A transcriptional insulator at the imprinted H19/Igf2 locus

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Igf2 and H19 exhibit parent-of-origin-specific monoallelic expression. H19 is expressed from the maternal chromosome and Igf2 from the paternal. The two genes share enhancer elements and monoallelic expression of both genes is dependent on cis-acting sequences upstream of the H19 promoter. In this work we examine the mechanisms by which this region silences the maternal Igf2 allele and we demonstrate that deletion of this region can result in high levels of activation of both H19 and Igf2 from a single chromosome. Moreover, by inserting this cis element between a promoter and its enhancer at a heterologous position, we demonstrate that the sequences carry both insulator activity and the ability to be stably imprinted. We also characterize the insulator in vitro and show that it is neither enhancer nor promoter specific.

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H19 and Igf2 are two monoallelically expressed genes that are part of a cluster of imprinted genes on the distal end of mouse chromosome 7 [Fig. 1]. H19 is expressed only from the maternal chromosome [Bartolomei et al. 1991], whereas Igf2 is generally expressed only from the paternal chromosome [DeChiara et al. 1991]. The two genes share complex patterns of developmental and tissue-specific gene expression, suggesting that their transcriptional activation is mechanistically linked. In fact, it has been demonstrated that enhancer elements, at least for endodermal-specific expression, are shared by the two genes [Yoo-Warren 1988; Leighton et al. 1995b]. These enhancers lie between 7 and 9 kb downstream of the H19 promoter and ~100 kb downstream of the Igf2 promoter, and are capable of directing high levels of transcriptional activation of each gene.

The mechanistic basis for monoallelic expression of H19 has been studied extensively by molecular and genetic approaches. One focus of research has been to detect epigenetic differences in the two chromosomes that might be the actual genomic imprint or that might play a role in maintaining monoallelic expression. Centered at −2.4 and −3.8 kb relative to the start of H19 transcription, lie regions showing nuclease hypersensitivity specific to the maternal chromosome [Hark and Tilghman 1998; Szabo et al. 1998; Khosla et al. 1999]. These regions of differential hypersensitivity were identified in somatic tissue and are located within a large domain of DNA showing hypermethylatyion specific to the paternal chromosome [Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1995, 1997; Olek 1997]. This differentially methylated region (DMR) is distinctly marked through all stages of development and thus has been considered a good candidate for the actual genomic imprint.

Multiple-copy insertions of H19 transgenes carrying these upstream sequences to ~4 kb exhibit parent-of-origin-dependent DNA methylation and RNA expression patterns like those at the endogenous locus [Bartolomei et al. 1993; Pfeifer et al. 1996; Elson and Bartolomei 1997; Brenton et al. 1999] However, the same constructs, when present in single copy, are not imprinted. The lack of parent-of-origin-specific expression of single copy transgenes indicates that all of the sequence information required for imprinting is not carried on these constructs.

By directly testing the necessity of the DMR for imprinting the endogenous H19 gene, Thorvaldsen and colleagues deleted the region located between −3.7 and −2.1 kb. Inheritance of the deletion on the paternal chromosome results in activation of the normally silent paternal H19 allele [Thorvaldsen et al. 1998]. Analysis of a conditional mutation in which the DMR was removed at varying stages of development demonstrates that the upstream region was required in the germ cells and in early embryonic development to mark the paternal chromosome. However, it was not required late in development to maintain transcriptional silencing of the paternal H19 [Srivastava et al. 2000]. Rather, the DMR directed epigenetic changes in the promoter region that were sufficient to silence the paternal H19. Once these epigenetic

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In this work, we address the mechanism for silencing the maternal Igf2 allele. Our work provides direct evidence for the presence of an insulator element upstream of the H19 promoter coincident with the DMR and nuclease hypersensitive sites. When active, as on the maternal chromosome, the insulator would function in a position-dependent manner to prevent activation of the Igf2 promoter by the downstream enhancers. On the paternal chromosome, epigenetic modifications would abrogate the insulating activity, permitting activation of the paternal Igf2 promoter. This model does not demand that expression of Igf2 from a single chromosome be mutually exclusive. It does predict that all enhancer elements will be located downstream of the insulator and that the DMR sequences have insulator activity. The insulator model is consistent with results from the four H19 deletion mutations described above.

An alternate model postulates that silencing of the maternal Igf2 is mediated by the action of an insulator element upstream of the H19 promoter coincident with the DMR and nuclease hypersensitive sites. When active, as on the maternal chromosome, the insulator would function in a position-dependent manner to prevent activation of the Igf2 promoter by the downstream enhancers. On the paternal chromosome, epigenetic modifications would abrogate the insulating activity, permitting activation of the paternal Igf2 promoter. This model does not demand that expression of Igf2 from a single chromosome be mutually exclusive. It does predict that all enhancer elements will be located downstream of the insulator and that the DMR sequences have insulator activity. The insulator model is consistent with results from the four H19 deletion mutations described above.

