Analysis of Sequence Upstream of the Endogenous *H19* Gene Reveals Elements Both Essential and Dispensable for Imprinting

Joanne L. Thorvaldsen, Melissa R. W. Mann, Okechukwu Nwoko, Kristen L. Duran,
and Marisa S. Bartolomei*

Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received 10 October 2001/Returned for modification 14 November 2001/Accepted 12 January 2002

Imprinting of the linked and oppositely expressed mouse *H19* and *Igf2* genes requires a 2-kb differentially methylated domain (DMD) that is located 2 kb upstream of *H19*. This element is postulated to function as a methylation-sensitive insulator. Here we test whether an additional sequence 5' of *H19* is required for *H19* and *Igf2* imprinting. Because repetitive elements have been suggested to be important for genomic imprinting, the requirement of a G-rich repetitive element that is located immediately 3' to the DMD was first tested in two targeted deletions: a 2.9-kb deletion (Δ DMD Δ G) that removes the DMD and G-rich repeat and a 1.3-kb deletion (Δ G) removing only the latter. There are also four 21-bp GC-rich repetitive elements within the DMD that bind the insulator-associated CTCF (CCCTC-binding factor) protein and are implicated in mediating methylation-sensitive insulator activity. As three of the four repeats of the 2-kb DMD were deleted in the initial 1.6-kb Δ DMD allele, we analyzed a 3.8-kb targeted allele (Δ 3.8kb-5'*H19*), which deletes the entire DMD, to test the function of the fourth repeat. Comparative analysis of the 5' deletion alleles reveals that (i) the G-rich repeat element is dispensable for imprinting, (ii) the Δ DMD and Δ DMD Δ G alleles exhibit slightly more methylation upon paternal transmission, (iii) removal of the 5' CTCF site does not further perturb *H19* and *Igf2* imprinting, suggesting that one CTCF-binding site is insufficient to generate insulator activity in vivo, (iv) the DMD sequence is required for full activation of *H19* and *Igf2*, and (v) deletion of the DMD disrupts *H19* and *Igf2* expression in a tissue-specific manner.

Genomic imprinting is an epigenetic modification to differentiate the maternal and paternal alleles of a gene, the consequence of which is parent-specific gene expression. Differential DNA methylation, allele-specific nuclease hypersensitivity, and repetitive elements have been proposed to be important for the regulation of imprinted gene expression (5, 6, 43). The imprinted mouse *H19* gene, which is expressed from the maternal allele, possesses each of these characteristics. A 2-kb sequence between –4 and –2 kb relative to the *H19* transcription start site (designated as the differentially methylated domain [DMD] or the imprinting control region) is exclusively methylated on the paternal allele throughout development (40, 52, 54). Maternally specific hypersensitive sites have been mapped to this 2-kb region in embryonic and neonatal tissues (28, 34). Coincident with these hypersensitive sites are four 21-bp GC-rich repeats that are conserved in mouse, rat, and human tissue (25, 48). Finally, a 461-bp sequence harboring 32 copies of a G-rich element (GGGGTATA) resides immediately 3' to the DMD (48, 53).

The mouse *H19* gene is located 90 kb 3' to the oppositely imprinted insulin-like growth factor 2 (*Igf2*) gene. The expression of the *H19* and *Igf2* genes is mediated through shared enhancers that are located 3' to *H19* (1, 3, 32, 37). Perhaps the most critical part of the joint regulation of *H19* and *Igf2* is the DMD, which has been the focus of multiple targeted deletion

studies at the endogenous locus (32, 47, 51). While these studies are consistent with a role for the DMD in the silencing of *H19* on the paternal allele and *Igf2* on the maternal allele, the DMD is also hypothesized to function as a methylation-sensitive insulator. Insulators isolate a gene from enhancers, thereby inhibiting enhancer-induced expression of the gene (10). According to the insulator model, the DMD insulator is active on the maternal allele and prevents the expression of *Igf2* by blocking its access to downstream enhancers. On the paternal allele, hypermethylation of the DMD is postulated to inactivate insulator function by inhibiting critical protein-DNA interactions, as described for the ubiquitous zinc finger protein CCCTC-binding factor (CTCF) (9, 27, 39). The DMD insulator activity has been demonstrated by using in vitro enhancer blocking assays, and this activity is dependent upon the 21-bp repeats of the DMD sequence (9, 27, 29, 32, 33, 50). CTCF binds to these repeat elements in vitro in a methylation-sensitive manner.

It was previously reported that a 1.6-kb deletion of the 2-kb DMD, which is designated Δ DMD and removes three of the four CTCF-binding 21-bp conserved repeats, results in the loss of *H19* and *Igf2* imprinted expression and perturbations in allele-specific methylation (51). To further define the *cis* elements required for *H19* and *Igf2* imprinting at the endogenous locus, we have constructed three additional mutations 5' of *H19* and compared methylation and allelic expression of the mutant alleles to the original 1.6-kb Δ DMD allele (Fig. 1). To test the role of the G-rich repeat sequence in *H19* and *Igf2* imprinting, we generated the Δ G deletion that removes 1.3 kb of sequence, including the 461-bp G-rich repetitive element. In

* Corresponding author. Mailing address: Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. Phone: (215) 898-9063. Fax: (215) 573-6434. E-mail: bartolom@mail.med.upenn.edu.

FIG. 1. DMD and G-rich repeat sequence deletions generated at the *H19* endogenous locus. The top line shows the 5' *H19* sequence with boxes indicating the 2-kb DMD, the 461-bp G-rich repeat element (G), and the first *H19* exon. The gray triangles within the DMD depict the 21-bp repeats (R1 to R4) that are conserved in mouse, rat, and human 5' *H19* sequences. These sites also function as CTCF-binding sites. The maternal allele-specific hypersensitivity sites (HS1 and HS2) are indicated. The crosshatched boxes correspond to sequences that are removed in each of the targeted deletions. The deleted sequences are as follows: a 1.6-kb *KpnI-HindIII* fragment in the Δ DMD deletion, a 2.9-kb *KpnI-XbaI* fragment in the Δ DMD Δ G deletion, a 1.3-kb *HindIII-XbaI* fragment in the Δ G deletion, and a 3.8-kb *SacI-XbaI* fragment in the Δ 3.8kb-5'*H19* deletion.

addition, to determine whether there is a synergistic effect of the DMD and G-rich repeat sequence on *H19* and *Igf2* imprinting, the Δ DMD Δ G deletion was generated by removal of 2.9 kb of sequence, including part of the DMD and all of the G-rich repeat element. We also tested the role of the remaining seven CpG dinucleotides and the CTCF-binding site within the DMD in directing *H19* and *Igf2* imprinted expression and *H19* allele-specific methylation in the Δ 3.8kb-5'*H19* deletion. This deletion removes a region that encompasses all of the DMD, including the fourth 21-bp conserved element that remained in the initial Δ DMD allele, and the G-rich repeat sequence. Finally, we tested the tissue-specific effects on *H19* and *Igf2* expression that these deletions confer. While the Δ G deletion had no effect on expression or methylation, the Δ DMD and Δ DMD Δ G deletions similarly disrupted *H19* and *Igf2* expression and *H19* parent-specific methylation. These perturbations were tissue specific, suggesting that the DMD interacts differentially with enhancers and other regulatory elements. Finally, the Δ 3.8kb-5'*H19* allele, from which the entire DMD and the last CTCF-binding site were removed, exhibited tissue-specific disruption of imprinting similar to that which was detected from the Δ DMD and Δ DMD Δ G alleles. This result demonstrated that deletion of the 1.6-kb DMD sequence was sufficient to perturb fully the insulator and silencing activity conferred by the 2-kb DMD. Our findings complement those of other studies which assay the DMD function either in vitro by transfection assays or in vivo by deleting the DMD together with several additional kilobases of 5' *H19* sequence from the endogenous locus (9, 27, 29, 32, 33, 47, 50). Taken together, these experiments show that the DMD is a complex element that exhibits methylation-dependent silencing and methylation-sensitive insulator activities that are required for allele-specific silencing of *H19* and *Igf2*, respectively, in all tissues.

