Deletion of a silencer element disrupts H19 imprinting independently of a DNA methylation epigenetic switch

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SUMMARY

The H19 imprinted gene is silenced when paternally inherited and active only when inherited maternally. This is thought to involve a cis-acting control region upstream of H19 that is responsible for regulating a number of functions including DNA methylation, asynchronous replication of parental chromosomes and an insulator. Here we report on the function of a 1.2 kb upstream element in the mouse, which was previously shown to function as a bidirectional silencer in Drosophila. The cre-loxP-mediated targeted deletion of the 1.2 kb region had no effect on the maternal allele. However, there was loss of silencing of the paternal allele in many endodermal and other tissues. The pattern of expression was very similar to the expression pattern conferred by the enhancer elements downstream of H19. We could not detect an effect on the expression of the neighbouring imprinted Igf2 gene, suggesting that the proposed boundary element insulating this gene from the downstream enhancers was unaffected. Despite derepression of the paternal H19 allele, the deletion surprisingly did not affect the differential DNA methylation of the locus, which displayed an appropriate epigenetic switch in the parental germlines. Furthermore, the characteristic asynchronous pattern of DNA replication at H19 was also not disrupted by the deletion, suggesting that the sequences that mediate this were also intact. The silencer is therefore part of a complex cis-regulatory region upstream of the H19 gene and acts specifically to ensure the repression of the paternal allele, without a predominant effect on the epigenetic switch in the germline.

Key words: H19, Silencer, Genomic imprinting, DNA methylation, Igf2, Epigenetic

INTRODUCTION

Genomic imprinting is fundamental to normal mammalian development. It involves the selective expression of an imprinted gene from a single parental allele resulting in functional differences between the parental genomes (Reik and Walter, 1998; Tilghman, 1999). The mechanism responsible for silencing of the reciprocal parental allele is thought to involve chromatin modifications (Ferguson-Smith et al., 1993; Hark and Tilghman, 1998; Khosla et al., 1999) and DNA methylation (Li et al., 1993). Specific cis control elements are apparently required to regulate the imprinting process, possibly by initiation of appropriate germ-line restricted epigenetic modifications (Surani, 1998). However, the precise mechanism by which these elements act is largely unknown.

The distal mouse chromosome 7 contains at least 12 imprinted genes, including H19 and Igf2, within a 1 Mb domain (Beechey and Cattanach, http://www.mgu.har.mrc.ac.uk). A 130 kb YAC from this domain, containing only the maternally expressed H19 and paternally expressed Igf2 genes, can imprint appropriately at ectopic loci (Ainscough et al., 1997), indicating that all the cis-acting control elements critical for imprinting are present within this region. Data from targeted deletion studies and H19 transgenic experiments have indicated that an imprinting control element lies in the 4 kb region immediately upstream of H19 (Srivastava et al., 2000; Elson and Bartolomei, 1997; Leighton et al., 1995a; Ripoche et al., 1997). This region contains a 2 kb differentially methylated domain (DMD), located between −2 and −4 kb from the start of transcription, which is required for parental-origin-specific silencing in the mouse (Thorvaldsen et al., 1998; Tremblay et al., 1997). We previously showed that a 1.2 kb element from the DMD functions as a cis-acting silencer of transgenic reporter genes in Drosophila (Lyko et al., 1997) and mice (Brenton et al., 1999).

To elucidate the role of this element in the imprinting of H19 and Igf2 in the context of the endogenous locus, we generated a targeted deletion of the 1.2 kb element, located between −1.7 and −2.9 kb from the transcription start site of H19. We carried out comprehensive in situ analysis on the expression of the H19 and Igf2 genes to determine the extent to which expression was affected in specific tissues. The deletion results in a relaxation of H19 silencing after paternal inheritance, without disrupting
expression of Igf2. Strikingly, the deletion does not alter the DNA methylation status or asynchronous pattern of DNA replication of the H19 locus.

