate a distinct histone modification status of H19-ICR on the two parental alleles relative to each other (12, 34). Consistent with these studies, we observed similar allele-specific differences in H19-ICR with regard to the association of modified histones. Further, we analyzed the histone modifications upstream of H19-ICR in addition to those at H19-ICR. Our results demonstrate a clear demarcation of chromatin states specifically on the maternally inherited chromosome.

The region upstream of H19-ICR was associated with dimethyl-H3K9 and devoid of activating modifications dimethyl-H3K4 and acetyl-H3K9, while the reverse was true for the H19-ICR region. These differences were noted specifically on the maternal chromosome, while the paternal allele was associated primarily with dimethyl-H3K9 in the entire region investigated. With such distinct chromatin organizations between the paternal and maternal H19-ICR, it is intriguing that intergenic transcripts are observed from both the alleles (25). It will be interesting to discern the biological role of these transcripts.

The demarcation in chromatin structure on the maternal chromosome is consistent with the observed H19 expression and Igf2 transcriptional silence on the maternal allele. The peak of dimethyl-H3K4, in combination with the juxtaposed silent and active chromatin structures, is suggestive of the presence of a chromatin barrier (35). However, the observed functional chromatin boundary was clearly not a consequence of a barrier activity. In a strict test for the barrier function, its deletion is predicted to lead to altered histone modifications in the flanking regions. Since the 6.5-kb deletion of the DMR did not alter the histone modification profile either upstream or downstream or the deletion, it was obviously not responsible for establishing and/or maintaining the chromatin barrier. This is in contrast to the boundary element present at the mat locus in yeast, whose deletion allows the repressive histone modifications to spread to adjoining regions (22).

While we observed no alteration in the histone modification pattern at the H19 promoter subsequent to deletion of the H19-ICR region and continuation of “active chromatin” status, an entirely distinct observation was made when maternal

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**FIG. 4.** Parental allele-specific histone modification pattern in the region encompassing the DMR as analyzed by ChiP in postnatal day 4 neonatal liver. (A) Positions of the amplicons used for the ChiP analysis placed relative to the H19 transcriptional start site (forward arrow) and the DMR. (B) ChiP using anti-dimethyl-H3K4 antibodies. (C) ChiP using anti-acetyl-H3K9 antibodies. Enrichment at different tissues. While transcription of maternal

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[Diagram of H19 expression and histone modifications]
FIG. 5. Histone modification pattern in the regions upstream and downstream of the DMR as analyzed by Chip in DMR-deleted alleles in PMEF. (A) Positions of the amplicons used for the Chip analysis placed relative to the H19 transcriptional start site (forward arrow) and deletion DMRdelG (gray rectangle). (B) Chip using anti-dimethyl-H3K9 antibodies. (C) Chip using anti-acetyl-H3K9 antibodies. (D) Chip using anti-dimethyl-H3K9 antibodies. Shown are the DMR-deleted maternal allele (red solid line, DMRdelG/H9del13), DMR-deleted paternal allele (blue solid line, H9del13/DMRdelG), and no-antibody controls (color-matched dotted lines). In each case, the y axis shows enrichments relative to the β-actin locus and the x axis shows the amplicons used. Bar diagrams show the enrichment at the β-actin (act) and Ig control loci. Red bar, DMRdelG/H9del13; blue bar, H9del13/DMRdelG; black bars (adjacent to red and blue), corresponding no-antibody controls.

Inheritance of the CTCF binding site mutation was analyzed. In this case, the downstream region encompassing the H19 promoter exhibited an increase in dimethyl-H3K9 and trimethyl-H3K27, the hallmarks of silent chromatin [12]. The different consequences of the deletion mutation and site-specific mutations are not surprising since site-specific mutagenesis of CTCF binding sites retains the rest of functional H19-ICR, although in a paternalized epigenetic state capable of acting as a silencer. Consequently, the silencer may recruit specific chromatin modifiers responsible for creation of silent chromatin at the H19 promoter.