MATERIALS AND METHODS

Targeting vectors. The Δ DMD targeting vector was previously described (51). The other vectors were designed to delete 2.9 kb (Δ DMD Δ G), 1.3 kb (Δ G), and 3.8 kb (Δ 3.8kb-5'*H19*) of sequence 5' to the *XbaI* site at -0.8 kb relative to the *H19* transcription start site (Fig. 1). For each vector, a *loxP*-flanked PGK-*neo* cassette and an *H19* sequence isolated from a 129Sv/J mouse genomic library (Stratagene) were cloned into pBKSII (Stratagene) (Fig. 2A). The same 2.6-kb *XbaI-BamHI* *H19* fragment was used for the right arm of each vector. A 6.6-kb *BamHI-KpnI* (Δ DMD Δ G) fragment, an 8.2-kb *BamHI-HindIII* (Δ G) fragment, and a 5.6-kb *BamHI-SacI* (Δ 3.8kb-5'*H19*) *H19* fragment were used for the left arms. The Δ DMD Δ G and Δ 3.8kb-5'*H19* vectors also include a 2.3-kb *SacI* fragment containing a diphtheria toxin A cassette for negative selection (38). The vectors were linearized by *NotI* digestion.

Targeted disruption in ES cells and detection of mutant alleles. Embryonic stem (ES) cells, embryonic day 14.1 (35), were used for the *H19* gene targeting experiments. The cell culture and electroporation methods, Southern blot analysis, generation of chimeric and germ line mice, and genotyping of tail biopsies were performed as previously described (51) with the following exceptions. Targeted *H19* Δ DMD Δ G Δ and *H19* Δ G Δ clones were transiently transfected with a Cre recombinase-expressing plasmid (45) for removal of the PGK-*neo* cassette. Mice harboring the *H19* Δ 3.8kb-5'*H19* Δ allele were mated to transgenic mice expressing Cre recombinase for germ line excision of the PGK-*neo* cassette (14). Mutant alleles were identified by Southern analysis (Fig. 2) and PCR amplification with primers that flank the deleted sequences (Table 1; Fig. 2A and B). Primer pairs G1 and G5 were used to assay *H19* Δ DMD (400 bp), G1 and G6 were used to assay *H19* Δ DMD Δ G (390 bp), G2 and G6 were used to assay *H19* Δ G (320 bp), G3 and G6 were used to assay *H19* Δ 3.8kb-5'*H19* Δ (350 bp), and G4 and G6 were used to assay *H19* Δ 3.8kb-5'*H19* (360 bp). PCR amplification was performed on 50 to 200 ng of tail and liver DNA with Ready-To-Go PCR beads (Amersham) and primers at a final concentration of 0.4 μ M as follows: 2 min of denaturation at 95°C; 32 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and 10 min of extension at 72°C.

Mutant mice were further bred to C57BL/6J mice. Allelic expression and methylation patterns were analyzed in the progeny of heterozygous mutant mice that were bred to the B6(CAST-*H19*) strain of mice (53). These mice have *Mus musculus castaneus* (CAST) *H19* and *Igf2* alleles on a C57BL/6J background. The maternal parent is designated first in hybrid mice. Heterozygous mutant mice were intercrossed to generate homozygous mutant mice.

RNA isolation and analysis. Tissues (liver, tongue, kidney, gut, heart, lung, and brain) were collected from neonatal (0 to 5 day) mice, and total RNA was prepared by the lithium chloride method (4). RNase protection assays (RPAs) were performed to assess *H19* (8, 13), *Igf2* (36), and *rpL32* (20) RNA levels. Products were resolved on 7% polyacrylamide-7 M urea gels.

***H19* allele-specific expression assay.** The *H19* allelic expression assay was conducted on cDNA (cDNA Cycle kit; Invitrogen) prepared from 1 μ g of total RNA by using the LightCycler Real Time PCR system (Roche Molecular Biochemicals). Primers were designed to amplify a coding region of the *H19* gene (642 bp, GenBank accession number AF049091). The forward primer lay over an exon-exon boundary at intron 3 of the *H19* gene (RT1) while the reverse primer was localized within exon 5 (RT2) (Table 1). Fluorescence resonance energy transfer hybridization probes were designed to the C57BL/6J amplicon: *H19* sensor probe, 5'-CCACCTGTCGTCCATCTCC-3'; and *H19* anchor probe, 5'-TCTGAGGCAACTGGGTGTGG-3'. The *H19* sensor probe spans a single nucleotide polymorphism at nucleotide 7954 between C57BL/6J (G) and CAST (A) and was labeled with fluorescein at the 3' end. The *H19* anchor probe was labeled with LC-Red640 at the 5' end and was phosphorylated at the 3' end. To a Ready-To-Go PCR bead, 6.22 μ l of sterile water and 0.38 μ l of TaqStart Antibody (Clontech) were added, and the reaction mixture was incubated at room temperature for 5 min. After incubation, a final concentration of 3.0 mM MgCl₂, a 0.3 μ M concentration of each primer, and a 0.15 μ M concentration of each probe were added to the mix and the volume was brought to 12.5 μ l. From this reaction mix, 10 μ l was removed and added to a LightCycler glass capillary (Roche Molecular Biochemicals) and 10 μ l of cDNA (1/100 to 1/250 diluted in H₂O) and H₂O were added for a final reaction volume of 20 μ l. Samples were denatured at 95°C for 2 min and then amplified for 38 to 45 cycles, depending on tissue type, at 95°C for 0 s, 55°C for 15 s, and 72°C for 25 s. A single fluorescence acquisition occurred at the end of each annealing step. After amplification, a final denaturation step was conducted at 95°C for 0 s, followed by a single annealing step at 50°C for 15 s and a melting curve analysis with fluorescence acquisition occurring continuously as the temperature was increased from 50 to 85°C in increments of 0.2°C. A cooling step at 40°C for 30 s was performed at the end of the melting curve analysis. The data were analyzed by using the Light-

FIG. 2. Generation of the $H19^{\Delta DMD\Delta G}$, $H19^{\Delta G}$, and $H19^{\Delta 3.8kb-5'H19}$ alleles. The $H19$ locus is illustrated before and after homologous recombination of the target vectors and Cre-mediated excision of the PGK-*neo* cassette. The DMD, G-rich repeat sequence (G), and $H19$ exons are depicted in boxes. (A) Endogenous $H19$ locus. Positions of restriction sites (in kilobases) are relative to the $H19$ transcription start site. Correctly targeted clones were identified with the use of external probes A (1.7-kb *EcoRV-EcoRI* fragment) and B (0.75-kb *BamHI-StuI* fragment). (B) Targeting vectors. The PGK-*neo* cassette (*neo*) (boxed) flanked by *loxP* sites (wide arrowheads), $H19$ DNA (thin line), and pBKSII (thick line) is illustrated. The directions of the *neo* and $H19$ transcripts are indicated (narrow arrowheads). Targeting vectors Δ DMD Δ G and Δ 3.8kb-5' $H19$ also included the diphtheria toxin A (DTA) negative selection cassette (boxed). The sequence that is deleted from each vector is described in the legend to Fig. 1. (C) Targeted alleles generated from homologous recombination of each of the targeting vectors. A *loxP*-flanked PGK-*neo* cassette replaced the deleted 5' $H19$ sequences. (D) PGK-*neo*-excised targeted alleles following Cre-mediated recombination. A *loxP* site remains at each targeted locus. (E) Southern analysis of the targeted alleles before and after Cre-mediated removal of the PGK-*neo* cassette. Probes A and B were hybridized to *EcoRV*- and *StuI*-digested DNA, respectively. The sizes of the DNA fragments are shown to the right. The primers (G1 to G6) (Table 1) used to assay the targeted alleles are shown below the schematics in panels A and B.