**MATERIALS AND METHODS**

**H19 silencer targeting construct**

Genomic fragments were isolated from a λ2001 mouse 129/Sv library (a kind gift of A. Smith). The targeting vector (Fig. 1) carries a 1177 bp deletion of the BspEI-BamHI fragment located between −2.9 kb and −1.7 kb from the H19 transcription start site. It consists of a left arm of homology of 1.4 kb (SpH-BspEI), the loxP-flanked PGK-tkneo cassette (Schwartz et al., 1991) and a right arm of homology, encompassing the H19 gene, of 9.0 kb (BamHI-Xbal). The vector was linearised by NotI digestion.

**Targeting in ES cells**

Transformation of ES cells was by electroporation with linearised targeting construct. Optimal transformation was achieved using 8×10⁶ R1 ES cells (Nagy et al., 1993) and 90 μg of targeting vector DNA at 250 V, 500 μF (Biorad Gene Pulser apparatus). ES cells were grown on neomycin-resistant feeders and selected for neomycin resistance with G418 (250 μg/ml) for 8 days. The surviving clones were expanded and passaged in 24-well plates. One plate was frozen and the other harvested for genomic DNA preparation as previously described (Torres and Kuhn, 1997).

**Fig. 1.** Targeting of a 1.2 kb region upstream of H19. (a) Genomic structure of the mouse H19 locus. The H19, Igf2 and Rpl23 genes (white boxes) and their allele-specific transcription are shown (maternal chromosome is indicated above the line and the paternal chromosome below the line). An enlarged view of the H19 gene and surrounding sequence are also shown. Individual methylation-sensitive CfoI restriction enzyme sites in the H19 promoter and upstream regions are shown as unmethylated on the maternal chromosome (open circles) or methylated on the paternal chromosome (filled circles). Vertical arrows indicate DNAse I hypersensitivity sites. Four clustered maternal chromosome-specific hypersensitivity sites are located in the H19 upstream region. The two known H19 enhancers are located downstream of the H19 gene (large black circles). The 2 kb differentially methylated domain (DMD) and 1.2 kb region deleted in this study are also shown. B, BamHI; Bs, BspEI; H, HindIII; R, EcoRI. (b) Targeting construct used to replace a 1.2 kb region, between 1.7 and 2.9 kb upstream of H19. The transcriptional orientation of the PGK-tkneo (Neo) and H19 genes are indicated by horizontal arrows. The loxP sites flanking the Neo cassette are directionally represented by triangles. Dashed lines join the regions of homology shared by the endogenous locus and the targeting construct. Positions of restriction enzyme sites used and the size of fragments generated to detect a targeted replacement event are indicated. PCR primers for genotyping are also shown. P, PstI; H, HindIII; M, MluI; (MfeI); Bs, BspEI; B, BamHI; X, Xbal; Bg, BglII. (c) Southern hybridisation to confirm H19Neo allele was identified by amplification from RADKO3 and a primer hybridising to the 5’ end of the deleted 1177 bp region (H19-NotI=5'-GGCTCCCTGGATGTTCTCACATCC-3’) and at the 3’ end of the neomycin gene (RADKO1=5’-CTATGGCTTGTGAAGTTTC-3’) generating a 2.2 kb product. The wild-type H19 allele was identified by amplification from RADKO3 and a primer hybridising to the 3’ end of the deleted region (H19-NotI=5'-GGCTCCCTGGACATTGTCCAG-3’) generating a 1.9 kb product. The transcriptional start site. It consists of a left arm of homology of 1.4 kb (SpH-BspEI), the loxP-flanked PGK-tkneo cassette (Schwartz et al., 1991) and a right arm of homology, encompassing the H19 gene, of 9.0 kb (BamHI-Xbal). The vector was linearised by NotI digestion.

**cre-mediated deletion of the selection cassette**

The plasmid PGK-cre (a kind gift of Sam Aparicio) was used to linearise the targeting construct. Optimal transformation was achieved using 8×10⁶ R1 ES cells (Nagy et al., 1993) and 90 μg of targeting vector DNA at 250 V, 500 μF (Biorad Gene Pulser apparatus). ES cells were grown on neomycin-resistant feeders and selected for neomycin resistance with G418 (250 μg/ml) for 8 days. The surviving clones were expanded and passaged in 24-well plates. One plate was frozen and the other harvested for genomic DNA preparation as previously described (Torres and Kuhn, 1997).

