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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 bi-directional 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 *Bsp*EI-*Bam*HI 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*I-*Bsp*EI), the *loxP*-flanked *PGK-tkneo* cassette (Schwartz et al., 1991) and a right arm of homology, encompassing the *H19* gene, of 9.0 kb (*Bam*HI-*Xba*I). The vector was linearised by *Not*I digestion.

Targeting in ES cells

Transformation of ES cells was by electroporation with linearised targeting construct. Optimal transformation was achieved using 8×10^6 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). Homologous recombination was analysed in resistant ES clones initially using a multiplex PCR reaction, with annealing at 60°C. The homologous recombination event was detected with primers 5′ to the left arm of homology (RADKO3=5′-GGCTCCCCTGGATGTTTCACTTCC-3′) and at the 3′ end of the neomycin gene (RADKO1=5′-CTATCGCCTTCTTGACGAGTTC-TTC-3′) generating a 2.2 kb product. The wild-type *H19* allele was identified by amplification from RADKO3 and a primer hybridising to the 5′ end of the deleted 1177 bp region (H19-*Not*I=5′-GCCCTTGGACATTGTCATGG-3′) generating a 1.9 kb product. Positive clones (designated $H19^{SAS}$) were further screened using a 5′ external probe (probe A – 473bp *Hin*dIII fragment) and a 3′ external probe (probe B – 800 bp *Xba*I fragment) by Southern blot analyses (Fig. 1), as previously described (Ainscough et al., 1997). Hybridisation signals were analysed using a Fuji PhosphorImager or on Kodak XAR film.