TABLE 1. Primers used for genotyping, bisulfite analysis, and reverse transcription-PCR

Primer no. (name) ^a	<i>H19</i> position no. ^b	Sequence (5'-3')
G1 (H19-3.9F)	-3953 (1303)	TGCAAGGAGACCATGCCTATTCTTG
G2 (H19-2.3F)	-2286 (2971)	CAATGTTTCATAAGGGTCATGGGGTG
G3 (PGK- <i>neo</i> F)	NA ^c	CCACTTGTGTAGCGCCAAAGTGCC
G4 (H19-4.8F)	-4811 (445)	CCAACTGAGAGGGCCATAGTGTGAG
G5 (H19-2.0R)	-2031 (3227)	CGTAAAGTGTACAAATGCCTGATCCC
G6 (HSEQ1R)	-750 (4551)	CCACAGAGTCAGCATCCAC
B1 (BMsp2t1)	-3978 (1278)	GAGTATTTAGGAGGTATAAGAATT
B2 (BMsp2t2)	-3952 (1304)	GTAAGGAGATTATGTTTATTTTGG
B3 (BHhalt4)	-3532 (1726)	CCTCATTAATCCCATAACTAT
B4 (BHhalt3)	-3507 (1751)	ATCAAAAACATAACATAAACCCCT
B5 (BHha7t)	-2031 (3225)	TAAAATATCACAAATACCTAATCCCT
B6 (BHSEQm1)	-709 (4551)	CCATTACTCTTAACCTTATTAA
B7 (BHSEQm2)	-733 (4527)	AATTATACAACTAAAAATCCACAAAATCAAC
B8 (BTV3-1)	-2310 (2938)	GGTAAATTTATGGGTTATTTAAGG
B9 (BTV3-2)	-2286 (2972)	AATGTTTATAAGGGTTATGGGGTGG
B10 (BTV3-3)	-1861 (3397)	CCTAAATTCATAAAACATTACAA
B11 (BTV3-4)	-1884 (3374)	CCCAACCTCTACTTTTATAAC
RT1 (HI3)	+1722 (7242) ^d	CCTCAAGATGAAAGAAATGGT
RT2 (HE5)	+2528 (8048) ^d	AACACTTTATGATGGAACCTG

^a G, genotyping; B, bisulfite analysis; RT, reverse transcription-PCR.

^b Specifies the position of the first nucleotide of the primer relative to the *H19* transcription start site (position in parentheses according to GenBank accession no. U19619, unless otherwise noted).

^c NA, not applicable, primer for *neo*^R sequence.

^d Position in parentheses according to GenBank accession no. AF049091.

Cycler Software Data Analysis function. After background subtraction, the contribution of each allele was calculated as the peak height or peak area of the melting curve generated at the allele-specific temperature, approximately 67.5°C for C57BL/6J and 61.5°C for CAST. The *H19* allelic expression pattern was calculated as the fraction of expression from the paternal mutant allele relative to the expression from the maternal CAST allele. A *Cac8I* restriction site unique to CAST DNA was used to confirm the efficacy of the LightCycler Data Analysis by digesting amplified sample and resolving the products on a 7% polyacrylamide gel.

DNA isolation and methylation: Southern analysis. DNA was isolated from tissues as previously described (7). Genomic DNA (10 µg) was digested with *Pvu*II and *Stu*I in combination with *Hpa*II to analyze the methylation of the *H19* transcription unit. To analyze methylation of the DMD, DNA was digested with *Sac*I (*H19*^{ΔDMD}) or *Sac*I and *Eco*RV (*H19*^{ΔDMDΔG} and *H19*^{ΔG}) in combination with *Hha*I. The probes used for the analyses were a 2.5-kb *Eco*RI-*Stu*I fragment (*H19* transcription unit) and a 0.9-kb *Sac*I-*Kpn*I fragment (DMD) (see Fig. 5).

DNA bisulfite modification and sequencing analysis. Neonatal liver and kidney DNA isolated from heterozygous neonates that had inherited the mutant alleles maternally (*H19*^{ΔDMD}/+, *H19*^{ΔDMDΔG}/+) and paternally (+/*H19*^{ΔDMD}, +/*H19*^{ΔDMDΔG}) were subjected to bisulfite modification. Briefly, 5 µg of DNA was digested with *Xba*I (*H19*^{ΔDMD}) or *Xba*I and *Eco*RV (*H19*^{ΔDMDΔG}, *H19*^{ΔG}) for 1 h, extracted with phenol-chloroform, ethanol precipitated with 1 M NH₄ acetate, and resuspended in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was then subjected to bisulfite modification, subcloning, and sequencing as described previously (17, 52) with modification. PCR amplification was performed with Ready-To-Go PCR beads with primers (0.5 µM) specific for the top strand of bisulfite-mutagenized DNA (Table 1) (primers B1 to B11). Primers for the mutant alleles flanked the 5' DMD and the *loxP* sequence. Primer pairs used for the first and second rounds of PCR amplification of the 5' DMD sequence were B1-B4 and B2-B3 for the wild-type allele, B1-B5 and B2-B5 for the *H19*^{ΔDMD} allele, and B1-B6 and B2-B7 for the *H19*^{ΔDMDΔG} allele, and primer pairs used for the 3' DMD sequence were B8-B10 and B9-B11 for the wild-type allele and B8-B6 and B9-B7 for the *H19*^{ΔG} allele (see Fig. 6). The data obtained for each set of primers were compiled from two or more independent PCRs.

RESULTS

Generation of the ΔDMDΔG, ΔG, and Δ3.8kb-5'H19 mice.

It was previously demonstrated that the DMD is required for the imprinted expression of *H19* and *Igf2* by deleting 1.6 kb of this 2-kb sequence from the endogenous locus (51). The

ΔDMD (Fig. 1) deletion removes the majority of the nuclease hypersensitive regions (28, 34) and three of the four 21-bp conserved repeats that confer CTCF-dependent in vitro insulator activity on the maternal allele (9, 27, 33). To determine if an additional sequence 5' to *H19* is required for *H19* and *Igf2* imprinting, three other deletion alleles were generated (Fig. 1). A similar targeting strategy was used to generate each of the ΔDMDΔG, ΔG, and Δ3.8kb-5'H19 alleles in ES cells and in mice (Fig. 2). Progeny from reciprocal mating of B6(CAST-*H19*) mice and mutant mice lacking the PGK-*neo* cassette were assayed for parent-specific expression of *H19* and *Igf2* as well as parent-specific methylation at the *H19* locus.

The G-rich repeat element can be deleted without loss of imprinted expression. One of the hallmark characteristics of imprinted genes is a repetitive element. Given that repeats may assume alternative chromatin structure, one can envision a role for repetitive elements in imprinted gene regulation. Alternatively, this presence of repetitive elements in imprinted loci may be purely coincidental, and therefore, the role of these elements must be tested in vivo. To this end, we generated the ΔG deletion that removes a 1.3-kb region containing the 461-bp G-rich repetitive element that resides between the DMD and the *H19* promoter (*H19*^{ΔG}) (Fig. 1). In contrast to results from the ΔDMD deletion, analysis of the *H19*^{ΔG} allele revealed that the ΔG deletion had no effect on the repression of the paternal *H19* and the maternal *Igf2* alleles (Fig. 3A and 4B and data not shown). Strict imprinted expression of these genes was maintained. In addition, maternal transmission of the *H19*^{ΔG} allele had no effect on relative *H19* expression levels (Fig. 4A and data not shown) and paternal transmission of the *H19*^{ΔG} allele had no effect on relative *Igf2* expression levels (Fig. 3B and data not shown), demonstrating that the deleted sequence is not required for wild-type levels of *H19* and *Igf2* expression. This study confirms the *H19* transgenic