PCR primers for genotyping are also shown. P, PstI; H, HindIII; M, MluI; (MfeI); Bs, BspEI; B, BamHI; X, Xbal; Bg, BglII. (c) Southern hybridisation to confirm H19Neo and hybridised to the external probes A or B, respectively. (d) Genotyping by Southern hybridisation to confirm H19Neo genotype after deletion of the PGK-tkneo cassette from wild-type (+/-) and heterozygous (+/-) ES cells. Genomic DNA was digested with PstI and hybridised to probe A, which detected a 5.8 kb wild-type fragment, a 4.6 kb targeted deletion (SilK) fragment and a 3.3 kb fragment from the targeted replacement allele (SAS).
transiently transfected correctly targeted H19SAS ES cells. Superoiled plasmid (30 μg) was electroporated into 5×10⁶ ES cells as described earlier. The transfected cells were cultured without selection. Colonies were picked on day 4 postelectroporation and expanded for characterisation as previously described. Genomic DNAs were probed with the 5′ external probe A (HindIII fragment) on a Southern blot, to identify cre-mediated recombination between theloxP sites flanking the selection cassette (designated H19SILK).

**Derivation and genotyping of targeted mice**

Mutant ES cells were used to generate chimeras by aggregation (Wood et al., 1993). MF1 morulae were co-cultured with ES cells, and Mutant ES cells were used to generate chimeras by aggregation. Derivation and genotyping of targeted mice.

**Plasmid (30 μmloxP site (RADKO11=5′-GTCATGGCTTCATAGGGCCAG-3′ and RADKO10=5′-CTCTGGGCTTAAAGCTAAG-3′) annealing at 58°C and generating a 0.6 kb product, on 1% agarose gel, transferred to a Hybond N+ membrane and hybridised as described (Boggs and Chinault, 1997). Biotinylated probes were described (Boggs and Chinault, 1997). Cosmid clones were used to generate sense and antisense probes by in vitro transcription using the digoxigenin RNA labelling kit (Boehringer Mannheim). Counterstaining was with biotinylated goat anti-avidin antibody (Vector Laboratories) and a final layer of Alexa488 anti-rabbit antibody (Molecular Probes). Images were obtained using a Zeiss Axiophot epifluorescence microscope and SmartCapture VP (Vysis) digital imaging software.

**RESULTS**

**Targeting the H19 upstream region**

The targeting construct (Fig. 1 and Methods) was used to create a deletion between −1.7 kb and −2.9 kb relative to the H19 transcriptional start site by homologous recombination. The H19 gene and the region immediately 5′ to the gene remained intact, including part of the differentially methylated region at the promoter (Bartolomei et al., 1993; Ferguson-Smith et al., 1993) and a G-rich repeat containing region (Tremblay et al., 1997). A region 5′ to the deletion was also preserved, which forms part of the upstream DMD (Tremblay et al., 1997; Fig. 1a). A correct targeting event replaced the 1.2 kb region with a 2.9 kbloxP-flanked selection cassette (Schwartz et al., 1991; H19SILK locus) which was subsequently deleted in ES cells by transient expression of cre recombinase (Fig. 1). The resulting deletion, termed H19SILK, was transmitted into 129/Sv mice.

**RNA and expression analysis**

RNA was prepared from embryos at day 13.5 of gestation or from neonatal tissues and allele-specific RT-PCR analysis performed as previously described (Ainscough et al., 1997). PCRs were performed for 30 cycles. 25 μl of each reaction was digested to completion withMspI, the fragments separated on a 2% agarose gel, transferred to nylon and hybridised as described earlier.

**In situ hybridisation**

Embryos at day 13.5 of gestation were wax embedded, sectioned at 10 μm, fixed in 4% paraformaldehyde in PBS for 30 minutes and subjected to ISH as described (Wilkinson and Nieto, 1993). A 2 kb mouse H19 (Poirier et al., 1991) and a 139 bp mouse Igf2 exon 4 cDNA clone were used to generate sense and antisense probes by in vitro transcription using the digoxigenin RNA labelling kit (Boehringer Mannheim). Detection of the signal was with the BM Purple substrate (Boehringer Mannheim). Counterstaining was with 0.5% Eosin.