cre-mediated deletion of the selection cassette

The plasmid *PGK-cre* (a kind gift of Sam Aparicio) was used to

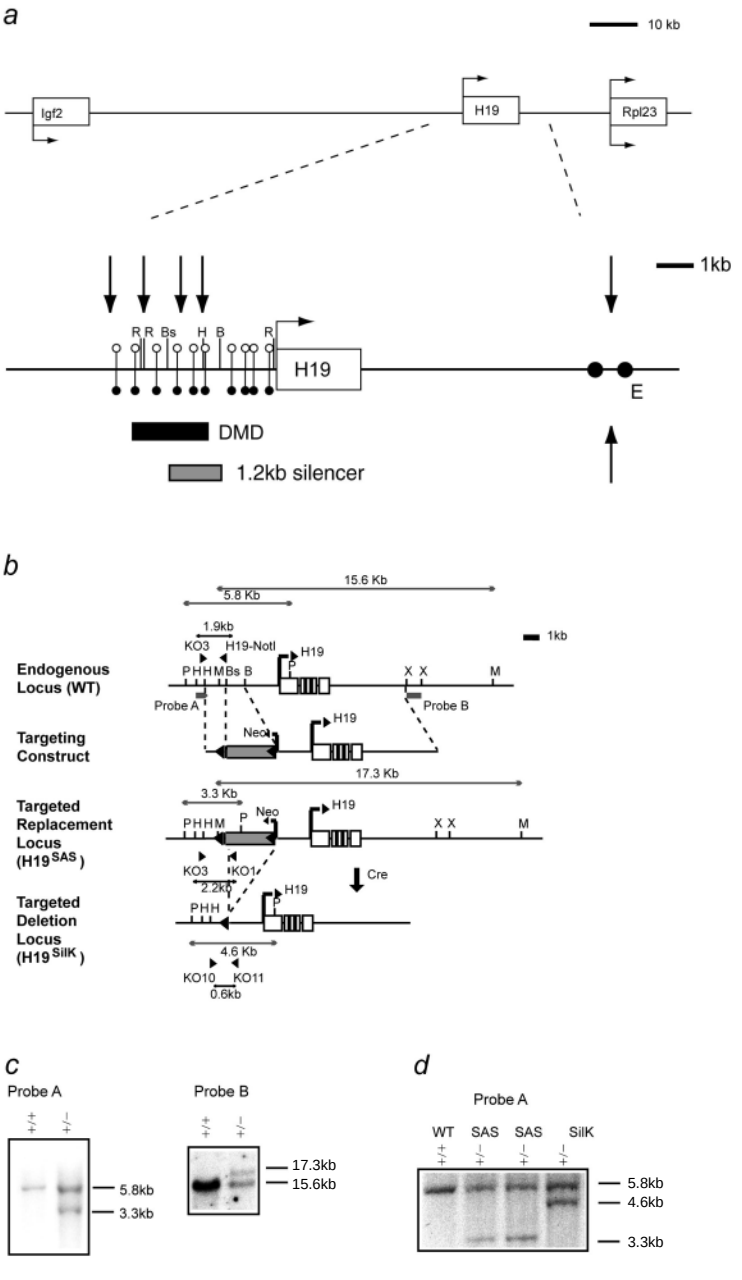

Fig. 1. Targeting of a 1.2 kb region upstream of *H19*. (a) Genomic structure of the mouse *H19* locus. The *H19*, *Igf2* and *Rpl23* genes (white boxes) and their allele-specific transcription are shown (maternal chromosome is indicated above the line and the paternal chromosome below the line). An enlarged view of the *H19* gene and surrounding sequence are also shown. Individual methylation-sensitive *Cfo*I 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 DNaseI 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, *Bam*HI; Bs, *Bsp*EI; H, *Hin*dIII; R, *Eco*RI. (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, *Pst*I; H, *Hin*dIII; M, *Mun*I; *(Mfe*I*)*; Bs, *Bsp*EI; B, *Bam*HI; X, *Xba*I; Bg, *Bgl*II. (c) Southern hybridisation to confirm $H19^{SAS}$ genotype (+/–). Genomic DNA was digested with *Pst*I or *Mun*I and hybridised to the external probes A or B, respectively. (d) Genotyping by Southern hybridisation to confirm $H19^{SilK}$ genotype after deletion of the *PGK-tkneo* cassette from wild-type (+/+) and heterozygous (+/–) ES cells. Genomic DNA was digested with *Pst*I 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 transfect correctly targeted $H19^{SAS}$ ES cells. Supercoiled plasmid (30 μg) was electroporated into 5×10^6 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 (*Hin*dIII fragment) on a Southern blot, to identify *cre*-mediated recombination between the *loxP* sites flanking the selection cassette (designated $H19^{SilK}$).

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 embryos transferred to pseudopregnant (C57BL/6 × CBA/Ca) F_1 females. The chimeras derived were 100% 129/Sv strain by coat colour and GPI analysis and were propagated on the 129/Sv inbred background. Typing of the $H19^{SilK}$ mutant mice was by PCR, using primers that amplified across the remaining *loxP* site (RADKO11=5′-GTCATGGGCTTCATGAGGCCAG-3′ and RADKO10=5′-TCCTG-CTTCACTTCAAACTAAG-3′) annealing at 58°C and generating a 0.6 kb product, on 1 μl crude lysates from neonatal toe DNA obtained by incubation in 50 μl Tris-HCl (50 mM, pH 8), NaCl (2 mM), EDTA (1 mM), 0.5% Tween-20, 0.5% NP40 and Proteinase K (0.4 μg/μl) for 1-3 hours at 65°C, or by Southern analysis (Fig. 1d).

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 with *Msp*I, the fragments separated on a 2% agarose gel, transferred to Hybond N+ 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, day 3 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, 20 μg DNA was digested to completion with appropriate restriction enzymes, separated on a 1.0% agarose gel, transferred to a Hybond N+ membrane and hybridised with the appropriate probe. Bisulphite genomic sequencing was performed as described previously (Olek and Walter, 1997).

Fluorescence in situ hybridisation

FISH was performed on cultured splenocytes from age-matched adult mice as previously described (Boggs and Chinault, 1997). Cosmid DNA was labelled by nick translation with biotin (cAH) or digoxigenin (c17.2) and hybridisation performed as previously described (Boggs and Chinault, 1997). Biotinylated probes were detected with Neutravidin-Alexa™ 488 (Molecular Probes), amplified with biotinylated goat anti-avidin antibody (Vector Laboratories) and a further layer of Neutravidin-Alexa™ 488. Digoxigenin-labelled probes were detected with sheep anti-digoxigenin antibody (Boehringer Mannheim), followed by rabbit Texas Red-conjugated anti-sheep antibody (Vector Laboratories) and a final layer of Alexa™ 468 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 kb *loxP*-flanked selection cassette (Schwartz et al., 1991; $H19^{SAS}$ locus) which was subsequently deleted in ES cells by transient expression of *cre* recombinase (Fig. 1). The resulting deletion, termed $H19^{SilK}$, was transmitted into 129/Sv mice.