FIG. 4. *H19* and *Igf2* expression in mice inheriting the maternal *H19*^{ΔDMD}, *H19*^{ΔDMDΔG}, *H19*^{ΔG}, and *H19*^{Δ3.8kb-5'H19} alleles. Tissues were isolated from neonatal mice generated from the mating of female heterozygous mutants (+/Δ) on a C57BL/6J (B) background and B6(CAST-*H19*) (C) males. The wild-type C and B alleles are designated with a plus sign and the deletion (Δ) alleles are designated with a minus sign above each lane. Total liver (3 μg), tongue (2 μg), and kidney (10 μg) RNA from wild-type (+/+) and mutant (-/+) littermates was analyzed by RPAs for the indicated deletions. (A) Relative levels of expression of *H19* compared to *rpL32*. RNA was assayed simultaneously with the *H19* and *rpL32* probes. In neonatal liver and tongue RNA, *H19* expression from the *H19*^{ΔDMD}, *H19*^{ΔDMDΔG}, and *H19*^{Δ3.8kb-5'H19} alleles was reduced by 40 to 65% relative to that of wild-type littermates (lanes 1 to 16 and data not shown). In contrast, *H19* was more severely affected by the maternal *H19*^{ΔDMD}, *H19*^{ΔDMDΔG}, and *H19*^{Δ3.8kb-5'H19} alleles in kidney (>90% reduction) (lanes 17 to 24 and 29 to 32) and in heart and lung (75 to 90% reduction) (data not shown) RNA. *H19*^{ΔDMD} and *H19*^{Δ3.8kb-5'H19} results are summarized in Table 3. The ΔG deletion had no effect on *H19* expression in the neonatal kidney (lanes 25 to 28) and other (data not shown) tissues assayed. Northern analysis confirmed the RPA results (data not shown). Control neonatal liver RNA was assayed with *H19* (lanes 33) and *rpL32* (lane 34) probes. (B) Allele-specific expression of *Igf2*. The distinct C and common B and deletion allele-protected fragments are designated. Biallelic expression of *Igf2* was detected in liver, tongue, and kidney RNA (lanes 2, 3, 5, 6, 11, 12, 14, 15, 17, 18, 23, 24, 26, 27, 29, 30, 35, and 36), and heart, lung, brain, and gut RNA (data not shown) upon maternal inheritance of the *H19*^{ΔDMD}, *H19*^{ΔDMDΔG}, and *H19*^{Δ3.8kb-5'H19} alleles. Relative levels of *Igf2* expression from the mutant maternal allele to the wild-type paternal allele were 25 to 40% in liver tissue and >50% in all other tissues. *H19*^{ΔDMD} and *H19*^{Δ3.8kb-5'H19} results are summarized in Table 2. *Igf2* expression was not detected from the maternal *H19*^{ΔG} allele in neonatal liver, tongue, and kidney RNAs (lanes 8, 9, 20, 21, 32, and 33) nor in heart and lung RNA (data not shown). Control C (lane 37) and B (lane 38) neonatal liver RNA was assayed. Size markers (in base pairs) are indicated to the left in lane M.

imprinting was disrupted in all tissues, the extent of derepression of the normally silent *H19* and *Igf2* alleles varied among tissues (Fig. 3A and 4B and data not shown). *H19* was most highly activated on the mutant paternal allele in liver tissue, slightly less activated in tongue and brain tissue, and significantly less activated in kidney, heart, lung, and gut tissue (Fig. 3A, values for ΔDMD in Table 2, and data not shown). It should be noted, however, that *H19* is typically expressed at low levels in kidney, heart, lung, and brain tissue. In contrast, *Igf2* was least activated on the mutant maternal allele in liver tissue (Fig. 4B, ΔDMD values in Table 2, and data not shown). The biallelic *Igf2* expression pattern that is normally observed in brain tissue was unaffected by the deletions (Table 2). These data indicate that *H19* and *Igf2* expression are regulated either directly by tissue-specific elements within the DMD or via

other regulatory elements that may interact with the DMD in a tissue-specific manner.

Tissue-specific reduction of maternal *H19* and paternal *Igf2* expression from the ΔDMD and ΔDMDΔG mutant alleles.

We assessed the tissue-specific effect of the ΔDMD and ΔDMDΔG deletions on the normally active maternal *H19* and paternal *Igf2* alleles. Maternal inheritance of the DMD deletions caused tissue-specific reductions in *H19* expression (Fig. 4A, ΔDMD values in Table 3, and data not shown). *H19* RNA was 40 to 65% lower in liver, tongue, and brain tissue and dramatically reduced (>90%) in kidney tissue. Heart and lung tissue exhibited intermediate effects from the DMD deletions. Hence, in most tissues, the level of *H19* RNA from the mutant maternal alleles was similar to the level observed from the mutant paternal alleles. In tongue tissue, however, we did

TABLE 2. Activation of paternal *H19* and maternal *Igf2* expression from mutant alleles

Tissue type	Deletion	Mutant paternal/wild-type maternal <i>H19</i> ratio ^a	Mutant maternal/wild-type paternal <i>Igf2</i> ratio ^a
Liver	Δ DMD	0.51 ± 0.03 (6) ^b	0.29 ± 0.06 (27)
	Δ 3.8kb-5'H19	0.53 ± 0.02 (6) ^b	0.30 ± 0.05 (6)
Tongue	Δ DMD	0.37 ± 0.02 (6) ^b	0.52 ± 0.07 (8)
	Δ 3.8kb-5'H19	0.32 ± 0.04 (6) ^b	0.56 ± 0.09 (6)
Kidney	Δ DMD	$<0.06 \pm \text{NA}$ (4) ^b	0.51 ± 0.06 (6)
	Δ 3.8kb-5'H19	$<0.06 \pm \text{NA}$ (6) ^b	0.68 ± 0.09 (6)
Heart	Δ DMD	0.10 ± 0.022 (3) ^b	0.62 ± 0.12 (6)
	Δ 3.8kb-5'H19	0.14 ± 0.014 (3) ^b	0.77 ± 0.22 (5)
Lung	Δ DMD	0.14 ± 0.07 (3) ^b	0.54 ± 0.07 (6)
	Δ 3.8kb-5'H19	0.23 ± 0.16 (3)	0.96 ± 0.06 (3)
Brain	Δ DMD	0.38 ± 0.05 (3) ^b	0.94 ± 0.28 (3) ^c
	Δ 3.8kb-5'H19	0.48 ± 0.05 (3)	ND ^d
Gut	Δ DMD	0.19 ± 0.09 (3) ^b	0.35 ± 0.04 (9)
	Δ 3.8kb-5'H19	0.19 ± 0.06 (3)	ND ^d

^a Numbers are ratios of expression from the mutant to wild-type allele averaged for number of animals assayed $\pm$ standard deviation. Values in parentheses represent the number of animals assayed. *H19* expression levels were measured by LightCycler Real-Time PCR, and *Igf2* expression levels were measured by RPA. In wild-type control samples, there was no observed activation of paternal *H19* and no or very low (<0.04 maternal/paternal ratio) levels of activation from the maternal *Igf2*.

^b A similar level of expression was also measured by RPA (Fig. 3A).

^c Biallelic expression was detected in both wild-type and mutant littermates.

^d ND, not determined.

detect a slightly higher level of *H19* RNA from the mutant maternal allele than from the mutant paternal allele (Δ DMD values in Tables 2 and 3 and data not shown).

Paternal inheritance of the deletions also disrupted *Igf2* expression in a tissue-specific manner (Fig. 3B). The *Igf2* RNA was on average 70% lower in liver tissue, $<30\%$ lower in tongue tissue, and unchanged in all other tissues (Fig. 3B, Δ DMD values in Table 3, and data not shown). We detected the most significant reduction of *Igf2* expression in liver tissue, the tissue in which *Igf2* was least activated from the mutant maternal allele (compare Fig. 3B and 4B). Consequently, the paternal and maternal *H19* ^{Δ DMD} and *H19* ^{Δ DMD Δ G} alleles expressed similar levels of *Igf2*.

The parent-specific methylation pattern of the *H19* ^{Δ DMD}, *H19* ^{Δ DMD Δ G}, and *H19* ^{Δ G} alleles. The paternally specific methylation that is acquired on the DMD in sperm is regarded as a mark that is critical for the regulation of *H19* and *Igf2* imprint-

ing (18, 40, 52). In contrast, paternally specific methylation of the *H19* promoter and 5' end of the *H19* transcription unit is acquired during embryogenesis so that in all somatic tissues paternally specific methylation extends from the DMD to the 5' end of the *H19* transcription unit (52, 53). It was previously determined that the *H19* ^{Δ DMD} allele acquires sperm-specific methylation and that in neonatal liver tissue both mutant parental alleles are partially methylated at the remaining DMD sequence and unmethylated at the *H19* transcription unit (51).