In this work, we address the mechanism for silencing the maternal Igf2 allele. Our work provides direct evidence for the presence of an insulator element upstream of the H19 that, when maternally inherited, can suppress interactions between downstream enhancers and upstream promoter elements. Specifically, we characterize a mutation removing sequences between −10 and −0.8 kb upstream of the H19 and demonstrate that, in contrast to predictions of the competition model, coexpression of H19 and Igf2 from a single chromosome is possible. Second, we use transgenes and transfection experiments to map the H19 mesodermal enhancers to a position downstream of the previously mapped endodermal enhancers, a location con-
sistent with the insulator model. Coincidentally, we identify upstream sequences sufficient to imprint even single-copy \( H19 \) transgenes. Third, we directly demonstrate insulator function in the sequences upstream of the \( H19 \) promoter by inserting them into a heterologous position in the chromosome. We confirm independently the presence of insulator activity on these sequences using an in vitro assay. Finally, we demonstrate that these upstream sequences carry information that defines their parental origin.

**Results**

**Deletion of the DMR sequences**

The competition model predicts that activation of the \( Igf2 \) promoter is a reflection of the silencing of the cis \( H19 \) promoter. Thus, \( Igf2 \) and \( H19 \) should not be expressed from a single chromosome at the same time. In liver cells, consistent with the competition model, parental inheritance of a deletion of the DMR results not only in activation of the normally silent paternal \( H19 \) allele, but also reduced levels of \( Igf2 \) expression (Thorvaldsen et al. 1998). However, \( Igf2 \) was also down-regulated in liver upon paternal inheritance of the \( H19^{*13} \) mutation that removed the entire \( H19 \) gene, including the \( H19 \) promoter, in which intergenic competition was unlikely. Therefore, we decided to examine the effects of paternal inheritance of a DMR deletion chromosome in additional tissues at various developmental stages to see whether we could identify conditions in which coexpression of \( H19 \) and \( Igf2 \) at high levels was possible. We used mice that carried a simple deletion of sequences from −10 to −0.8 kb (Fig. 1). This deletion, \( K519 \), is similar to the Thorvaldsen deletion, but extends 5’ to remove all of the known maternal-specific DNase I hypersensitive sites [Hark and Tilghman 1998; Szabo et al. 1998; Khosla et al. 1999] and all of the upstream sequences required for imprinting of single-copy transgenes [see below].

Paternal inheritance of the \( K519 \) mutation led to activation of paternal \( H19 \) in all embryonic tissues as determined by in situ hybridization (Fig. 2a,b,c). The relative contribution of the paternal allele was quantitated by single nucleotide primer extension (SNuPE) assay (Kuppuswamy et al. 1991; Singer-Sam et al. 1992). The ability of this assay to quantitate the relative contributions of maternal and paternal alleles to the total \( H19 \) and \( Igf2 \) RNA levels within a single sample have been established previously (Szabo and Mann 1995; Srivastava et al. 2000). In liver, muscle, heart, and gut, the paternal \( K519 \) chromosome contributes \( \sim 50\% \) of the total \( H19 \) expression (Fig. 3a; Table 1). The high degree of \( H19 \) activation in these tissues was confirmed by Northern analysis of RNA from neonatal pups carrying a paternal \( K519 \) chro-
mosome and a maternal chromosome with the H19^{A13} mutation [Fig. 3c]. In other tissues, the activation of H19 was reduced compared with that seen from wild-type maternal chromosomes (data not shown), consistent with previous reports (Thorvaldsen et al. 1998).

Transcription of the paternal H19 allele did not always lead to a concomitant loss of Igf2 expression as seen by in situ hybridization [Fig. 2e,f]. Northern hybridization confirmed that levels of Igf2 from the paternal K519 chromosome were like those noted from wild type in skeletal muscle and gut [Fig. 3c] and in heart, kidney, brain, and lung (data not shown). Liver RNA was reduced in total Igf2 levels as reported previously (Thorvaldsen et al. 1998). We conclude that activation of paternal H19 was not at the expense of paternal Igf2 expression and our results, therefore, are not well explained by the competition model.

When maternally inherited, the K519 mutation led to activation of the normally silent maternal Igf2 promoter in all tissues tested. Total Igf2 RNA increased approximately twofold in all non-hepatic tissues from mutant neonates (Fig. 3d), consistent with activation of both the mutant maternal and the wild-type paternal Igf2 alleles. SNuPE assays confirm that the maternal chromosome contributed ∼50% of the total Igf2 RNA in all tissues except the liver (Fig. 3b, Table I). These results were consistent with the presence of a transcriptional insulator element upstream of H19. Our subsequent experiments were therefore directed toward directly testing the insulator model for monoallelic expression of Igf2.

### Table 1. The effect of the K519 mutation on allele specific expression

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Contribution of the paternal chromosome to total H19 mRNA</th>
<th>Contribution of the maternal chromosome to total Igf2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>20 ± 3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Muscle</td>
<td>40 ± 4</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Heart</td>
<td>49 ± 3</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Kidney</td>
<td>47 ± 5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lung</td>
<td>37 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Gut</td>
<td>40 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Brain</td>
<td>61 ± 5</td>
<td>52 ± 18</td>
</tr>
</tbody>
</table>

Samples were analyzed by SNuPE assay as described in Fig. 3. For the H19 assay, control experiments indicate that pure DNA samples give a background of 10 ± 3% due to misincorporation during the SNuPE protocol. For the Igf2 assay, the background is estimated at 6 ± 4%. The numbers shown are not corrected for this background.