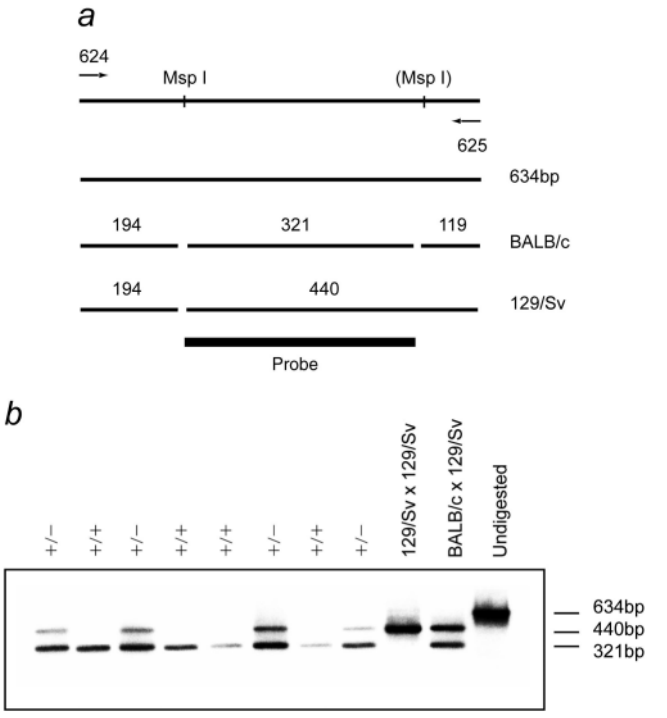

Fig. 2. Expression of the $H19^{SilK}$ allele after paternal and maternal inheritance by RT-PCR. **(**a) An *Msp*I polymorphism in exon 1 of *H19* between 129/Sv and BALB/c strains was used to analyse gene expression. The $H19^{SilK}$ allele was from the 129/Sv strain (440 bp band), while the wild-type *H19* allele was from BALB/c, which has the additional *Msp*I 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 $H19^{SilK}$ allele. Heterozygous embryos (+/–) exhibited bi-allelic *H19* expression. *Msp*I 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.

H19^{SilK} deletion reactivates *H19* expression after paternal inheritance

To analyse allele-specific expression of *H19*, we first used a *Msp*I polymorphism in exon 1 that detects a 321bp BALB/c-specific fragment, which was not present in the *H19*^{SilK} allele on the 129/Sv genetic background (Fig. 2a; Ainscough et al., 1997). The expression of the *H19*^{SilK} paternal allele was therefore examined in (*H19*^{SilK} × 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 *H19*^{SilK} 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 *H19*^{SilK} deletion does not disrupt *H19* expression

The reciprocal mating (Balb/c × *H19*^{SilK}) 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 (–/+) and wild-type (+/+) 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 *H19*^{SilK} locus, we also used in situ hybridisation analysis in embryos carrying a null *H19* allele from *H19*$^{\Delta 3}$ 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 *H19*^{SilK} 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 *H19*^{SilK} maternally inherited alleles.

