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Imprinting of Human H19: Allele-specific CpG Methylation, Loss of the Active Allele in Wilms Tumor, and Potential for Somatic Allele Switching

Yonghui Zhang,* Thomas Shields,* Taria Crenshaw,* Yue Hao,* Thomas Moulton,† and Benjamin Tycko*

*Divisions of Oncology and Neuropathology, Department of Pathology, and †Division of Pediatric Hematology-Oncology, Department of Pediatrics, and College of Physicians and Surgeons, Columbia University, New York

Summary

Genomic imprinting and monoallelic gene expression appear to play a role in human genetic disease and tumorigenesis. The human H19 gene, at chromosome 11p15, has previously been shown to be monoallelically expressed. Since CpG methylation has been implicated in imprinting, we analyzed methylation of H19 DNA. In fetal and adult organs the transcriptionally silent H19 allele was extensively hypermethylated through the entire gene and its promoter, and, consistent with a functional role for DNA methylation, expression of an H19 promoter-reporter construct was inhibited by in vitro methylation. Gynogenetic ovarian teratomas were found to contain only hypomethylated H19 DNA, suggesting that the expressed H19 allele might be maternal. This was confirmed by analysis of 11p15 polymorphisms in a patient with Wilms tumor. The tumor had lost the maternal 11p15, and H19 expression in the normal kidney was exclusively from this allele. Imprinting of human H19 appears to be susceptible to tissue-specific modulation in somatic development; in one individual, cerebellar cells were found to express only the otherwise silent allele. Implications of these findings for the role of DNA methylation in imprinting and for H19 as a candidate imprinted tumor-suppressor gene are discussed.

Introduction

Genomic imprinting is a phenomenon in which some genes are silenced when transmitted through a particular parental germ line. Genes subject to imprinting are therefore monoallelically expressed, and it is this aspect of imprinting which may have important consequences both in certain human genetic diseases, where imprinting conveys a unique pattern of inheritance (Hall 1990), and in embryonal tumors, in which observations of a parental bias in loss of heterozygosity (LOH) have suggested the existence of imprinted tumor-suppressor genes. In particular, in Wilms tumor (WT) and embryonal rhabdomyosarcoma (ER), there is a strong bias toward loss of maternal 11p15 markers (Schroeder et al. 1987; Scrable et al. 1989; Williams et al. 1989; Pal et al. 1990).

Four native genes have been found to be imprinted in mice. Two of them, IGF2R and H19, are expressed exclusively from the maternal allele (Barlow et al. 1991; Bartolomei et al. 1991), while the other two, IGF2 and SmRNP, were found to be expressed only from the paternal allele (DeChiara et al. 1991; Leff et al. 1992). We showed elsewhere that human H19, located at chromosome 11p15, is also monoallelically expressed (Zhang and Tycko 1992). However, the parental source of the expressed allele was not established in that study.

The H19 gene codes for a spliced and polyadenylated RNA which is highly expressed in a large variety of fetal tissues at a stage when cells are differentiating (Pachnis et al. 1988; Wiles 1988; Poirier et al. 1991; Rachmilewitz et al. 1992*a;* T. Shields and B. Tycko, unpublished observations). Transcription of H19 remains detectable in differentiated myoblasts in culture (Davis et al. 1987) and, at a level somewhat reduced from that in the

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Address for correspondence and reprints: Benjamin Tycko, M.D. Ph.D., Department of Pathology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.
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corresponding fetal organs, in several adult mouse and human organs (Brunkow and Tilghman 1991; Leibovitch et al. 1991; Poirier et al. 1991; Han and Liau 1992; Rachmilewitz et al. 1992*a;* T. Shields and B. Tycko, unpublished observations and this report). Because of the apparent lack of evolutionarily conserved open reading frames, the protein-coding potential of H19 RNA is uncertain. In part for this reason it has been proposed that this gene may act directly at the level of its RNA, perhaps through the formation of a ribonucleoprotein particle (Brannan et al. 1990). While one report has suggested that the human H19 gene might give rise to a polypeptide in vivo (Leibovitch et al. 1991), this preliminary study has not yet been substantiated. In spite of these enigmatic aspects, as a highly expressed endogenous gene and the only locus which has yet been demonstrated to show conservation of monoallelic expression between mice and humans, the H19 gene is one of the best available models for studying the mechanism and consequences of genomic imprinting.

Analysis of imprinted transgenes in mice, showing allele-specific CpG methylation correlating with the parent of origin, has supported a mechanism of imprinting involving DNA methylation (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). Strong a priori considerations supporting a role for methylation in imprinting include the facts that DNA methylation is a transmissible epigenetic modification which can have profound effects on gene expression (Cedar 1988; Bird 1992) and that DNA is known to be differentially methylated in paternal versus maternal gametes (Monk et al. 1987; Sanford et al. 1987; Howlett and Reik 1991; Kafri et al. 1992). Although at nonimprinted loci these methylation differences may be largely erased in early development (Monk et al. 1987; Sanford et al. 1987; Frank et al. 1991; Howlett and Reik 1991; Kafri et al. 1992), the simplest explanation for imprinting would be the persistence, at imprinted loci, of allelic methylation differences at critical CpGs, with subsequent effects on allelic gene expression. Reasonable theoretical arguments can also be made for more complicated models, e.g., those which involve spread of gene inactivation along the chromosome from hypothetical distant imprinting controlling elements, perhaps in a process analogous to heterochromatinization or X-chromosome inactivation.