### Figure 3. Expression of H19 and Igf2 in K519 neonates. Allele specific expression patterns of H19 (a) and of Igf2 (b) were analyzed by SNuPE assay. (a) H19 expression is analyzed in neonates carrying a mutated paternal allele (P) on a domesticus chromosome and a maternally inherited wild-type castaneous allele. Control littersmates (+) have inherited a wild-type domesticus allele paternally and a wild-type castaneous allele maternally. (b) Igf2 expression is analyzed in neonates carrying a mutated maternal allele (M) on a domesticus chromosome and a paternally inherited wild-type castaneous allele. Control littersmates (+) have maternally inherited a wild-type domesticus allele and paternally inherited a wild-type castaneous allele. For both a and b, lanes C, D, and F1 depict results for a pure castaneous DNA template, a pure domesticus DNA template, and DNA from a castaneous x domesticus F1 animal, which therefore represents a 50:50 mix of the two DNAs. Total RNA levels for H19 and Igf2 were analyzed by Northern blot upon paternal inheritance of the K519 chromosome to total H19 mRNA and upon maternal inheritance of the K519 chromosome to total Igf2 mRNA. For this background.

Mapping mesodermal enhancers of H19

To prevent blocking of H19 expression from the maternal chromosome, in which the putative insulator is predicted to be active, the insulator model demands that all H19 enhancer elements be located 3’ of the DMR region. Consistent with the model, endodermal enhancers are located between +7 and +9.5 kb (Leighton et al. 1995b; Yoo-Warren et al. 1988). However, the exact location of the mesodermal enhancers has been unknown. Previous transgenic experiments have mapped skeletal muscle enhancers to either upstream of −4 kb or between +12.5 and +36 kb (Brunkow and Tilghman 1991; Pfeifer et al. 1996; Ainscough et al. 1997). Likewise, these same experiments indicate that enhancers responsible for cardiac muscle expression lie either downstream of +36 kb or upstream of the Igf2 gene. To help distinguish between these possible locations, we generated transgenic lines using a bacterial artificial chromosome (BAC) clone that spans from −7 to about +130 kb. We analyzed two lines.
in which a single copy of the entire BAC clone was inserted at sites unlinked to the H19/Igf2 locus (data not shown). In situ hybridization experiments demonstrate a pattern of expression from the H19–BAC1 transgene that is highly similar to that of the endogenous locus (Fig. 2d). Furthermore, Northern analyses of RNA prepared from skeletal muscle of day 5 pups confirm that the BAC transgene contains sequences sufficient for this mesodermal expression of H19 (Fig. 4). Taken together with the previously published transgenic results and the continued skeletal muscle expression in the K519 deletion mutant, these BAC transgenic analyses place the skeletal muscle enhancers between +12 and +36 Kb. This location is consistent with predictions of the boundary model.

As seen in Figure 4, H19 is expressed from the BAC transgene only upon maternal inheritance. Maternal specific expression was noted in all of the following tissues tested: liver, heart, lung, and kidney. Thus we conclude that upstream sequences to −7.0 kb are sufficient for consistent imprinting of even single-copy H19 transgenes.

Our in situ analyses demonstrate expression of the BAC transgene in embryonic cardiac tissue (Fig. 2). These results were confirmed by Northern analysis of RNAs extracted from neonatal hearts (data not shown). These results, together with previous transgenic analyses (Ainscough et al. 2000), indicate that the cardiac enhancers lie between +36 and +130 kb. However, we did note that H19–BAC1 expression in heart and in kidney was reduced about three- to fourfold compared with endogenous expression (data not shown).

In vitro mapping of skeletal muscle enhancers

To confirm the presence of skeletal muscle enhancers in the downstream region and to define their location more precisely, in vitro transfection analysis was performed in two muscle cell lines. Mouse C2C12 and Sol8 cell lines have provided an excellent system for studying musclespecific gene expression (Calvo et al. 1999). Differentiation of these myoblast cell lines into myotubes can be accomplished by the transfer of cells into medium containing reduced levels of serum supplement and results in high-level expression of H19 RNA (data not shown). DNA fragments spanning the +10 to +42 kb downstream of the H19 gene were cloned into the multiple cloning site of a reporter vector, pBgal-Promoter. This vector carries an SV40 promoter inserted just upstream of the Escherichia coli lacZ gene. We assayed expression of the lacZ gene in pools of stably transfected cell lines as described in the Materials and Methods section. These results, summarized in Figure 5, show that the insert on Constructs 6 and 7 was able to increase expression from the SV40 promoter by ~400- to 500-fold. Construct 8, with an insert partially overlapping that of Constructs 6 and 7, showed a relatively very small enhancement of ~30-fold. All other constructs demonstrated essentially background levels of gene activation. Given the lack of enhancement from Constructs 4, 5, 9, and even 8, we conclude that the skeletal muscle enhancers for H19

![Figure 4](image.png)

**Figure 4.** Expression of H19 in skeletal muscle from the H19–BAC1 transgene. RNA was prepared from tissues dissected from day 5 pups carrying the H19–BAC1 transgene in an H19+/−H9004 background. RNA was assayed by Northern analysis comparing pups in which the transgene was inherited maternally (M) or paternally (P) with wild-type (Wt) littermates. Results for one of two independent lines are depicted.