In the gut, for example, *H19* is normally expressed in both

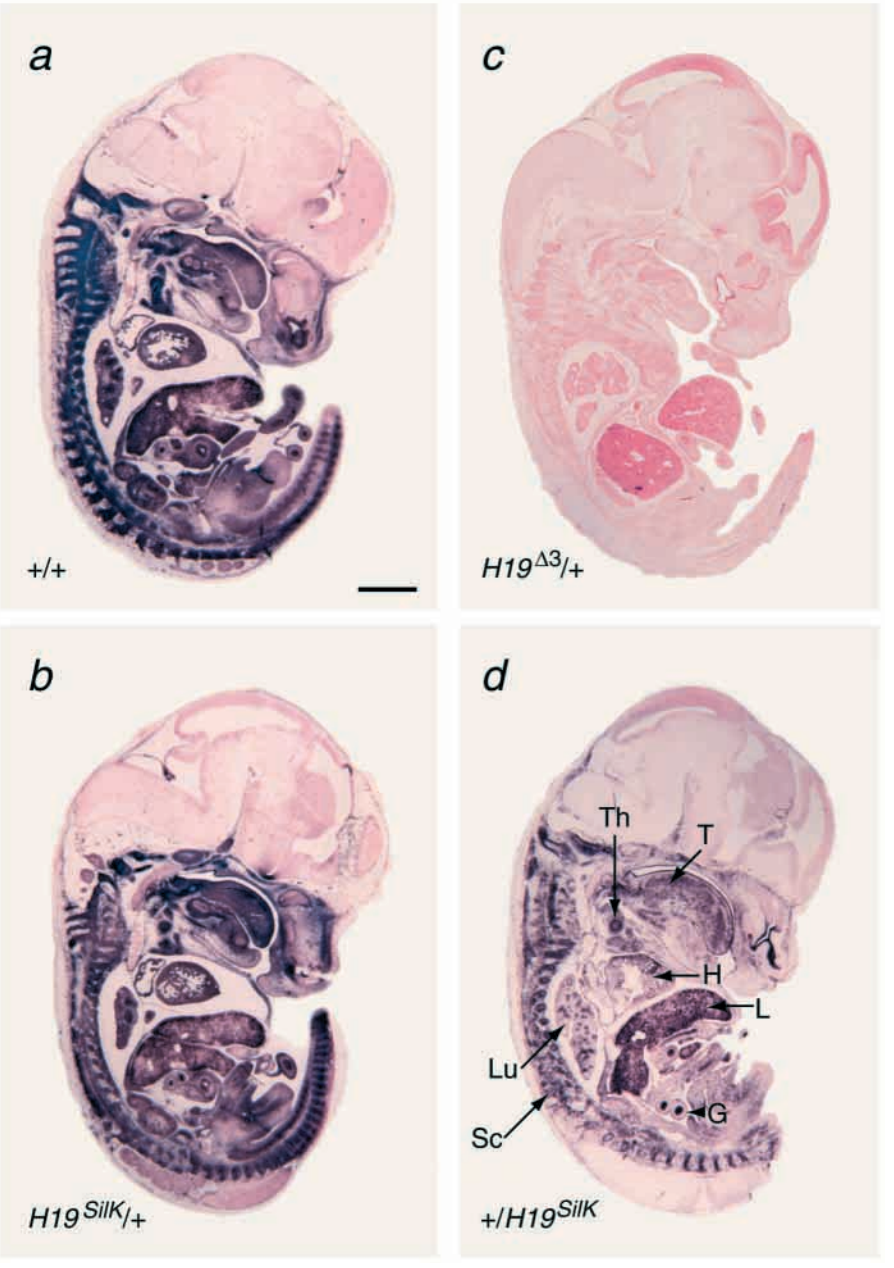

Fig. 3. Expression of mutant *H19* alleles in day 13.5 embryos. Expression of *H19* in sections of embryos at day 13.5 of gestation was examined by in situ hybridisation. (a) *H19* expression in wild-type (+/+) embryo. (b) *H19* Expression in embryos from the maternally inherited *H19*^{SilK} allele in heterozygous (*H19*^{SilK}/+) embryos; expression in these embryos is indistinguishable from the pattern in wild-type litter mates shown in a. (c) Expression from the *H19* null (*H19*$^{\Delta 3}$) allele inherited maternally (*H19*$^{\Delta 3}$/+); there is no detectable expression of H19. (d) Expression from the paternally inherited *H19*^{SilK} allele examined in embryos with a maternally inherited *H19*$^{\Delta 3}$ null allele (*H19*$^{\Delta 3}$/*H19*^{SilK}); the expression of the *H19*^{SilK} paternal allele is restricted to specific tissues, unlike the maternally inherited allele shown in b. G, gut; H, heart; L, liver; Lu, lung; Sc, Sclerotome; T, tongue; Th, thymus. Scale bar in a = 1 mm.