**DNA and methylation analysis**

DNA was prepared, using standard procedures, from: (a) decapitated embryos at day 13.5 of gestation or from neonatal tissues or adult tissues for methylation analysis, (b) embryonic or neonatal heads for genotyping by Southern analysis and (c) embryonic or neonatal limbs for rapid PCR genotyping. For methylation analysis, (b) embryonic or neonatal heads for embryos at day 13.5 of gestation, day 3 neonatal tissues or adult DNA was prepared, using standard procedures, from: (a) decapitated tissues for methylation analysis, (b) embryonic or neonatal heads for embryos at day 13.5 of gestation, day 3 neonatal tissues or adult DNA and methylation analysis.

**Fig. 2. Expression of the H19SILK allele after paternal and maternal inheritance by RT-PCR. (a) An MspI polymorphism in exon 1 of H19 between 129/Sv and BALB/c strains was used to analyse gene expression. The H19SILK allele was from the 129/Sv strain (440 bp band), while the wild-type H19 allele was from BALB/c, which has the additional MspI site (321 bp band). Genotypes are indicated with the maternal allele listed first. RT-PCR analysis was performed on day 13.5 embryos and the probe used for Southern analysis is indicated by thick black bar. (b) Paternal transmission of the H19SILK allele. Heterozygous embryos (+/−) exhibited bi-allelic H19 expression. MspI digested reaction products were hybridised with the 321 bp probe shown in A. Wild-type littermates (+/+) only expressed the maternally inherited allele. Control lanes contain genomic DNA.
To analyse allele-specific expression of H19, we first used a MspI polymorphism in exon 1 that detects a 321bp BALB/c-specific fragment, which was not present in the H19SilK allele on the 129/Sv genetic background (Fig. 2a; Ainscough et al., 1997). The expression of the H19SilK paternal allele was therefore examined in (H19SilK × BALB/c) embryos. Compared to the wild-type controls (+/+) that expressed only the maternally inherited (BALB/c) allele of H19, expression of both parental alleles was detected in the embryos with a mutant locus (+/−; Fig. 2b). Although not strictly quantitative, Southern analysis combined with phosphorimager densitometry suggested that the paternally inherited H19SilK allele was expressed at a level of approximately 50% of the wild-type maternally inherited allele (Fig. 2b). Since the 321 bp probe used was internal to both of the allele-specific fragments, it should hybridise to each with equal affinity, which provides a reasonable measure of the relative amounts of each transcript. It was therefore possible that activation of the mutant paternal allele was either partial or restricted to a subset of cells (see below).

Maternal inheritance of the H19SilK deletion does not disrupt H19 expression

The reciprocal mating (Balb/c × H19SilK) was used to examine H19 expression after maternal inheritance of the targeted deletion allele using RT-PCR analysis on day 13.5 embryos. Expression was only detected from the maternal allele in both mutant (−/+ or −/−) and wild-type (+/+ or +/−) embryos (data not shown). This observation was confirmed by in situ hybridisation, which revealed a pattern of expression indistinguishable from a wild-type maternal locus allele (Fig. 3a,b).

Paternal H19 reactivation is restricted

To gain further insight into the nature of the derepression of the paternal H19SilK locus, we also used in situ hybridisation analysis in embryos carrying a null H19 allele from H19A3 females (Ripoche et al., 1997). As expected, almost no expression was detected from the wild-type paternal H19 locus (Fig. 3c), except in some embryos where expression was detected from a small subset of cells in the liver, as previously described (Jouvenot et al., 1999). By contrast, paternal inheritance of the H19SilK locus showed widespread, but not full, H19 expression (Fig. 3d). Detailed examination of this restricted reactivated expression revealed that, in many tissues, H19 was only expressed in a subset of cells, when compared to the normal expression pattern of the wild-type or H19SilK maternally inherited alleles.