![Figure 5](image.png)

**Figure 5.** β-Galactosidase activity from stable transfection of the Sol8 cell line. (a) DNA fragments spanning the +10 to +35.5-kb region downstream of H19 were cloned into the multiple cloning site of the reporter vector, pβgal-Promoter. This vector carries an SV40 promoter inserted upstream of the lacZ gene. The top line depicts an EcoRI (R) and partial BamHI (B) map of the region. The subcloned fragments are indicated below along with the construct number. The arrow indicates the fragment orientation. (b) Sol8 myoblasts were cotransfected with the reporter constructs and pTK-Hyg (Clontech) and resistant colonies were selected in hygromycin B. Resistant cells were replated and grown 72 hr in differentiation medium to induce myotube formation. Cell lysates were assayed for β-galactosidase activity with enzyme activity normalized to total protein levels. The levels of induction relative to that generated by an enhancerless control construct are depicted.
centers in the sequences between ~22 and 28 kb downstream of the promoter (Fig. 5).

We mapped enhancer activity to this same interval using transient transfection of both Sol8 and C2C12 cell lines. In both cell lines, Constructs 6 and 7 were able to enhance expression of the SV40 promoter ~10-fold [data not shown]. No enhancer activity was detected in this assay system using Construct 8. Thus, in both C2C12 and Sol8 cell lines, and measured by both transient and stable transfection, we map skeletal muscle enhancer activity to DNA sequences found in Constructs 6 and 7.

Effect of the CRK10 insertion on H19 expression

To test directly the ability of the upstream element to prevent enhancer-promoter interactions, we generated a mutation, CRK10, in which the potential insulator element was inserted between the endodermal and the skeletal muscle enhancers located downstream of H19 [Fig. 1]. If, in fact, the region acts as an insulator, its effect on transcription will be position dependent. Endodermal enhancers will remain free to interact with the H19 promoter. Skeletal muscle enhancers now separated from the promoter by the insulator will be prevented from activating H19 transcription. The maternally inherited mutant allele will thus express H19 in tissues of endodermal origin but not in tissues of mesodermal origin. RNA from pups maternally inheriting the CRK10 mutation was prepared from liver and skeletal muscle. H19 expression in livers from mutant mice was equal to that seen in wild-type mice. In contrast, skeletal muscle expression of H19 in mutant pups is drastically reduced when compared with wild-type littermates, demonstrating that this element acts in a position-dependent manner to prevent enhancer activity [Fig. 6].

Our experiment cannot directly exclude the possibility that transcription in muscle is reduced due to the increase in distance of the enhancers to the H19 promoter. However, enhancers are known to work over large distances. The endodermal enhancers, for example, direct high levels of expression on both H19 and Igf2 promoters that are 9 and 100 kb away, respectively. The skeletal muscle enhancers are >20 kb distant from the H19 promoter. Although not yet demonstrated experimentally, we expect, on the basis of the nearly identical expression patterns of Igf2 and H19 in tissues of both endodermal and mesodermal origins, that Igf2 also shares the same mesodermal enhancers with H19 [see also Ainscough et al. 2000]. We noted no changes in the levels of Igf2 expression upon paternal inheritance of the CRK10 insertion [C.R. Kaffer and K. Pfeifer, unpubl.].

In vitro demonstration of insulator activity

We used a colony assay [Chung et al. 1993] to test independently whether DMR-containing sequences could insulate the H19 promoter from the H19 endodermal enhancers. Constructs in which potential insulators were inserted between the H19 endodermal enhancers and the H19 promoter (~800 to +6 bp) were transfected into the human hepatoma cell line, Hep3B, generating cell lines with multicopy insertions of the DNA constructs. In all constructs, the neomycin resistance gene was fused to the H19 promoter so that activation of the promoter could be assayed by counting the number of G418-resistant colonies. If the DMR sequences contain insulator elements, we expected to see a reduction in the number of resistant colonies toward the level noted for control constructs that have no enhancer elements.

When the 4.7-kb Xbal–Xbal fragment covering from −5.5 to −0.8 kb of the H19 upstream region was inserted on both sides of the endodermal enhancer [pKP-4], the number of G418-resistant colonies was, in fact, reduced to below those seen in constructs in which no enhancer at all was present [Fig. 7]. The same fragment, when inserted only upstream of the enhancer, had no effect on the number of colonies [pKP-3]. Thus, its ability to silence the H19 promoter appears to be position dependent. A smaller BglII–BglII fragment carrying sequences between −4.3 and −1.9 kb upstream of the H19 start site also showed strong insulator activity [pKP-5].

Next, we tested whether the activity was enhancer specific by assaying constructs in which the SV40 enhancer replaced the H19 endodermal enhancer elements. This assay system was also advantageous because the difference in colony numbers noted with and without enhancer activity was much greater [Fig. 7B]. Again in this system, even the 2.4-kb BglII–BglII fragment showed significant insulator activity. The decreased number of colonies was not due to an increased distance between the promoter and the enhancer, as we observed no such decrease when we tested a nonspecific 2.3-kb genomic fragment in the same assay.