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 littermates, mice inheriting the mutation maternally were on average 12% heavier (±s.e.m. 1.26%, n=44, P<0.003) while mice inheriting the mutant allele paternally were 12% smaller (±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

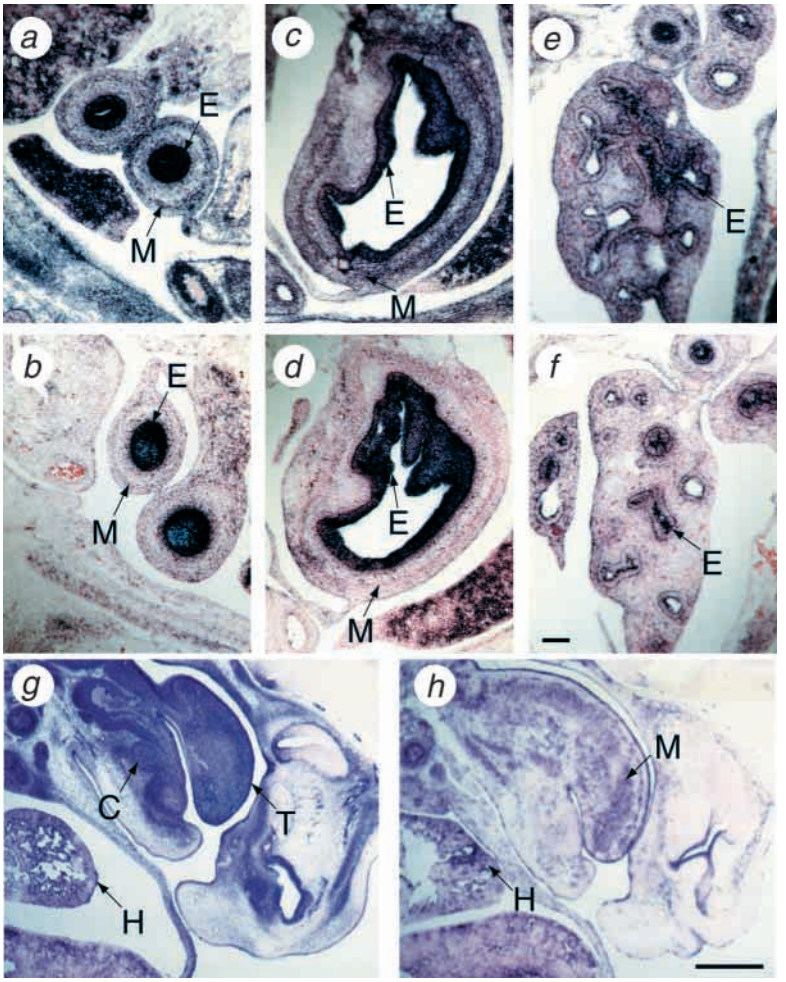

Fig. 4. Detailed expression pattern of the $H19^{SilK}$ maternal and paternal alleles. Expression from the maternally inherited $H19^{SilK}$ allele in ($H19^{SilK}$/+) heterozygous embryos (a,c,e,g); this expression is comparable to that from the wild-type maternal *H19* allele (see Fig. 3). Expression from the paternally inherited $H19^{SilK}$ allele in embryos with the maternally inherited $H19^{\Delta 3}$ null allele in ($H19^{\Delta 3}/H19^{SilK}$) heterozygous embryos (b,d,f,h). The panels show expression in the gut (a,b), stomach (c,d), lung (e,f) and tongue (g,h). C, Meckel's cartilage; E, epithelium; H, heart; M, muscle; T, tongue. Scale bar in f = 0.1 mm for a-f; in h = 1.0 mm for g,h.