In the gut, for example, H19 is normally expressed in both
the endodermally derived gastric epithelium and the mesodermally derived smooth muscle cells (Fig. 4a). However, the paternal \( H19^{SilK} \) allele exhibited strong expression in the epithelial cells only (Fig. 4b). Similarly, in the stomach, there was no expression from the paternal \( H19^{SilK} \) allele in the smooth muscle (Fig. 4c,d). Restricted expression was also observed in the epithelial cells of bronchi in the lung, but not in the surrounding mesenchymal cells (Fig. 4e,f). Endoderm-derived epithelial cell expression was also seen in the mesonephric tubules in the developing kidney, in the oesophagus and in the olfactory cavity (data not shown). Subsets of cells within the liver, tongue and the developing oral cavity also all showed expression. \( H19 \) appeared to be active in the muscle cells of the tongue, but absent from the interspersed adipose and neural tissues and Meckel’s cartilage in the developing lower jaw (Fig. 4h). High-level expression was also detected in the sclerotome. This expression pattern was consistently observed in all embryos without evidence of variegated expression. These observations revealed that the mutant \( H19^{SilK} \) locus was expressed in specific cell types and tissues. The observations argue against an equal overall lower level reactivation in all tissues. Furthermore, the restricted reactivated expression pattern is similar to the expression conferred by the two previously characterised enhancers downstream of \( H19 \) (Leighton et al., 1995b; Brenton et al., 1999; see Discussion).

### The effect of the \( H19^{SilK} \) deletion on growth and \( Igf2 \) expression

It has been shown previously that expression of the closely associated and reciprocally imprinted \( Igf2 \) gene is virtually identical to \( H19 \) as both genes share the same enhancers (Leighton et al., 1995b; Webber et al., 1998) and that targeted deletions at the \( H19/Igf2 \) domain can have an effect on the growth of mice (Leighton et al., 1995a,b; Ripoche et al., 1997; Thorvaldsen et al., 1998). We therefore examined the effect of the \( H19^{SilK} \) deletion on growth and \( Igf2 \) expression. A study of the mass of animals carrying the silencer deletion allele, revealed a weight phenotype on the inbred 129/Sv genetic background. Mice inheriting the mutant allele were weighed 2 days after birth and then approximately every 30 days up to 3 months of age. When compared to wild-type littersmates, mice inheriting the mutation maternally were on average 12% heavier (\( \pm \text{s.e.m.} \) 1.26%, \( n=44, \ P<0.003 \)) while mice inheriting the mutant allele paternally were 12% smaller (\( \pm \text{s.e.m.} \) 2.09%, \( n=90, \ P<0.05 \)).

In order to determine whether this growth phenotype was a result of changes in \( Igf2 \) expression, we examined \( Igf2 \) expression from the \( H19^{SilK} \) locus by in situ hybridisation and northern analysis in day 13.5 embryos, using a probe specific to exon 4 of \( Igf2 \). To ensure that we only detected expression from the \( Igf2 \) gene in \( cis \) to the \( H19^{SilK} \) allele, heterozygous \( H19^{SilK} \) mice were crossed to \( Igf2^{+/+} \) mice (DeChiara et al., 1990). There was no detectable effect on \( Igf2 \) expression in \( cis \) to the \( H19^{SilK} \) allele after maternal or paternal transmission (data not shown). The phenotypic growth effect that we observe could be a result of a very minor, and hence undetectable, modulation of \( Igf2 \) levels. Indeed, in one targeted deletion of the \( H19 \) gene, an increase in \( Igf2 \) levels of only 17% specifically in skeletal muscle was associated with an overgrowth phenotype (Schmidt et al., 1999). It is, however, of interest to note that in other models in which changes in \( Igf2 \) levels are associated with growth phenotypes, \( H19 \) transcript levels are also altered (Jones et al., 1998; Leighton et al., 1995a; Ripoche et al., 1997; Thorvaldsen et al., 1998). Our data may therefore raise the possibility of a functional role for the \( H19 \) RNA in an unknown growth pathway.