To determine if removing an additional sequence 5' to *H19* further perturbed the methylation imprint, we compared the methylation status of the *H19* ^{Δ DMD}, *H19* ^{Δ DMD Δ G}, and *H19* ^{Δ G} alleles in sperm and neonatal tissues. Analysis of the methylation status of the remaining DMD sequence and the 5' end of the *H19* transcription unit in sperm revealed that the wild-type methylation pattern was acquired on the mutant alleles: the DMD was hypermethylated (Fig. 5B) and the 5' end of the

TABLE 3. Reduction of maternal *H19* and paternal *Igf2* expression from mutant alleles

Tissue type	Deletion	Mutant maternal/wild-type maternal <i>H19</i> ratio ^a	Mutant paternal/wild-type paternal <i>Igf2</i> ratio ^a
Liver	Δ DMD	0.44 ± 0.05 , 0.41 ± 0.11	0.28 ± 0.04 , 0.35 ± 0.03
	Δ 3.8kb-5'H19	0.26 ± 0.05 , 0.37 ± 0.03	0.26 ± 0.05 , 0.38 ± 0.10
Tongue	Δ DMD	0.59 ± 0.12	0.75 ± 0.12
	Δ 3.8kb-5'H19	0.53 ± 0.06	0.71 ± 0.06
Kidney	Δ DMD	≤ 0.10	$\sim 1.0^b$
	Δ 3.8kb-5'H19	≤ 0.10	$\sim 1.0^b$
Heart	Δ DMD	0.26 ± 0.04	$\sim 1.0^b$
	Δ 3.8kb-5'H19	0.15 ± 0.05	$\sim 1.0^b$
Lung	Δ DMD	0.11 ± 0.03	$\sim 1.0^b$
	Δ 3.8kb-5'H19	≤ 0.10	$\sim 1.0^b$
Brain	Δ DMD	0.56 ± 0.13	$\sim 1.0^b$
	Δ 3.8kb-5'H19	0.53 ± 0.11	ND ^c

^a Expression was standardized to *rpL32* and averaged in 2 or 3 wild-type and 2 or 3 mutant littermates. Results from one or two litters are presented. Numbers are the ratios of the averaged mutant to averaged wild-type level of expression as detected by RPA and/or Northern analysis $\pm$ deviation. The noted deviations reflect the variation between the independent mutant and the independent wild-type samples that were assayed. There is no paternal *H19* and maternal *Igf2* expression from the wild-type (CAST) alleles.

^b No effect was detected; wild-type and mutant paternal *Igf2* RNA levels were similar.

^c ND, not determined.

FIG. 6. Methylation profile of individual DNA strands in the 5' DMD region in neonatal liver. (A) Schematic of the wild-type, *H19* Δ DMD, and *H19* Δ DMD Δ G alleles analyzed by the bisulfite mutagenesis and sequencing assay. The seven CpG dinucleotides at the 5' DMD region that are common to the wild-type and deletion alleles are depicted as open circles. CpG dinucleotides 1 (1330), 2 (1360), 3 (1362), 4 (1382), 5 (1384), 6 (1391), and 7 (1397) are located between -3926 and -3729 relative to the *H19* transcription start site of the wild-type allele (the GenBank accession no. U19619 position is given in parentheses). CpG dinucleotide 6 is within the *Hha*I site assayed by the genomic Southern blot in Fig. 5. The primers used to amplify the 5' DMD region are shown above each allele (B1 to B7) (Table 1). (B) The methylation status of individual DNA strands of the wild-type, *H19* Δ DMD, and *H19* Δ DMD Δ G alleles. Filled circles correspond to methylated cytosines, and open circles correspond to unmethylated cytosines. The absence of a circle indicates that the residue was not assayed. The number of times a given profile was observed is indicated to the left of each strand. (C) Summary of the methylation profiles of the seven CpG dinucleotides on the wild-type, *H19* Δ DMD, and *H19* Δ DMD Δ G alleles. Gray and black bars represent the fraction of methylated cytosines on the maternal and paternal alleles, respectively. In contrast to wild-type alleles that are methylated exclusively on the paternal alleles, mutant Δ DMD and Δ DMD Δ G alleles show an intermediate level of methylation with more methylation on the mutant paternal allele than on the mutant maternal allele.

H19 transcription unit was unmethylated (Fig. 5C, lanes 1 to 5) in Δ DMD, Δ DMD Δ G, and Δ G deletion sperm DNA. Similarly, examination of neonatal liver DNA isolated from Δ G heterozygous and homozygous mutant mice revealed that the DMD and *H19* transcription unit possessed the wild-type pattern of methylation (Fig. 5C and data not shown). In contrast, neonatal liver tissue derived from heterozygous and homozygous mutant *H19* Δ DMD and *H19* Δ DMD Δ G mice exhibited hypomethylation of the *H19* transcription unit and partial methylation of the remaining DMD sequence on the mutant alleles (Fig. 5C and D and data not shown). Thus, although the wild-type pattern of methylation is acquired on the mutant alleles in sperm DNA, the *H19* Δ DMD and *H19* Δ DMD Δ G alleles fail to maintain the methylation pattern in somatic tissues. Additionally, no striking differences in methylation were revealed between Δ DMD and Δ DMD Δ G DNA.

To determine if tissue-specific methylation differences were associated with the tissue-specific differences in *H19* expression observed for the Δ DMD and Δ DMD Δ G mice (Fig. 3 and 4 and Tables 2 and 3), we assayed genomic DNA methylation in neonatal liver, tongue, kidney, heart, and brain tissue. The

mutant parental alleles were equally hypomethylated at the 5' end of the *H19* transcription unit (data not shown) and equivalently methylated at the remaining 5' DMD sequence in all *H19* Δ DMD- and *H19* Δ DMD Δ G-derived tissues (Fig. 5D, lanes 3 to 10 and 13 to 20, and data not shown). We conclude that differential DNA methylation patterns neither cause nor reflect the tissue-specific *H19* expression patterns observed for the mutant alleles. These methylation patterns were maintained after six generations of crossing with C57BL/6J mice (data not shown).

Parent-specific methylation is detected at each of the remaining seven DMD CpG dinucleotides on the *H19* Δ DMD and *H19* Δ DMD Δ G alleles. While the DMD deletions remove most of the DMD sequence, seven DMD CpG dinucleotides remain on the Δ DMD and Δ DMD Δ G alleles. A closer examination of the CpG dinucleotide within the remaining *Hha*I site 5' to the Δ DMD and Δ DMD Δ G deletions revealed slightly more methylation on the mutant paternal alleles than on the mutant maternal alleles (Fig. 5D, lanes 3 to 10 and 13 to 20). To quantify this methylation difference at all seven CpGs, we used the bisulfite mutagenesis and sequencing assay. The wild-type

and mutant alleles were independently amplified from bisulfite-modified DNA (Fig. 6A and Table 1). As suggested by Southern analysis, the *H19* ^{Δ DMD} and *H19* ^{Δ DMD Δ G} alleles were methylated at each of the seven CpG dinucleotides on both the maternal and paternal mutant alleles in liver tissue (Fig. 6). While the level of methylation for the mutant alleles was intermediate between the maternal and paternal wild-type alleles, the seven CpG dinucleotides were more likely to be methylated on the mutant paternal alleles than on the mutant maternal alleles (Fig. 6). Similar results were obtained when kidney DNA was assayed (data not shown). Thus, the *H19* ^{Δ DMD} and *H19* ^{Δ DMD Δ G} alleles retained some parentally specific methylation in the remaining DMD sequence. In addition, no tissue-specific methylation at the 5' DMD sequence was observed.

The analysis of the *H19* ^{Δ DMD} and *H19* ^{Δ DMD Δ G} alleles revealed that the CpG dinucleotides of the *loxP* vector sequence were also methylated on both the paternal and maternal mutant alleles (data not shown). Because the introduced sequence could contribute to the observed methylation on the mutant alleles, we assayed the methylation status of the 3' DMD and *loxP* vector sequence of the *H19* ^{Δ G} alleles. As with the wild-type 3' DMD CpG dinucleotides, the *H19* ^{Δ G} DMD and *loxP* vector CpG dinucleotides were also methylated exclusively on the paternal allele in neonatal liver DNA (*H19* ^{Δ G}/+, +/*H19* ^{Δ G}) (data not shown). These results show that rather than causing the disruption in allelic methylation, the introduced *loxP* vector sequence acquired the methylation imprint of the adjacent sequence. It should be noted that the analysis of the wild-type allele also revealed that six CpG dinucleotides external to the previously defined 3' end of the DMD (–2083 *Hind*III site in Fig. 1) (52) were also methylated exclusively on the paternal allele (data not shown). Hence, the deleted DMD sequence as opposed to the *loxP* vector sequence likely accounts for the loss of differential methylation at the *H19* locus.

