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upstream of *H19* required to establish the methylation imprint at the *H19*

lective medium, cells were incubated for 8 days with G418 medium (200 μg/μl) to select for neomycin resistance. The 380 surviving colonies were picked and grown up in four 96-well plates. DNA was extracted and digested with AccIII and StuI to screen for correct integration of the 5′ and 3′ ends by Southern blotting as described below. The 5′ probe consists of a 2.8-kb PCR fragment (90437 to 93289 from AP03184) and the 1.3-kb AvaI-AvaI *H19* cDNA 3′ probe is previously described (15). The neomycin resistance cassette (*neo*) was removed by transiently transfecting Cre recombinase. Correctly excised clones were verified by digestion with StuI, injected into C57BL/6J blastocysts, and transferred into pseudopregnant C57BL/6J females. Male chimeras produced by these injections were paired with C57BL/6J females, and germ line pups (as evidenced by coat color) were screened for the targeted allele by using the StuI digest as described above and subsequently the following PCR assay. PCR amplification was performed with Roche Taq DNA polymerase and buffer with 2.25 mM (50 μl) of $MgCl_2$, using the primers ΔKf (ACACTTCCTCTGAACAAGG) and ΔKr (TA CAATGAGGGCAGTAAGC) and a program of 94°C for 2 min followed by 10 cycles consisting of 94°C for 15 s, 55°C for 30 s, and 68°C for 3 min, and then 20 cycles consisting of 94°C for 10 s, 55°C for 30 s, and 68°C for 3 min plus 20 extra s for each cycle at 68°C for 5 min.

[illegible]. Genomic DNA was extracted from ES cells or tissues as previously described (21). Twenty micrograms of DNA was digested with 65 U of each restriction enzyme, loaded onto a 0.8 to 1% agarose gel, transferred to Hybond N+ membrane (Amersham Pharmacia) under alkaline conditions, and hybridized with specific radiolabeled probes (8, 16).

[illegible]. Wet weights of placenta, whole fetuses, and newborn mice were recorded. Data were analyzed by means of two-way analyses of variance with litters and genotype as the two factors.

[illegible]. Total RNA was isolated from mouse tissues with the RNeasy mini kit (QIAGEN). For expression analysis by Northern blotting, 10 μg of RNA was separated in low-percentage formaldehyde gels and transferred to Hybond N+ membrane (Amersham Pharmacia) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Radiolabeling of probes and hybridization of filters were as described for DNA analysis. Hybridization was carried out with a 0.9-kb KpnI-BamHI probe specific for *Igf2*, a 1.3-kb AvaI-AvaI probe specific for *H19*, and a 0.25-kb HindIII-PstI probe specific for *Gapdh* as described by Feil et al. (15). Quantifications of expression levels were performed with a phosphorimager (Fujifilm FLA-3000 and AIDA software), and a *t* test was carried out with the Microsoft Excel statistical function. The reverse transcriptase PCR (RT-PCR) assays used to detect allele-specific expression of *Igf2* and *H19* were performed as described previously (12).

[illegible]. DNA was prepared from mouse tissues and sperm, extracted with phenol-chloroform, precipitated with 100% ethanol, and digested with BamHI. Digested DNA wasdigested
with BamHI.with BamHIrc]TJT5.3o3(was[(with)-3953(was)-3co 1237(prob-3co 1bTD[((lters)T

the Omiga sequence analysis package) showed positional conservation of the K repeat and some conservation of the sequence (Fig. 1a and c) but no conservation of the downstream G-rich repeat. The human CTCF-dependent boundary element contains additional repeat elements as previously described (39). There are also two smaller regions of conservation upstream of the repeat, one of which contains a retroviral long terminal repeat element. The K repeat consists of a 30-bp unit repeated 22 times in mice and a 31-bp unit repeated 19 times in humans. In pigs, the repeat consists of a 19-bp unit repeated 48 times, but there is no signi

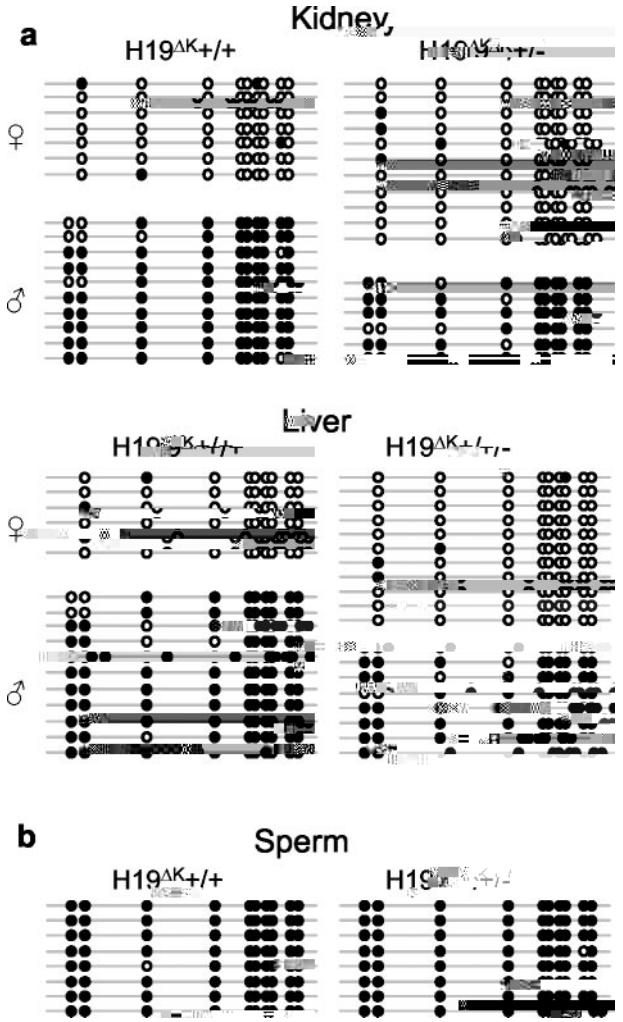

FIG. 4. Analysis of methylation levels at the *H19* DMR after paternal transmission of *H19*$^{\Delta K}$ deletion. (a) Heterozygous *H19*$^{\Delta K}$ males were mated to homozygous SD7 females. Methylation of the *H19* DMR was analyzed in tissues from newborn *H19K*