**\( H19^{SilK} \) deletion does not affect differential methylation**

One of the key aspects concerning imprinting is how the imprint is initiated and maintained. We therefore examined
Fig. 5. Methylation analysis of the maternally and paternally inherited \( H19_{\text{SilK}} \) allele. (a) The \( H19 \) locus with the location of \( CfoI \) sites (open circles) known to show differential methylation between parental alleles. \( H19 \) transcription start site is represented by a horizontal arrow and the two probes used are indicated by bold lines (RBs and RX). The positions of restriction enzyme sites used to generate probes are indicated. The dashed line represents the deleted region in the \( H19_{\text{SilK}} \) allele and the black triangle the remaining \( loxP \) site. Restriction endonuclease recognition sites are abbreviated as follows: R, \( EcoRI \); Bs, \( BspEI \); B, \( BamHI \); X, \( XbaI \). Scale bar indicates 1 kb. (b) Methylation state of the maternally (Mat) or paternally (Pat) inherited \( H19_{\text{SilK}} \) allele, and in wild-type (WT) day 13.5 embryos in the upstream DMD region (RBs probe). Methylation analysis in neonatal or adult tissues were; liver (L), brain (B), heart (H), skeletal muscle (M) and sperm (S). The DNAs were digested with \( EcoRI \) (−) or \( EcoRI \) and the methylation sensitive enzyme \( CfoI \) (+). Two independent samples are shown for digests on whole embryos and sperm. The wild-type allele generates a methylated band at 3781 bp and the methylated \( H19_{\text{SilK}} \) allele a band at 2604 bp. A completely unmethylated state in this region would allow digestion by \( CfoI \) to produce bands at 386 bp, 376 bp and 180 bp in size. (c) Methylation analysis of the promoter region of \( H19 \) (RX probe). The same size bands as in B are generated for methylated alleles. A completely unmethylated state would produce \( CfoI \) generated bands of 959 bp and 79 bp (not detectable on blot). Quantitation of single bands was performed using Scion Image software and the percentage of DNA in each lane which is found in the demethylated band of 959 bp compared to the methylated \( H19_{\text{SilK}} \) allele band is indicated. For maternal inheritance a fully demethylated allele would therefore give a value of 100% and for paternal inheritance a fully methylated allele gives a value of 50%. (d) Bisulphite genomic sequencing analysis of upstream DMD region. The region analysed in detail in this study (grey box) extends from −4000 to −3440 relative to the \( H19 \) transcriptional start site (horizontal arrow). CpG methylation patterns of individual chromosomes derived from sequencing are shown as single lines. Methylated and unmethylated CpG dinucleotides are represented by filled and open circles, respectively. CpGs that were not read unambiguously (dashed lines) were omitted from the figure. Methylation patterns of chromosomes from wild-type (WT), paternal \( H19_{\text{SilK}} \) heterozygotes (Pat) and maternal \( H19_{\text{SilK}} \) heterozygotes (Mat) are shown.
whether the 1.2 kb deletion affected this process. It has been demonstrated that the CpG residues in the promoter region and upstream of the H19 transcription unit are differentially methylated on parental chromosomes throughout development (Bartolomei et al., 1993; Ferguson-Smith et al., 1993; Tremblay et al., 1995, 1997). The upstream region (termed DMD) extends from –2 to –4 kb upstream of H19. Therefore, in the H19SilK locus, some 1.1 kb of the DMD remains (Fig. 1a). We analysed these regions using the methylation sensitive restriction enzyme CfoI (Fig. 5a). We found that the methylation pattern of the paternal H19SilK locus was in fact similar to the wild-type locus, both in tissues where H19 expression is high (liver) as well as in tissues where the expression is confined to very few cells (brain; Fig. 5b,c). Maternal inheritance of the H19SilK allele revealed a consistent and appropriate hypomethylated epigenotype, which was comparable to the wild-type maternal locus (Fig. 5b,c).

To confirm the ability of the hypomethylated H19SilK allele to undergo re-methylation in the male germline, we examined adult male heterozygotes that had inherited the deletion through the female germline. As expected, the H19SilK allele in this animal was predominantly hypomethylated in all somatic tissues examined (Fig. 5b,c). However, in mature sperm, both the upstream DMD and promoter-proximal regions were methylated (Fig. 5b,c). The combined evidence showed that the mutant locus was therefore capable of an appropriate epigenetic switch as demonstrated by its hypomethylated state after transmission through the female germline and the acquisition of methylation in the male germline.