The 3.8-kb deletion shows no additional effect on imprinting when compared to the 1.6-kb DMD deletion. Both the Δ DMD and Δ DMD Δ G deletions retained some differential methylation at the 5' portion of the DMD. Therefore, we tested whether a larger deletion that eliminates the entire 2-kb DMD and all four 21-bp repeats (CTCF sites), would lead to a greater impact on *H19* and *Igf2* imprinting (Fig. 1). We did not detect significant differences in *H19* and *Igf2* expression from the paternal allele by deleting the entire 2 kb of DMD (*H19* ^{Δ 3.8kb-5'*H19*}) compared to deleting 1.6 kb of the DMD sequence (*H19* ^{Δ DMD}) (Fig. 3 and Tables 2 and 3). In addition, the single remaining CTCF site did not appear to confer residual insulator activity since its deletion on the maternal *H19* ^{Δ 3.8kb-5'*H19*} allele did not result in a significant increase in *Igf2* expression relative to the *H19* ^{Δ DMD} and *H19* ^{Δ DMD Δ G} alleles in most tissues, with the exception of lung tissue (Fig. 4 and Tables 2 and 3). Likewise, *H19* expression from the *H19* ^{Δ DMD} and *H19* ^{Δ 3.8kb-5'*H19*} maternal alleles was similar in most tissues. Moreover, the 5' end of the *H19* transcription unit was equally hypomethylated on both of the parental *H19* ^{Δ 3.8kb-5'*H19*} alleles in somatic tissues (data not shown). Consequently, the deleted 1.6-kb DMD sequence likely accounts for the loss of *H19* and *Igf2* imprinting that is detected in this and other targeting deletion studies that remove the entire DMD sequence (32, 47, 51).

DISCUSSION

A previous study of the 1.6-kb DMD deletion (Δ DMD) demonstrated that the DMD was required for both *H19* and *Igf2* imprinting in neonatal liver tissue and that the DMD functions to silence *H19* on the paternal allele and *Igf2* on the maternal allele (51). In addition, the DMD was shown to be required for complete activation of the maternal *H19* and paternal *Igf2* alleles and for paternally specific methylation at the *H19* locus. Here, we further analyze the Δ DMD deletion and three additional deletions of the DMD region in multiple neonatal tissues. We show that the Δ DMD perturbs imprinting in all tissues and that relative expression levels from both parental alleles in heterozygous mutant mice vary in a tissue-specific manner. In addition, this expression is not further affected by removing more sequence 3' to the DMD or an additional upstream sequence, including the remaining 5' DMD sequence. We conclude that the original 1.6-kb Δ DMD deletion is sufficient to cause deregulation of *H19* and *Igf2* imprinted expression in all tissues.

Similar to what we report above, a 9.2-kb targeted deletion at the *H19* locus spanning the sequence 5' to the DMD, the DMD, and the G-rich repetitive element also resulted in perturbation of *H19* and *Igf2* imprinting in all tissues assayed (32). Interestingly, maternal transmission of a conditional allele that removed a 6.2-kb segment from –7.0 to –0.8 kb 5' of *H19* in terminally differentiated muscle activated the silent *Igf2* allele while *H19* remained unaffected after paternal transmission of this deletion (47). Deregulated expression resulting from these larger deletions was suggested to be the actual consequence of removing the 2-kb DMD (32, 47). The question that arises from these and similar studies is whether the roles attributed to the DMD in *H19* and *Igf2* imprinting can be associated with the same or different regions of the DMD.

The first indication that the DMD functions as an insulator element in regulating *Igf2* imprinting was demonstrated by movement of the endodermal enhancers between the endogenous *Igf2* gene and the DMD (55), resulting in *Igf2* expression from the normally silent maternal allele. Through the use of in vitro enhancer-blocking assays, the DMD has since been shown to function as an insulator that is dependent upon the conserved CTCF-binding repeat sequences (9, 27, 32, 33). As CTCF binding and in vitro DMD enhancer-blocking activity are methylation sensitive (9, 27, 29) and the DMD repeat sequences footprint specifically on the maternal allele in vivo (50), CTCF is an excellent candidate for regulating *H19* and *Igf2* imprinting. Although DMD repeat element R1 (Fig. 1) confers insulator activity in an in vitro enhancer-blocking assay (9) and the 5' R1-spanning DMD sequence acquires more methylation on the paternal than on the maternal Δ DMD allele, the R1 element alone does not confer significant insulator activity at the endogenous locus, as is indicated by the nonimprinted expression of *Igf2* in Δ DMD mice. Thus in vitro assessment of insulator activity of individual DMD repeat elements may not reflect the sequence requirements for insulator function at the endogenous *H19* and *Igf2* locus, which is governed by complex long-range enhancer-mediated interactions. In a mouse transgenic assay, the sequence spanning three of the four DMD repeats (R2 to R4) (Fig. 1) mediated enhancer-blocking activity; however, the sequence which spans

R2 alone did not exhibit insulator activity (27). Furthermore, a 1.2-kb targeted deletion of the DMD sequence spanning repeats R3 and R4 did not appear to perturb *Igf2* imprinting (19), indicating that repeats R1 and R2 together may confer insulator activity at the endogenous locus. Thus, it appears that at least two CTCF-binding repeats are required for insulator activity at the *H19* and *Igf2* imprinting domain. It remains to be determined if any two CTCF-binding repeats will mediate insulator activity at the endogenous *H19* and *Igf2* locus and whether a sequence other than the repeats is required for this activity. In support of sequence requirements other than the CTCF-binding repeats, *in vitro* assays indicated that an additional 5' flanking sequence enhanced the insulator activity of the DMD (9, 32).

At other loci, the number of CTCF sites varies. Only one CTCF site is present at the chicken β -globin insulator element (11). In contrast, two methylation-sensitive CTCF-binding sites flank the CTG repeats of the human DM1 locus that is associated with myotonic dystrophy (23). These sites, in combination with CTG repeats, are proposed to function as an insulator. In the mouse, a single CTCF-binding site has been reported within an intron of the *Gtl2* locus, a maternally expressed nontranslated gene that is linked to the oppositely imprinted *Dlk1* gene (22, 46). In the human, two CTCF-binding sites have been found within the *GTL2* promoter (56). Thus, it will be important to determine if these CTCF sites can function as an insulator or if these sites have one of the other regulatory functions (such as transcriptional activation or repression) that have been ascribed to CTCF (39).

The DMD sequence requirements for *H19* silencing versus *Igf2* insulation may differ. Paternal transmission of a 1.2-kb deletion that removed DMD repeats R3 and R4 caused activation of the paternal *H19* allele, even though the remaining DMD sequence acquired paternally specific methylation (19). In contrast, *Igf2* was not activated after maternal transmission of the deletion. From these studies, the authors concluded that the DMD sequence requirements for *H19* silencing differ from those for *Igf2* insulator activity. Our data indicate that the DMD sequence spanning repeats R2, R3, and R4 is required for both *H19* silencing and *Igf2* insulation. The amount of DMD sequence deleted, rather than the specific sequence itself, could be responsible for the distinct imprinting defects observed for the different deletions. Additional gene targeting experiments are required to resolve this issue.

While the DMD provides allele-specific silencing and insulator activities, it also appears to play a role in modulating tissue-specific expression of *H19* and *Igf2*. Paternal transmission of the 1.2-kb 5' *H19* deletion was reported to perturb *H19* silencing in a subset of tissues (19). The 9.2-kb 5' *H19* deletion exhibited tissue-specific perturbations in *H19* and *Igf2* imprinting (32). Our data show that the DMD deletions cause tissue-specific changes in *H19* and *Igf2* activation from the normally imprinted allele. We also detect reduced levels of *H19* and *Igf2* expression from the normally expressed alleles relative to their wild-type counterparts, and the levels of expression from the mutant maternal and paternal alleles are nearly equivalent in all tissues assayed. Although the DMD is required for imprinting in all tissues, it is unclear how the tissue-specific effects are conferred. One possibility is that the DMD interacts differently with tissue-specific enhancers within this locus (1, 30, 32, 37).