These results confirm that there is position-dependent insulator activity in sequences from the upstream region of the H19 gene. This activity is enhancer nonspecific. The activity is also species nonspecific in that the mouse sequences can function as insulators in a human cell line. The smallest fragment we have successfully tested for insulator function includes the DMR and the sites of the maternal-specific nuclease hypersensitivity.

Methylation analysis of the CRK10 allele

In its normal location upstream of H19, the insulator element sequences are hypermethylated on the paternal
chromosome and are essentially unmethylated on the maternal chromosome. To determine whether the inserted element contained sequences sufficient for this imprint, genomic DNAs extracted from the livers of CRK10 mice and their wild-type littermates were subjected to restriction digestion with methylation-sensitive enzymes. When the mutation is inherited from the mother, our displaced insulator, seen as a 3.0-kb EcoRI fragment, is completely sensitive to digestion with ClaI and HhaI (Fig. 8) or with HpaII (data not shown), indicating that the element is unmethylated at these sites. When inherited from the father, however, the inserted element is completely resistant to digestion with these same enzymes, indicating that the sequences are methylated at these sites. We conclude that the inserted element contains the information that is sufficient for marking the H19/Igf2 region as paternal or as maternal in origin.

Discussion

We provide evidence that maternal silencing of Igf2 is a consequence of the presence of a cis-acting insulator element that acts in a position-dependent manner to prevent enhancers from interacting with the maternal Igf2 promoter. We first show that a deletion of a 9-kb region upstream of the H19 promoter permits coexpression of H19 and Igf2 from a single chromosome, a result incongruent with the competition model. We then map mesodermal enhancer elements to sequences downstream of the H19 gene, a position consistent with determination of Igf2 monoallelicism via a maternal-specific insulator. Third, we directly demonstrate position-dependent insulator function by inserting the region containing the putative insulator between the liver and skeletal muscle-enhancer elements located downstream of H19. In vitro analyses independently confirm the presence of insulator activity and provide additional evidence that loss of enhancer function is not due to changes in the distance between the promoter and enhancer elements. Finally, we demonstrate that this region contains sufficient information to mark its own parental origin. When paternally inherited, the inserted insulator sequences are hypermethylated just as they are at their normal location. We presume that this DNA methylation inactivates the endogenous insulator function and thus permits Igf2 expression from the wild-type paternal chromosome.

Tissue-specific expression of H19 is determined by at least three independent enhancer elements. Expression in endodermal tissues and in skeletal muscle is directed by cis-acting elements centered ∼8 and 25 kb, respectively, downstream of the H19 gene. Our results also indicated that cardiac muscle enhancers lie down-
stream of +35 kb. It has been demonstrated that Igf2 shares the endodermal enhancers (Leighton et al. 1995). We are currently testing the proposition that the other enhancer elements are also shared. On the basis of transgenic analysis (Ainscough et al. 1997, 2000) and on the matched patterns of tissue-specific and temporally regulated patterns of gene expression, we expect that the elements are shared. In that case, activation of the Igf2 promoter is dependent upon cis elements that lie between 110 and >135 kb downstream.

Because enhancers can often work across long distances and thus have the potential to affect many different promoters, it seems necessary for the cell to have the ability to limit enhancer accessibility and prevent promiscuous gene expression. Over the past few years, novel regulatory elements, called insulators, have been identified, which restrict access of enhancers to distal promoters and thus do provide a mechanism to stop inappropriate enhancer activity [for reviews, see Bell and Felsenfeld 1999, Sun and Elgin 1999]. Insulator elements were first described in Drosophila. Scs and scs' elements were identified as chromatin boundary elements that flank two hsp70 genes [Udvardy et al. 1985]. The gypsy retrotransposon carries an insulator element that is responsible for the mutant phenotypes associated with insertion of the transposon [Corces and Geyer 1991]. Both gypsy and scs/scs' elements are able to block enhancers in a position-dependent manner: They prevent promoters from being activated when placed between the promoters and their enhancers [Geyer and Corces 1992; Kellum and Schedl 1992]. They are also able to insulate transgenes against both positive and negative position effects [Kellum and Schedl 1991; Roseman et al. 1993]. In vertebrates, regulation of the chicken β-globin locus by insulators is well established [Chung et al. 1993]. The β-globin insulator, like the Drosophila elements, can prevent enhancer-promoter interactions in a position-dependent manner and can provide position independence to transgenes. It has been suggested that the insulator may play a role in chromatin organization, organizing the switch from heterochromatin to euchromatin, perhaps through attracting proteins that regulate histone acetylation [Hebbes et al. 1994].