The germline-specific changes in methylation were confirmed by a study of differential methylation on individual chromosomes by bisulphite genomic sequencing of a 560 bp region of the remaining DMD (Fig. 5d). This region harbours 16 CpG dinucleotides that have been previously shown to exhibit extensive differential methylation (Olek and Walter, 1997; Tremblay et al., 1997). As expected, individual chromosomes from this region were either predominantly hypermethylated or hypomethylated in wild-type embryos (Fig. 5d). No variation from this pattern was detected on chromosomes from embryos inheriting the paternal H19SilK or maternal H19SilK locus (Fig. 5d), confirming that the H19SilK allele was methylated after patriarchal transmission and demethylated after maternal transmission.

**Asynchronous DNA replication is not disrupted at the H19SilK locus**

Another characteristic epigenetic feature of imprinted domains is asynchronous DNA replication, with early replication of the paternal chromosome (Kitsberg et al., 1993; Simon et al., 1999). At the wild-type H19 locus, a cosmid covering the H19 gene and upstream region detects an asynchronous pattern of DNA replication between the two chromosomes (Greally et al., 1998; Fig. 6a). Conversely, a cosmid that maps to the Rpl23 gene approximately 30 kb downstream of H19 and outside of the imprint domain, detects a synchronous DNA replication pattern (Fig. 6a). The asynchronous pattern of replication is disrupted when the entire 10 kb region upstream of H19 is deleted (Greally et al., 1998). In contrast, we found that our 1.2 kb deletion did not affect the asynchronous replication pattern at H19 after paternal or maternal inheritance of the H19SilK locus (Fig. 6b), demonstrating that the epigenetic mark regulating asynchronous replication was unaffected.

**DISCUSSION**

We have addressed the function of a 1.2 kb region located between 1.7 kb and 2.9 kb upstream of the imprinted H19 gene, by targeted deletion at the endogenous murine locus. This element was originally identified as a silencer in *Drosophila* (Lyko et al., 1997), but its function in imprinting was unknown. Using extensive in situ analysis at the level of single cells, we demonstrated that its deletion in the mouse results in a tissue-specific relaxation of silencing after paternal transmission, but it has no effect after maternal inheritance. Strikingly, this loss of H19 silencing occurred without a detectable change in the methylation status or asynchronous DNA replication pattern of the locus. This suggests that the imprinted epigenotype of H19 may be
initiated by a different cis-acting element to that responsible for transcriptional silencing of the gene.

**Identification of a cis-acting silencer at H19**

The deletion of a 1.2 kb element from the region upstream of the imprinted H19 gene resulted in reactivation of the normally silent paternally inherited H19 allele. One possibility was that reactivation of the paternal H19 gene occurs in all tissues to the same extent. However, close examination revealed that the loss of silencing appears to be restricted to specific cells and tissues. Furthermore, the pattern of expression resembles that conferred by the two known enhancers downstream of H19 (Ainscough et al., 1997; Brenton et al., 1999; Leighton et al., 1995b), as well as by some unknown enhancers that are also present on a 130 kb YAC (Ainscough et al., 2000). The two H19 enhancers downstream of H19 were previously shown to have an open chromatin configuration regardless of their parental origin, indicating that they have the potential to function on both parental chromosomes (Bartolomei et al., 1993). In the absence of the silencer, these enhancers are apparently able to activate expression of the paternal H19 allele. The apparent tissue-specific effect of the silencer and its potential interaction with only a subset of enhancers is intriguing. The identification of a mesoderm-specific silencer at the Igf2 gene suggests that tissue-specific expression of imprinted genes may be regulated by specific interactions between silencers and enhancers (M. Constancia and W. Reik, personal communication). The mechanism by which such interactions could occur is unknown at present.

**Loss of silencing is independent of DNA methylation**

DNA methylation is closely associated with silencing of the paternally inherited wild-type H19 allele. Examination of the methylation state of the H19silK allele after paternal inheritance revealed that methylation remained unchanged both upstream of the deletion and at the promoter-proximal region of H19, even in tissues where H19 was reactivated. The 1.2 kb deleted region constitutes a significant part of the H19 upstream DMD. However, its deletion apparently had no effect on many of the key functions of this region. Most striking amongst these was the ability of the mutant locus to undergo an appropriate epigenetic switch in the germline as demonstrated by differential DNA methylation, indicating that the signal for both the initiation and propagation of the imprint was unaffected. This observation dissociates the commonly held view of an intrinsic link between differential methylation and transcriptional regulation at imprinted loci. There is a precedent for allele-specific expression of an imprinted gene independent of methylation at the mouse Mash2 locus (Caspy et al., 1998), suggesting that differences in DNA methylation at some imprinted loci may not be essential to the imprinting process.