Additionally, the DMD may exhibit unique interactions with the mesoderm-specific silencer and repressor elements that regulate *Igf2* (2, 15), one of which coincides with the differentially methylated region (DMR) 5' of *Igf2* (DMR1). DMR1 and another DMR within the *Igf2* gene (DMR2) are exclusively methylated on the paternal allele; however, upon maternal inheritance of a 13-kb deletion that removes the DMD, maternal *Igf2* is expressed and the DMRs become methylated (24). A complete understanding of tissue-specific imprinted regulation will require the elucidation of the mechanism by which insulators and enhancers operate in vertebrates as well as the identification of additional tissue-specific regulatory elements in the *H19* and *Igf2* imprinting domain.

Our Δ DMD targeted deletion clearly demonstrates the necessity, but not the sufficiency, of the DMD sequence for regulating *H19* and *Igf2* imprinting. Targeted deletions have also characterized tissue-specific transcriptional regulatory elements required for *H19* and *Igf2* imprinting and expression (15, 37). The single-copy yeast and bacterial artificial chromosome transgenic models together suggest that the sequence from immediately upstream of *Igf2* to as far as 130 kb downstream of *H19* is required to recapitulate *H19* and *Igf2* imprinted expression in all tissues (1–3, 32). *H19* minitransgenes that consist of 5' *H19* sequence, the *H19* transcription unit or a reporter gene, and 3' *H19* sequence that spans the endodermal enhancers have been used to elucidate the sequence required for *H19* imprinting in liver tissue (12, 16, 21, 41, 48). To date, the shortest low-copy-number transgene that confers *H19* imprinted methylation and expression in liver tissue includes 5.5 kb of 5' *H19* sequence, the *H19* transcription unit, and 8 kb of 3' *H19* (16). Interestingly, the methylation of a low-copy-number enhancerless transgene containing 5.5 kb of 5' *H19* sequence and the *H19* transcription unit is imprinted, demonstrating that DMD can acquire imprinted methylation in the absence of enhancer-mediated transcription (16). It remains to be proven whether the entire 2-kb DMD alone is sufficient to imprint exogenous targets.

Repetitive elements are often associated with imprinted genes. Recently, a repetitive element within the *U2afbp-rs1* locus has been reported to be dispensable for imprinted methylation of this gene (49). In contrast, a repetitive element at the *Rasgrf1* locus is essential for its imprinting (P. D. Soloway, personal communication). Here we show that deleting a 1.3-kb segment between the DMD and *H19* promoter that contains the G-rich repeat does not affect *H19* and *Igf2* imprinting in any neonatal tissue tested. Hence, we corroborate the findings that the G-rich repeat element was not required for imprinting of an *H19* transgene in neonatal liver tissue (48) and that removal of the endogenous G-rich repeat element in combination with the *H19* transcription unit did not perturb *Igf2* imprinting (42). Curiously, our Δ G deletion also removed a sequence that acquires parent-specific DNA methylation (52, 53) and histone H4 acetylation (26), other hallmarks that are commonly associated with imprinted genes. Thus, since the Δ G deletion does not affect imprinting, these parent-specific characteristics of the sequence between the DMD and *H19* promoter are most likely a consequence of the spreading of epigenetic silencing signals from the DMD to the *H19* gene during postimplantation development (44, 52, 53).

In conclusion, further mutational analysis of the endogenous

DMD region is required to unravel the role of specific sequences in activation versus the silencing of parental alleles and for tissue-specific enhancer-mediated interactions that result in imprinted gene expression. For example, it will be interesting to determine whether eliminating or altering CTCF-binding repeats will disrupt *H19* as well as *Igf2* imprinting in vivo. Furthermore, it will also be crucial to determine whether the DMD harbors other parent-specific differences, such as histone modifications associated strictly with active or inactive chromatin (31), as they may play an integral part in establishing epigenetic signals at the DMD locus. Together, these studies at the *H19* and *Igf2* imprinting domain will elucidate the complex mechanisms of long-range tissue-specific enhancer-mediated interactions, insulation, and silencing, all of which regulate imprinted gene expression.

ACKNOWLEDGMENTS

We thank J. Richa and the University of Pennsylvania Transgenic Core Facility for the production of chimeric mice. We thank Nora Engel and Raluca Verona for comments on the manuscript.

This work was supported by U.S. Public Health Service grant GM51279 and the Howard Hughes Medical Institute. J.L.T. was supported by National Research Service Award postdoctoral fellowship GM18458. M.R.W.M. was supported by the Lalor Foundation.