Deleting sequences carrying the DMR and the maternal-specific hypersensitive sites on the maternal chromosome causes full activation of the maternal Igf2 promoter. To determine whether the region upstream of H19 has insulator function, we generated mice carrying an insertion of this region between the endodermal and mesodermal enhancers located downstream of the H19 promoter. The insertion was made at +10.7 kb, a site at which insertion of a NeoR gene does not interfere with H19 expression in skeletal muscle [Leighton et al. 1995b]. As predicted by the insulator model, CRK10 mice carrying the insertion exhibited drastically reduced levels of H19 in skeletal muscle. In contrast, endodermal expression of H19 in mutant pups was essentially normal, indicating that the insertion did not show a generalized silencing of the H19 promoter. Thus, the inserted element does not act in a nonspecific way to silence the H19 promoter. Rather, the effect is enhancer specific, only expression dependent upon enhancers that are separated from the promoter by the insulator element is blocked. We believe that the upstream element shows a similar position-dependent insulator activity at its endogenous location, and that this activity is responsible for preventing activation of the maternal Igf2 promoter.

We independently assayed the upstream element for insulator activity using a colony assay developed to examine insulator function of the chicken β-globin locus [Chung et al. 1993] and confirmed the ability of the upstream element to suppress promoter-enhancer interactions in a position-dependent manner. Using this assay, we were able to define a 2.4-kb region containing insulator function. The ability of the insulator to function is not enhancer specific, as it was able to block activation of the H19 promoter by both the SV40 enhancer and by H19 endodermal-specific enhancer elements. Likewise, we surmise that the insulator is not promoter specific, because it works on the H19 promoter and, in its normal location upstream of H19, on the Igf2 promoter. Finally, the in vitro assay system allowed us to demonstrate directly that loss of enhancer activation is not explained by the increased distance between the enhancer and promoter elements that is necessitated by our assay systems.

The colony assay alone cannot rule out that the insulator may also show some silencing activity. The ability of the DMR to act as a silencer when assayed by use of constructs similar to those tested in vitro in this study was demonstrated previously in Drosophila [Lyko et al. 1997]. Using constructs in which expression was driven by the H19 endodermal enhancers [Fig. 7A], we noted that presence of the insulator actually reduced the number of G418 resistant clones to ~75% of that seen with the promoter alone. In fact, we postulate that DMR, in its normal function, does act to unidirectionally silence the H19 promoter via the direction of epigenetic modifications of the promoter postnatally. However, clear evidence that the DMR does carry sequences that do function as insulators comes from the in vivo analysis of mice maternally inheriting the CRK10 insertion. The concomitant activation of the H19 promoter in liver and the lack of activation in skeletal muscle is inconsistent with silencing the H19 promoter by the CRK10 insertion. Thus, in vivo analyses demonstrate that a biologically fundamental function of the DMR is its action as a transcriptional insulator.

Southern analysis of the CRK10 mutation shows identical patterns of methylation at the inserted insulator compared with that seen at the endogenous element. When displaced, it remains unmethylated on the maternal allele, but methylated on the paternal allele, demonstrating that the region contains sufficient information to stably maintain a genomic imprint. We presume that this imprint or the epigenetic changes it directs during development results in inactivation of the insulator function at its normal location, thereby allowing expression of paternal Igf2. The major significance of our results relative to insulator biology is this indication that
the insulator itself is subject to regulation. In the case of \( \text{Igf2} \), the regulation is via the epigenetic modifications induced by the paternal imprint. For other genes, one could anticipate that the modification be developmental or tissue specific, and thus would contribute to regulated gene expression.

The molecular mechanisms for insulator activity are not yet clear, and no current model explains all the experimental data (for reviews, see Bell and Felsenfeld 1999; Sun and Elgin 1999). In one model, proteins associated with the insulator interact with proteins associated with the enhancer and interrupt the ability of the latter to activate proteins interacting with the promoter. At its endogenous location upstream of the \( \text{H19} \) promoter, the \( \text{Igf2} \) insulator is preventing activation of the maternal \( \text{Igf2} \) gene by enhancers that are clearly functional in that they are directing high-level expression of the maternal \( \text{H19} \) allele. Thus, as demonstrated previously in transgenic studies of \( \text{gypsy} \) function, insulators do not actually inactivate enhancers, but only block their motion directionally (Cai and Levine 1995; Scott and Geyer 1995). In a second broad category of models, insulators play a role in higher-order chromatin organization. Any such model for insulator activity at the \( \text{H19} / \text{Igf2} \) locus must take into account the ability of the insulator to act upon enhancers and promoters that are >30 and 80 kb distant, respectively (see Bell and Felsenfeld 1999). Our results agree with a model that assumes that the enhancer is the origin of a progressive signal that must move in \( \text{cis} \) toward the promoter. However, the nature of such a signal is enigmatic.

In summary, our results support a model in which parent-specific expression of the \( \text{H19} \) and \( \text{Igf2} \) genes occurs via a shared parental imprint of the DMR region, but in which transcriptional silencing of the paternal \( \text{H19} \) and maternal \( \text{Igf2} \) alleles occurs via fundamentally different mechanisms. The DMR is required only temporarily during development to establish silencing of the paternal \( \text{H19} \) promoter (Srivastava et al. 2000). During development, the DMR directs epigenetic changes in the \( \text{H19} \) promoter and gene that are the direct cause of transcriptional silencing. In contrast, presence of the DMR is continually required for silencing of the maternal \( \text{Igf2} \) allele (Srivastava et al. 2000), consistent with sequences in the DMR region acting as a transcriptional insulator that blocks enhancer activation of promoters in a position-dependent manner. Both the maternal and paternal \( \text{Igf2} \) promoters are thus potentially functional, and only the lack of enhancement on the maternal chromosome prevents transcription of the maternal allele. This view of gene regulation at \( \text{Igf2} \) was actually suggested by very early experiments demonstrating that whereas the \( \text{H19} \) promoter was differentially accessible to nucleosome digestion on the paternal and maternal chromosomes (Bartolomei et al. 1993; Ferguson-Smith et al. 1993), the \( \text{Igf2} \) promoter behaved equivalently on both chromosomes (Sasaki et al. 1992). Similarly, moving the endodermal enhancers to a position upstream of the insulator allowed biallelic expression of \( \text{Igf2} \) (Webber et al. 1998).