It was surprising that despite a major loss in H19 transcription due to the H19-ICR deletion on the maternal chromosome in PMEF, the H19 promoter was found to be associated with activating modifications rather than silent modifications of the chromatin. In another recent study, the H19 promoter in PMEF with a maternal H19-ICR deletion was reported to have a considerable reduction in the maternal allele-specific prefer-
ential enrichment of activating histone modifications compared to that of the wild type (34). The observed discrepancy in the PMEF chromatin structure between the two studies may be due to different experimental designs; our experiments reported the histone modification status quantitatively, specifically from the allele under investigation, by ensuring that the other allele could not contribute toward the analysis. Promoters that are transcriptionally silent despite having activating histone modifications have been reported in other studies, and it has been suggested that these promoters are poised for transcription although not transcribing actively (24, 27). It is noteworthy that deletion of H19-ICR on the paternal allele leads to activation of H19 in several tissues, including neonatal liver, skeletal muscle, and heart (14, 30), but activation in PMEF is hardly detectable (Fig. 2B). However, deletion on the paternal allele also led to the presence of activating modifications on the H19 promoter in PMEF (Fig. 5). Thus, it appears that the H19 promoter, even when associated with activating histone modifications, is unable to transcribe effectively in the absence of H19-ICR from either maternal or paternal alleles. It is plausible that the mechanism for regulation of H19 expression in PMEF is slightly different than in other tissues and requires some additional regulatory factor dependent on H19-ICR.

Our analysis of the wild type and deletion mutants provides evidence that at the Igf2/H19 locus, silent and active chromatin can be maintained despite the absence of a chromatin barrier element actively protecting the distinct domains, suggesting that the silent chromatin state does not necessarily spread. Active and inactive chromatin have been reported to be interspersed without the ability to spread (23) at the Igf2 locus also. The generality of our observation regarding the absence of a chromatin barrier at the Igf2/H19 locus needs to be tested in other genomic contexts. Since CTCF does not contribute to chromatin barrier function at H19-ICR, its presence at a large number of sites in the genome might suggest that for defining functionally distinct chromatin domains, the enhancer-blocking function of CTCF and/or its ability to organize a higher-order chromatin structure, rather than barrier formation, is more important.

With chromosome conformation capture (3C) analysis, CTCF has also been shown to organize the higher-order chromatin structure, which may be relevant for gene regulation in a context-dependent manner (29). In accordance with this, the DMR organizes the higher-order chromatin structure at the Igf2/H19 locus. Distinct looped domains, established by the interaction of the DMR with other cis elements like DMR1, DMR2, and MAR3, influence enhancer-promoter interactions and thus regulate Igf2 expression in an allele-specific manner (18, 21). Subsequent studies show that the H19-ICR insulator can effectively interfere with promoter-enhancer interaction even at heterologous positions by forming associations with the promoter and the enhancer (38). The 3C analysis provided evidence for the final stable complex formation by the enhancer and the Igf2 promoter, but it could not explain the position dependence of the insulator for preventing these interactions.

The position dependence of insulator activity intuitively suggests interference with some form of tracking signal initiated at the enhancer. A few enhancers like Eβ and Eγβ, at the TCRβ and IgH loci, respectively, are responsible for creating a large chromatin domain with activating histone modifications (6, 28, 29) in accordance with the proposed tracking model of enhancer-based activation. In this context, the enhancers at the Igf2/H19 locus appeared different mechanistically. Our results demonstrate that a significant part of the intervening region, between the active Igf2 promoter and enhancers at the Igf2/H19 locus, has a silent chromatin structure. A region of at least 5 kb upstream of the H19-ICR insulator was observed to be rich in repressive histones and devoid of activating histone modifications. An identical pattern was seen on the wild-type and DMR-deleted maternal and paternal alleles, i.e., irrespective of the activation status of the Igf2 promoter. Thus, the enhancer-based activation of the Igf2 promoter was possible despite a significant part of the intervening chromatin being in a silent state as judged by histone modification status. Surprisingly, the DMRs on both the maternal and paternal alleles do generate sense and antisense transcripts (25). In view of the belief that the position dependence of insulator action must include some form of tracking or linking signal for promoter-enhancer interaction, we infer that the tracking of an enhancer-based signal at the Igf2 locus does not involve chromatin modifications investigated in our study, and hence the H19-ICR insulator does not act by preventing this form of tracking signal. What else may constitute a tracking signal and/or can explain the position-dependent activity of the insulators remains to be investigated. It will be interesting to investigate if enhancer-based loading of RNA polymerase on the Igf2 promoter is affected by the presence of the insulator, as observed for the hH54 insulator in minichromosome-based assays (40), despite the absence of histone modifications along the intervening chromatin domain.

Thus, the H19-ICR region, responsible for maintaining independent expression profiles of Igf2 and H19, acts as the transition point for distinct chromatin states on the maternal chromosome. However, there is no chromatin barrier element associated with the CTCF-dependent H19-ICR insulator.

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