REFERENCES

- Ainscough, J. F., L. Dandolo, and M. A. Surani. 2000. Appropriate expression of the mouse *H19* gene utilizes three or more distinct enhancer regions spread over more than 130 kb. *Mech. Dev.* **91**:365–368.
- Ainscough, J. F., R. M. John, S. C. Barton, and M. A. Surani. 2000. A skeletal muscle-specific mouse *Igf2* repressor lies 40 kb downstream of the gene. *Development* **127**:3923–3930.
- Ainscough, J. F.-X., T. Koide, M. Tada, S. Barton, and M. A. Surani. 1997. Imprinting of *Igf2* and *H19* from a 130 kb YAC transgene. *Development* **124**:3621–3632.
- Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**:303–314.
- Barlow, D. P. 1997. Competition—a common motif for the imprinting mechanism? *EMBO J.* **16**:6899–6905.
- Bartolomei, M. S., and S. M. Tilghman. 1997. Genomic imprinting in mammals. *Annu. Rev. Genet.* **31**:493–525.
- Bartolomei, M. S., A. L. Webber, M. E. Brunkow, and S. M. Tilghman. 1993. Epigenetic mechanisms underlying the imprinting of the mouse *H19* gene. *Genes Dev.* **7**:1663–1673.
- Bartolomei, M. S., S. Zemel, and S. M. Tilghman. 1991. Parental imprinting of the mouse *H19* gene. *Nature* **351**:153–155.
- Bell, A. C., and G. Felsenfeld. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature* **405**:482–485.
- Bell, A. C., A. G. West, and G. Felsenfeld. 2001. Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. *Science* **291**:447–450.
- Bell, A. C., A. G. West, and G. Felsenfeld. 1999. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* **98**:387–396.
- Brenton, J. D., R. A. Drewell, S. Viville, K. J. Hilton, S. C. Barton, J. F.-X. Ainscough, and M. A. Surani. 1999. A silencer element identified in *Drosophila* is required for imprinting of *H19* reporter transgenes in mice. *Proc. Natl. Acad. Sci. USA* **96**:9242–9247.
- Brunkow, M. E., and S. M. Tilghman. 1991. Ectopic expression of the *H19* gene in mice causes prenatal lethality. *Genes Dev.* **5**:1092–1101.
- Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw III, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* **292**:1728–1731.
- Constancia, M., W. Dean, S. Lopes, T. Moore, G. Kelsey, and W. Reik. 2000. Deletion of a silencer element in *Igf2* results in loss of imprinting independent of *H19*. *Nat. Genet.* **26**:203–206.
- Cranston, M. J., T. L. Spinka, D. A. Elson, and M. S. Bartolomei. 2001. Elucidation of the minimal sequence required to imprint *H19* transgenes. *Genomics* **73**:98–107.
- Davis, T. L., K. T. Tremblay, and M. S. Bartolomei. 1998. Imprinted expression and methylation of the mouse *H19* gene are conserved in extraembryonic lineages. *Dev. Genet.* **23**:111–118.
- Davis, T. L., G. J. Yang, J. R. McCarrey, and M. S. Bartolomei. 2000. The *H19* methylation imprint is erased and reestablished differentially on the parental alleles during male germ cell development. *Hum. Mol. Genet.* **9**:2885–2894.
- Drewell, R. A., J. D. Brenton, J. F. Ainscough, S. C. Barton, K. J. Hilton, K. L. Arney, L. Dandolo, and M. A. Surani. 2000. Deletion of a silencer element disrupts *H19* imprinting independently of a DNA methylation epigenetic switch. *Development* **127**:3419–3428.
- Dudov, K. P., and R. P. Perry. 1984. The gene encoding the mouse ribosomal protein L32 contains a uniquely expressed intron-containing gene and an unmutated processed gene. *Cell* **37**:457–468.
- Elson, D. A., and M. S. Bartolomei. 1997. A 5' differentially methylated sequence and the 3' flanking region are necessary for *H19* transgene imprinting. *Mol. Cell. Biol.* **17**:309–317.
- Ferguson-Smith, A. C., and M. A. Surani. 2001. Imprinting and the epigenetic asymmetry between parental genomes. *Science* **293**:1086–1089.
- Filippova, G. N., C. P. Thienes, B. H. Penn, D. H. Cho, Y. J. Hu, J. M. Moore, T. R. Klesert, V. V. Lobanenko, and S. J. Tapscott. 2001. CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. *Nat. Genet.* **28**:335–343.
- Forne, T., J. Oswald, W. Dean, J. R. Saam, B. Bailleul, L. Dandolo, S. M. Tilghman, J. Walter, and W. Reik. 1997. Loss of the maternal *H19* gene induces changes in *Igf2* methylation in both cis and trans. *Proc. Natl. Acad. Sci. USA* **94**:10243–10248.
- Frevel, M. A. E., J. J. Hornberg, and A. E. Reeve. 1999. A potential imprint control element: identification of a conserved 42 bp sequence upstream of *H19*. *Trends Genet.* **15**:216–218.
- Grandjean, V., L. O'Neill, T. Sado, B. Turner, and A. Ferguson-Smith. 2001. Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted *Igf2-H19* domain. *FEBS Lett.* **488**:165–169.
- Hark, A. T., C. J. Schoenherr, D. J. Katz, R. S. Ingram, J. M. LeVorse, and S. M. Tilghman. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the *H19/Igf2* locus. *Nature* **405**:486–489.
- Hark, A. T., and S. M. Tilghman. 1998. Chromatin conformation of the *H19* epigenetic mark. *Hum. Mol. Genet.* **7**:1979–1985.
- Holmgren, C., C. Kanduri, G. Dell, A. Ward, R. Mukhopadhyaya, M. Kanduri, V. Lobanenko, and R. Ohlsson. 2001. CpG methylation regulates the *Igf2/H19* insulator. *Curr. Biol.* **11**:1128–1130.
- Ishihara, K., N. Hatano, H. Furuumi, R. Kato, T. Iwaki, K. Miura, Y. Jinno, and H. Sasaki. 2000. Comparative genomic sequencing identifies novel tissue-specific enhancers and sequence elements for methylation-sensitive factors implicated in *Igf2/H19* imprinting. *Genome Res.* **10**:664–671.
- Jenuwein, T., and C. D. Allis. 2001. Translating the histone code. *Science* **293**:1074–1080.
- Kaffer, C. R., M. Srivastava, K.-Y. Park, E. Ives, S. Hsieh, J. Battle, A. Grinberg, S.-P. Huang, and K. Pfeifer. 2000. A transcriptional insulator at the imprinted *H19/Igf2* locus. *Genes Dev.* **14**:1908–1919.
- Kanduri, C., V. Pant, D. Loukinov, E. Pugacheva, C. F. Qi, A. Wolffe, R. Ohlsson, and V. V. Lobanenko. 2000. Functional association of CTCF with the insulator upstream of the *H19* gene is parent of origin-specific and methylation-sensitive. *Curr. Biol.* **10**:853–856.
- Khosla, S., A. Aitchison, R. Gregory, N. D. Allen, and R. Feil. 1999. Parental allele-specific chromatin configuration in a boundary/imprinting-control element upstream of the mouse *H19* gene. *Mol. Cell. Biol.* **19**:2556–2566.
- Kuhn, R., K. Rajewsky, and W. Muller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science* **254**:707–710.
- Leighton, P. A., R. S. Ingram, J. Eggenschwiler, A. Efstratiadis, and S. M. Tilghman. 1995. Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature* **375**:34–39.
- Leighton, P. A., J. R. Saam, R. S. Ingram, C. L. Stewart, and S. M. Tilghman. 1995. An enhancer deletion affects both *H19* and *Igf2* expression. *Genes Dev.* **9**:2079–2089.
- McCarrick, J. W. I., J. R. Parnes, R. H. Seong, D. Solter, and B. B. Knowles. 1993. Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. *Transgenic Res.* **2**:183–190.
- Ohlsson, R., R. Renkawitz, and V. Lobanenko. 2001. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet.* **17**:520–527.
- Olek, A., and J. Walter. 1997. The pre-implantation ontogeny of the *H19* methylation imprint. *Nat. Genet.* **17**:275–276.
- Pfeifer, K., P. Leighton, and S. M. Tilghman. 1996. The structural gene of *H19* is required for transgene imprinting. *Proc. Natl. Acad. Sci. USA* **93**:13876–13883.
- Reed, M. R., C. F. Huang, A. D. Riggs, and J. R. Mann. 2001. A complex duplication created by gene targeting at the imprinted *H19* locus results in two classes of methylation and correlated *Igf2* expression phenotypes. *Genomics* **74**:186–196.
- Reik, W., and J. Walter. 2001. Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet.* **2**:21–32.
- Sasaki, H., A. C. Ferguson-Smith, A. S. W. Shum, S. C. Barton, and M. A.

- Surani. 1995. Temporal and spatial regulation of *H19* imprinting in normal and uniparental mouse embryos. *Development* **121**:4195–4202.
45. Sauer, B., and N. Henderson. 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.* **2**:441–449.
 46. Schmidt, J. V., P. G. Matteson, B. K. Jones, X. J. Guan, and S. M. Tilghman. 2000. The *Dlk1* and *Gtl2* genes are linked and reciprocally imprinted. *Genes Dev.* **14**:1997–2002.
 47. Srivastava, M., S. Hsieh, A. Grinberg, L. Williams-Simons, S.-P. Huang, and K. Pfeifer. 2000. *H19* and *Igf2* monoallelic expression is regulated in two distinct ways by a shared cis acting regulatory region upstream of *H19*. *Genes Dev.* **14**:1186–1195.
 48. Stadnick, M. P., F. M. Pieracci, M. J. Cranston, E. Taksel, J. L. Thorvaldsen, and M. S. Bartolomei. 1999. Role of a 461 bp G-rich repetitive element in *H19* transgene imprinting. *Dev. Genes Evol.* **209**:239–248.
 49. Sunahara, S., K. Nakamura, K. Nakao, Y. Gondo, Y. Nagata, and M. Kat-suki. 2000. The oocyte-specific methylated region of the *U2afbp-rs/U2af1-rs1* gene is dispensable for its imprinted methylation. *Biochem. Biophys. Res. Commun.* **268**:590–595.
 50. Szabo, P. E., S.-H. Tang, A. Rentsendorj, G. P. Pfeifer, and J. R. Mann. 2000. Maternal-specific footprints at putative CTCF sites in the *H19* imprinting control region give evidence for insulator function. *Curr. Biol.* **10**:607–610.
 51. Thorvaldsen, J. L., K. L. Duran, and M. S. Bartolomei. 1998. Deletion of the *H19* differentially methylated domain results in loss of imprinted expression of *H19* and *Igf2*. *Genes Dev.* **12**:3693–3702.
 52. Tremblay, K. D., K. L. Duran, and M. S. Bartolomei. 1997. A 5' 2-kilobase-pair region of the imprinted mouse *H19* gene exhibits exclusive paternal methylation throughout development. *Mol. Cell. Biol.* **17**:4322–4329.
 53. Tremblay, K. D., J. R. Saam, R. S. Ingram, S. M. Tilghman, and M. S. Bartolomei. 1995. A paternal-specific methylation imprint marks the alleles of the mouse *H19* gene. *Nat. Genet.* **9**:407–413.
 54. Warnecke, P. M., J. M. Mann, M. Frommer, and S. J. Clark. 1998. Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted *H19* gene. *Genomics* **51**:182–190.
 55. Webber, A. L., R. S. Ingram, J. M. LeVorse, and S. M. Tilghman. 1998. Location of enhancers is essential for the imprinting of *H19* and *Igf2* genes. *Nature* **391**:711–715.
 56. Wylie, A. A., S. K. Murphy, T. C. Orton, and R. L. Jirtle. 2000. Novel imprinted *DLK1/GTL2* domain on human chromosome 14 contains motifs that mimic those implicated in *IGF2/H19* regulation. *Genome Res.* **10**:1711–1718.