Regulation of expression of \( \text{H19} \) and of \( \text{Igf2} \) has been studied extensively by a number of laboratories as a useful model system for understanding the molecular basis of genomic imprinting and the consequent monoallelic expression. As recent experiments demonstrate, the basis of monoallelic expression of the two genes is quite distinct, the lesson of the model system might be that there is no single model to explain parental-specific expression. The \( \text{H19} \) and \( \text{Igf2} \) genes share enhancer elements, although it is not clear that this is pertinent to regulatory mechanisms for either gene. Considerable experimental evidence now indicates that competition for the shared enhancers is not the basis for the reciprocal pattern of monoallelic expression of \( \text{H19} \) and \( \text{Igf2} \) in that monoallelic expression of each gene can be maintained independently of the other (Schmidt et al. 1999; Srivastava et al. 2000; this work). The most parsimonious mechanism for imprinting at this locus posits that the two genes share a common imprinting element. Strictly speaking, however, this fact remains to be established experimentally. Whether sequences that function as the imprinting box for \( \text{H19} \) and \( \text{Igf2} \) are identical, and whether the insulator and the actual imprinting box are distinct, and the molecular mechanisms by which the imprint inactivates the insulator and the \( \text{H19} \) promoter all remain to be established.

Materials and methods

Generation of \( \text{K519} \) and \( \text{CRK10} \) mutant mice

The targeting vectors were linearized and transfected into ES cells. To identify \( \text{K519neo} \) clones, DNAs were digested with \( \text{BamHI} \) and probed with a 2.2-kb \( \text{PstI–EcoRI} \) probe from just outside of the 5′ flank to identify a 7-kb fragment in wild-type cells and an additional 13-kb fragment in targeted clones. Likewise, digestion with \( \text{BglII} \) and probing with a 1.8-kb \( \text{XbaI–XbaI–BglII} \) probe from just beyond the 3′ flank results in an 11-kb fragment in wild-type cells and an additional 14.5-kb fragment in targeted clones. Two independent clones were each transiently transfected with pBS185, which contains the Cre recombinase driven by the \( \text{HCMV} \) promoter/enhancer (Sauer and Hendenson 1990). Successful excision of the NEO-TK sequences was assayed by identification of a 4.0-kb \( \text{Xbal} \) fragment from the 5′ probe described above. The wild-type and \( \text{K519neo} \) alleles show 8.0- and 7.5-kb bands, respectively. In addition, the region across the deletion was amplified and its DNA sequence confirmed. Positive cell lines were introduced into \( \text{C57BL6/J} \) blastocysts to generate chimeric founder mice.

To identify \( \text{CRK10neo} \) clones, DNAs were digested with \( \text{BamHI} \) and probed with a 1.3-kb \( \text{BamHI–SalI} \) fragment from just beyond the 5′ flank to identify a 10.5-kb fragment in wild-type cells and an additional 9.0-kb band in targeted lines. Likewise, digestion with \( \text{SacI} \) and probing with a 2.2-kb \( \text{BamHI–EcoRI} \) fragment results in a 5.1-kb fragment in wild-type cells and an additional 2.8-kb band in targeted lines. Two lines were used to generate chimeric founders as above. Founder males were mated with mice carrying an \( \text{Ella–cre} \) transgene (Lasko et al. 1996) to excise the \( \text{NEO} \) gene sequences. Successful excision of the \( \text{Neok} \) gene was assayed by digestion of genomic DNAs with \( \text{HindIII} \) and hybridization to the 5′ probe used to screen for \( \text{CRK10neo} \). Wild-type, \( \text{CRK10neo} \), and \( \text{CRK10} \) alleles generate 7.5-, 11.5-, and 10.0-kb bands, respectively.

For both \( \text{K519} \) and \( \text{CRK10} \) constructs, the targeting vector
include the *diptheria toxin A chain* gene for negative selection (McCarrick et al. 1993). Mice carrying the K519 or the CRK10 mutations were crossed with Dis7CAS mice or with *H19*13/Igf2 mice as described in the text. Dis7CAS mice are mostly *domesticus*, but are homozygous *castaneus* across the *H19/Igf2* locus (Gould and Pfeifer 1998).