tant and wild-type embryos and newborns. In addition, no difference was observed in placental weight at E18 after paternal transmission of the deletion. Similarly, maternal transmission of the *H19*$^{\Delta K}$ deletion had no significant effect on growth (data not shown).

H19

*Igf2***.** Substantial changes in the levels of *Igf2* expression do not always result in comparable changes in fetal growth (9, 40). We therefore analyzed the expression levels of *Igf2* and *H19* by Northern blot after paternal transmission of the deletion (Fig. 3a). No significant differences were seen between mutant and wild-type levels in newborn tissues or E18 placenta. Allelic expression in the locus was also unchanged, with both wild-type and *H19*$^{\Delta K+/-}$ animals expressing *Igf2* from only the paternal chromosome (except for some regions of the brain) and *H19* only from the maternal chromosome (except for low levels of paternal expression in skeletal muscle and liver) (Fig. 3b). Again, no changes were observed after maternal transmission of the deleted allele (data not shown).

. Methylation at the *H19* DMR was analyzed in tissues from newborn mice by bisulfite sequencing (Fig. 4a) and Southern blot (data not shown) after paternal transmission of the *H19*$^{\Delta K}$ allele. No differences were seen in paternal or maternal methylation levels. Paternal allele-specific methylation levels remained high even at the 5′ end of the DMR, which lies only 590 bp downstream of the deletion. In addition, sperm DNA was also found to be highly methylated in *H19*$^{\Delta K+/-}$ adult males by bisulfite sequencing (Fig. 4b) and Southern blot (data not shown). Methylation analysis by Southern blot also revealed no methylation changes after maternal transmission of the deletion (data not shown).

We have identified a direct repeat element 1 kb upstream of the mouse *H19* DMR, which is conserved in humans, rats, and pigs. We proposed that this repeat may be involved in regulation of methylation in the DMR, but targeted deletion did not affect imprinting or methylation in the *Igf2-H19* locus.

Given the dominant hypothesis in the imprinting field that associates tandem repeats with targeting of methylation to DMRs (27), the considerable evidence for a role for tandem repeats in heterochromatization, and the conservation of the K repeat across several species, this result was unexpected. However similar deletions of the G repeat upstream of the *H19* DMR and the direct repeat in the *U2afbp-rs1* locus also have no effect on imprinting (34, 37, 41). To date, only one repeat deletion in an imprinted region has given rise to aberrant DNA methylation and imprinting: the repeat downstream of the *Rasgrf1* DMR (47). (It should be noted that in mice carrying this deletion, the loss of methylation varies in a strain-dependent manner and in some cases methylation is at wild-type levels.) It is therefore possible that *Rasgrf1* is the exception rather than the rule. In support of this, a conditional deletion of the de novo methylase gene *Dnmt3a* in the male germ line causes a reduction in methylation at paternally methylated DMRs upstream of *H19* and *Gtl2* but has no effect on methylation at *Rasgrf1*, indicating that a different enzymatic pathway may be responsible for methylation of the *Rasgrf1* DMR in the male germ line (H. Sasaki, personal communication). Perhaps the pathway used at *Rasgrf1* is partly dependent on the presence of a direct repeat, whereas the *Dnmt3a* pathway used at *H19* and *Gtl2* require a different methylation targeting signal. Taken together, these results suggest that direct tandem repeats are not universal signals for methylation of DMRs.

The transgenic experiments described above indicate that an extra 1.5 kb of sequence upstream of the DMR enables transgenes to imprint as single-copy insertions. This extra sequence includes 400 kb (12 repeat units) of the K repeat, which led us to hypothesize that the repeat was important for imprinting. However, it must be noted that the original short transgenes

used a slightly truncated form of the DMR lacking about 80 bp of the DMR as defined by methylation studies (4, 13, 33, 42). Though relatively short in length, this sequence contains one of the four CTCF binding sites. We now know that that CTCF binding not only is important for boundary function but also is involved in protecting against de novo methylation on the maternal *H19* allele (14, 30, 36). Perhaps it is this small sequence element that enables the long transgenes to imprint as single-copy insertions rather than an incomplete copy of the K repeat.

Although the upstream region of *H19* is essential for imprinting, there are also downstream elements involved in the establishment of differential methylation (7, 11). Maternal transmission of the *Mnt* mutation, a chromosomal inversion beginning 25 kb downstream of *H19*, causes hypermethylation of the maternal *H19* DMR. This indicates that a number of *cis* elements are required to establish and maintain differential methylation and imprinting at *H19*. An interesting possibility is that there may be redundancy between some of these elements with the presence of one or two being sufficient to establish the methylation imprint at the endogenous DMR even if others are deleted.

In conclusion, our results taken together with others now suggests that the simple (and appealing) hypothesis that tandem repeats are necessary for targeting of methylation to DMRs in imprinted genes is not generally correct.

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