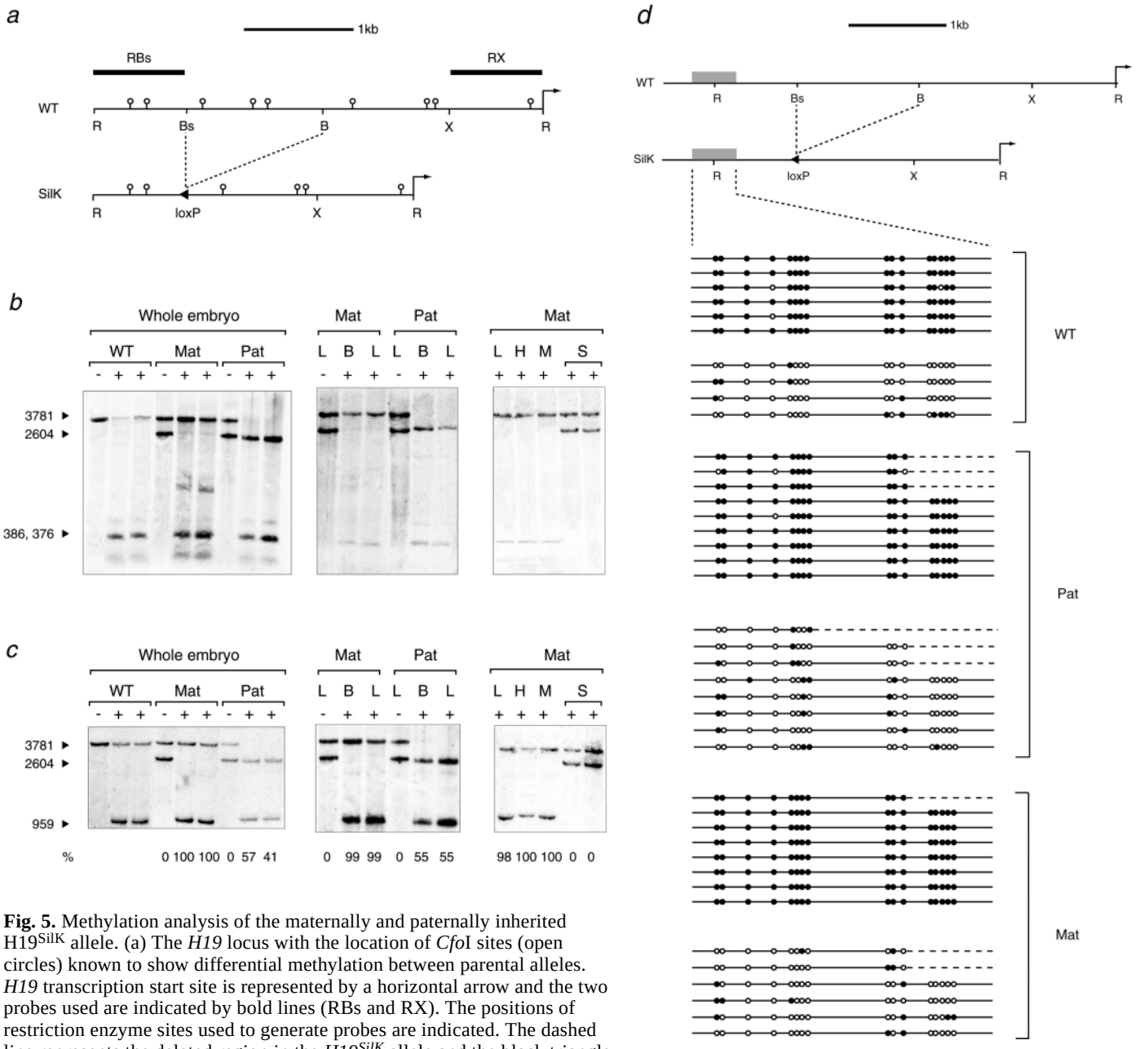

Fig. 5. Methylation analysis of the maternally and paternally inherited H19^{SilK} allele. (a) The *H19* locus with the location of *Cfo*I sites (open circles) known to show differential methylation between parental alleles. *H19* transcription start site is represented by a horizontal arrow and the 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^{SilK}* allele and the black triangle the remaining *loxP* site. Restriction endonuclease recognition sites are abbreviated as follows: R, *Eco*RI; Bs, *Bsp*EI; B, *Bam*HI; X, *Xba*I. Scale bar indicates 1 kb. (b) Methylation state of the maternally (Mat) or paternally (Pat) inherited *H19^{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 *Eco*RI (–) or *Eco*RI and the methylation sensitive enzyme *Cfo*I (+). 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^{SilK}* allele a band at 2604 bp. A completely unmethylated state in this region would allow digestion by *Cfo*I 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^{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^{SilK}* heterozygotes (Pat) and maternal *H19^{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 $H19^{SilK}$ locus, some 1.1 kb of the DMD remains (Fig. 1a). We analysed these regions using the methylation sensitive restriction enzyme *Cfo*I (Fig. 5a). We found that the methylation pattern of the paternal $H19^{SilK}$ 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 $H19^{SilK}$ 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 $H19^{SilK}$ 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 $H19^{SilK}$ 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 $H19^{SilK}$ or maternal $H19^{SilK}$ locus (Fig. 5d), confirming that the $H19^{SilK}$ allele was methylated after paternal transmission and demethylated after maternal transmission.