**Isolation and characterization of H19–BAC**

H19–BAC1 was identified in the Genome Systems Release I Library using a 1.7-kb EcoRI–HindIII probe spanning the DMR element upstream of the *H19* gene. Southern blot analyses and DNA sequencing demonstrated that the BAC insert contained sequences up to the −7-kb HindIII site. The size of the NotI fragment containing the entire insert (and several hundred base pairs of vector sequences) was measured as −140 kb by pulsed-field gel electrophoresis. A 500-bp probe isolated from the +140-kb end of the insert was able to detect a SacI polymorphism distinguishing *castaneus* and *domesticus* mice. This polymorphism cosegregated in 37 of 37 mice with a polymorphism from upstream of the *H19* gene, demonstrating genetic linkage of the DNAs from the two ends of the BAC insert, thus indicating that the BAC insert was not chimeric.

**H19–BAC1 transgenic mice**

A total of 40 µg of H19–BAC1 DNA was digested with NotI to release the mouse DNA insert and electroporated with an equimolar amount of plasmid pMC1neoPolyA (Stratagene) into RI embryonic stem cells. Drug-resistant colonies were selected and screened for the presence of both ends of the BAC insert and for absence of BAC vector sequences. Two of the 44 colonies tested met these criteria. Southern analysis demonstrated that a single copy of the H19–BAC1 was present in each cell line. The cell lines were injected into C57/BL6-J blastocysts to generate chimeric founder mice.

**RNA preparation**

Tissues were homogenized in TRIZOL Reagent (Life Technologies) with a power homogenizer (Polytron), and the RNA was isolated according to Life Technologies protocols.

**SNuPE**

cDNAs were synthesized with 2 µg of DNase I-treated RNA as a template by the Superscript Preamplification System ( Gibco BRL). cDNAs were amplified with *H19* or Igf2-specific primers to generate 164- and 574-bp fragments (Srivastava et al. 2000). Purified PCR products were analyzed as described (Srivastava et al. 2000) except that the extension primers used for *H19* and Igf2 were 5'-GGCGACATTGGCCAAAGAGG-3' and 5'-GCCCATATGGTGTTTGTGAA-3', respectively, and incorporation of radioactively labeled dCTP and TTP was monitored in each case. *Domesticus* H19 and castaneus Igf2 templates incorporate TTP, whereas castaneus H19 and domesticus Igf2 templates incorporate dCTP. Minus reverse transcriptase controls were included with each sample. Relative incorporation of the two nucleotides by SNuPE was used to calculate the contribution of the paternal allele to the total *H19* RNA pool [T/(C+T)] and the contribution of the maternal allele to the total Igf2 RNA pool [C/(C+T)].

**In vitro mapping of the H19 muscle enhancers**

DNA fragments spanning the +10- to +42-kb region downstream of *H19* were cloned into the multiple cloning site of the reporter vector, pβgal-Promoter (Clontech). This vector carries an SV40 promoter inserted upstream of the lacZ gene. Sol8 myoblasts were grown and transfected essentially as described (Calvo et al. 1999), except that for stable transfections, pTK-Hyg (Clontech) was cotransfected with the reporter constructs and resistant colonies were selected for 10 days in 360 µg/ml hygromycin B. Resistant cells were replated and grown 48 hr before switching to differentiation medium containing reduced levels of serum supplements. After 72 additional hr, cell lysates were prepared and β-galactosidase activity was measured using the Promega Enzyme assay system and was normalized to total protein in the cell extracts. To normalize plasmid uptake for each transient transfection, DNA extracted from 50% of the transfected cells was digested with BamHI and HindIII and analyzed by Southern blot using a probe specific to the pβgal-Promoter vector. The C2C12 cell line was grown and transfected by an identical procedure, except that only transient transfection was performed.

**In vitro mapping of the insulator**

Reporter plasmid pKP-1 carries the *H19* promoter from −800 to −6 bp upstream of the Neomycin-resistance gene. A 2.5-kb Xbal–Xbal–BglII fragment carrying the *H19* endodermal enhancers or a 256-bp fragment carrying the SV40 enhancers were cloned upstream of the *H19* promoter of pKP-2 to make pKP-2 and pKP-6, respectively. pKP-3 is derived from pKP-2 but carries an 4.7-kb Xbal–Xbal fragment inserted upstream of the *H19* enhancers. This fragment contains *H19* sequences from −5.5 to −0.8 kb. pKP-4 is identical to pKP-3 except that the Xbal–Xbal fragment is also inserted between the promoter and enhancer, thus, this Xbal fragment flanks the enhancer. pKP-5 is identical to pKP-4 except that a 2.4-kb BglIII–BglII fragment was cloned in to flank the enhancer element. pKP-7 is identical to pKP-5 except that the parent plasmid is pKP-6. Thus, the potential insulator element flanks the SV40 enhancer rather than *H19* enhancer sequences. pKP-8 is similar to pKP-7 except that a 2.3-kb BglIII–BamHI fragment from downstream of the *H19* gene was inserted to flank the SV40 enhancers.

The human hepatoma cell line, Hep3B, was grown in DMEM with 10% FBS. Cells were transfected with 10 µg of DNA using the Calcium Phosphate Transfection Kit (Invitrogen) according to the manufacturer’s instructions. After 72 hr, cells were trypsinized and replated at 1:10 dilution in growth medium containing 500 µg/ml G418. Resistant colonies were counted 10 days after selection.

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**Note added in proof**

References