Another characteristic epigenetic mark established in the germline is the asynchronous replication of imprinted domains, including the H19 region (Kitsberg et al., 1993; Simon et al., 1999). The sequences upstream of H19 are essential for the maintenance of this replication pattern (Greally et al., 1998). However, the 1.2 kb deletion at the H19silK locus did not disrupt this asynchronous replication, suggesting that the critical sequences for this epigenetic modification are also located elsewhere in the region. It would be of interest to examine the effect of the larger deletion of the DMD (Thorvaldsen et al., 1998) on the DNA replication pattern at the H19 locus, which may help to pinpoint the sequences required for asynchronous replication of the region.

**The fate of the insulator upstream of H19**

The weight phenotype that we observed in mice carrying the 1.2 kb silencer deletion possibly suggests an effect on the Igf2 gene. However, despite examining expression using a number of different methods, we could not detect a change in Igf2 expression. It is possible that a small, but undetectable modulation in Igf2 levels was sufficient to induce the growth phenotype, as has been shown previously (Schmidt et al., 1999). This is particularly likely in the inbred 129/Sv background mice that we have used here. However, the absence of a marked and detectable effect on Igf2 suggests that the proposed insulator or boundary element upstream of the H19 gene is unaffected in the H19silK allele (Greally et al., 1998; Khosla et al., 1999; Webber et al., 1998). As a result, there is no large-scale activation of the Igf2 gene on the maternal chromosome by the enhancers downstream of H19. There are nuclease hypersensitivity sites within the DMD on the maternal chromosome of unknown function (Hark and Tilghman, 1998; Khosla et al., 1999; Fig. 1a). The 1.2 kb deletion leaves 1.1 kb of the DMD intact (Fig. 1a), including some hypersensitive sites that may constitute the insulator. Indeed, recent studies have identified a number of CTCF protein binding sites that map close to some of these hypersensitivity sites, suggesting that at the endogenous locus these sequences are important for the insulator function (Szabo et al., 2000; Bell and Felsenfeld, 2000; Hark et al., 2000).

**Evidence for multifunctional cis elements upstream of H19**

The 5’ 1.1 kb region of the DMD may also be required for at least some of the critical imprinting functions, such as the initiation and/or propagation of the imprint and the asynchronous replication of the region. This notion is supported by comparing the 1.6 kb deletion of the DMD previously reported (Thorvaldsen et al., 1998), which apparently resulted in a loss of the appropriate epigenetic switch of the locus in the germline. This larger deletion also had a very marked effect on Igf2 expression, suggesting that the insulator may also have been deleted. The precise role of the region upstream of the 1.2 kb silencer element needs to be addressed by deleting it from the endogenous locus. In this way, it should be possible to dissect the multiple functions of the DMD region. We have also recently identified three transcripts at –10.5, –8.5 and –3.0 kb upstream of the H19 gene, but the role of these sequences in the imprinting mechanism is as yet unknown.

There is evidence suggesting that control elements are often complex and can display both functional redundancy as well as divergent functions within control regions (Zhou and Levine, 1999). A multifunctional role for the upstream region of H19, including a silencer, imprint initiation and insulator, is very reminiscent of regulatory systems in other organisms, such as the mating type locus silencing in yeast (Donze et al., 1999; Fourel et al., 1999) and Hox gene regulation in...
Drosophila (Hagstrom et al., 1997; Mihaly et al., 1997). We are particularly interested in the mechanism by which silencer elements from imprinting control regions also function as silencers in Drosophila. Such an activity has not only been detected for the 1.2 kb H19 upstream region, but also the imprinting centre from the SNRPN gene (Lyko et al., 1998), suggesting an evolutionary conserved epigenetic silencing mechanism in flies and mice.

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