Asynchronous DNA replication is not disrupted at the $H19^{SilK}$ 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 imprinted 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

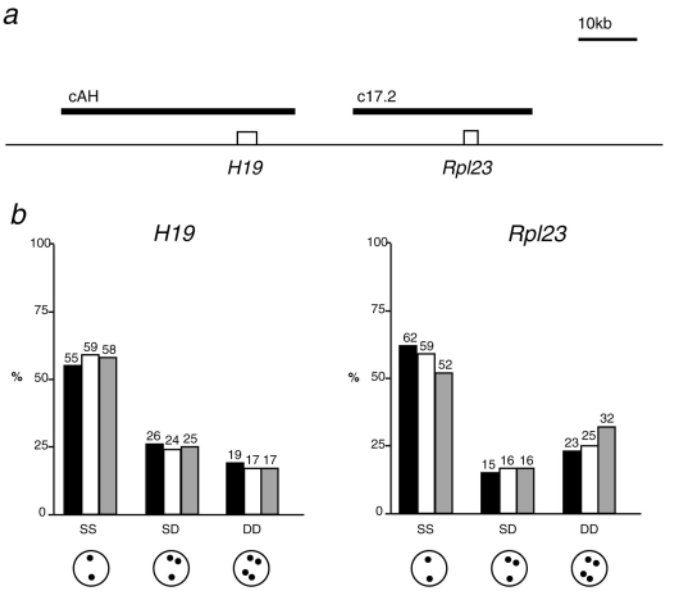

Fig. 6. DNA replication patterns at wild-type and mutant alleles. (a) Genomic map showing the location of cosmids, cAH and c17.2, used to analyse the timing of DNA replication at the *H19* and *Rpl23* genes, respectively. Scale bar indicates 10 kb. (b) The percentage of cells showing each pattern of hybridization, single/single (SS), single/double (SD) or double/double (DD), is illustrated for wild-type (black), paternal $H19^{SilK}$ heterozygous (white) and maternal $H19^{SilK}$ heterozygous (grey) cells, expressed as a percentage of BrdU-positive cells. The cAH cosmid upstream from *H19* detected asynchronous replication in wild-type and mutant cells, manifested by proportions of cells with single/double patterns of 24% or greater and cells with double/double patterns of less than 19%. This contrasts with the pattern for the downstream *Rpl23*-containing c17.2 cosmid which only detects a background rate of 15-16% single/double patterns and a proportion with double/double patterns of greater than 23% in both wild-type and mutant cells. This indicates that the transition in replication patterns between *H19* and *Rpl23* is not disrupted at the mutant $H19^{SilK}$ allele. Numbers of cells examined were; *H19*; WT (n=520), paternal $H19^{SilK}$ (n=287), maternal $H19^{SilK}$ (n=384). *Rpl23*; WT (n=260), paternal $H19^{SilK}$ (n=106), maternal $H19^{SilK}$ (n=110).

$H19^{SilK}$ 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 $H19^{SilK}$ 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 (Caspary 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 $H19^{SilK}$ 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 $H19^{SilK}$ 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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