Because it is difficult to exclude insertion-site artifacts in transgenic systems, models for imprinting require testing at endogenous loci. As a first step in this direction we have analyzed allelic methylation patterns of human H19 DNA. Here we describe an absolute correlation between allele-specific DNA methylation and monoallelic expression at this locus and show preliminary functional evidence for inhibition of H19 RNA expression by promoter methylation. We also describe a novel phenomenon of tissue-specific somatic allele switching, correlating with demethylation of the imprinted allele.

Certain human embryonal tumors are known to preferentially delete maternal alleles of 11p15 markers (Schroeder et al. 1987; Scrable et al. 1989; Williams et al. 1989; Pal et al. 1990), and therefore, a paternally imprinted tumor-suppressor gene is thought to exist in this chromosomal region. We have suggested elsewhere (Zhang and Tycko 1992) that H19 is a candidate for this gene. Here we assign the active H19 allele as maternal and describe loss of this allele in a case of WT.

Material and Methods

DNA and Southern Blotting

Genomic DNAs were prepared from human fetal and adult tissues by the proteinase K/SDS method (Sambrook et al. 1989). Six micrograms were digested overnight with 30 U of each of the indicated restriction enzymes, electrophoresed on 1.2% agarose gels, transferred to nylon membranes, and hybridized with the indicated DNA probes. To monitor completeness of digestion, control samples were spiked with 50 ng of DNA from an H19 phage clone, isolated from a human genomic library (human placental DNA in λFIX; Stratagene, La Jolla, CA) prior to digestion, and the Southern blots were evaluated for the presence of the limit-digest bands predicted from the published H19 sequence (Brannan et al. 1990) and the absence of partial digestion products.

DNA Probes and PCR

H19 genomic probes (fig. 1) were made by PCR using primers based on the published sequence. Probe A, spanning the last three exons, was made using the nested primers and PCR conditions described elsewhere (Zhang and Tycko 1992). Probe B, spanning the 5′ end of the first exon, was made using primers with the sequences GAAAAAGCCCGGGCTAGGAC (upstream) and GCGTCACCAAGTCCACTGTG (downstream). Genomic DNA (300 ng) was amplified in Tricine buffer with 2.5 mM $MgCl_2$ (Ponce and Micol 1992) for 30 cycles with initial denaturation for 4 min at 94°C, subsequent denaturations for 1.5 min at 94°C, annealing for 1 min at 60°C, and extension for 1.5 min at 73°C. IGF2 PCR was carried out with primers brack-

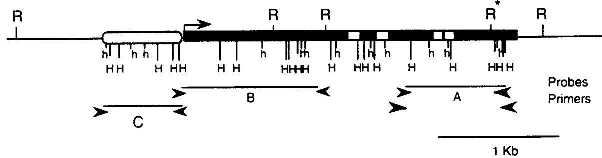

Figure 1 Map of the human H19 locus. Locations of enzyme sites, DNA probes (A and B), the region used in the promoter-CAT construct (C), and PCR primers (arrowheads) are indicated. Blackened boxes indicate exons; unblackened boxes indicate introns; and the oval indicates the promoter region. The asterisk indicates the polymorphic *Rsa*I site. *Hpa*II and *Hha*I sites are indicated only within the region spanned by the published sequence (Brannan et al. 1991). R = *Rsa*I; H = *Hpa*II; and h = *Hha*I.

etting an 800-bp region, including a large CA repeat in the last exon (Sussenbach 1989; Julier et al. 1991). Primers were CTCATACTTTATGCATCCCCG (upstream) and GCCTGATCCATACAGATATCG (downstream). PCR was in standard buffer with an initial denaturation for 4 min at 94°C and subsequent denaturation for 1.25 min at 94°C; annealing was for 45 s at 58°C and extension for 1.25 min at 72°C, with a final extension of 4 min at 72°C. The PCR product was then diluted 1:100 into fresh reagents containing ^{32}P-dCTP and was uniformly labeled by reamplification for 10 cycles. The radiolabeled product was digested with *Nsp*I, and the fragments were analyzed by electrophoresis through 6% polyacrylamide containing 7 M urea.

Determination of Allelic Representation in DNA and RNA

For DNA analysis, the appropriate PCR products were digested with the indicated restriction enzymes. For RNA analysis, tissues frozen in liquid nitrogen were pulverized, and RNA was prepared by the guanidinium/hot phenol method (Sambrook et al. 1989). RT-PCR was carried out as described elsewhere (Zhang and Tycko 1992), and allelic representation in cDNA PCR products was determined by restriction enzyme digestion.

H19 Promoter-CAT Construct, CAT Assays, and In Vitro Methylation

A region of 823 bp immediately upstream of the human H19 transcriptional start site (Brannan et al. 1990) was amplified by PCR from the H19 genomic phage DNA using primers AACAACCCTCACCAAAGGCC (upstream) and CCGGATCCTCGGTCCTAGCCCGG (downstream). The resulting PCR product was cloned in the pCR vector (Invitrogen, San Diego, CA), excised with *Nsp*I and *Bam*HI, and ligated into the *Sph*I and *Bam*HI sites of pBLCAT-2 (Luckow and Schütz 1987). A previously described (Luckow and Schütz 1987) herpes simplex virus thymidine kinase promoter (TK)-CAT construct (pBLCAT-3) was used as a positive control. In vitro methylation of the construct was carried out using universal CpG, *Hpa*II, or *Hha*I (*Cfo*I) methylases (New England Biolabs, Beverly, MA), and completeness of methylation was ascertained by assessing subsequent resistance to digestion with *Hpa*II or *Hha*I. Transfections were carried out using lipofectin reagent (GIBCO-BRL, Gaithersburg, MD) according to the recommendations of the manufacturer, and cells were harvested after 48 h. CAT assays were carried out as described by Sambrook et al. (1989).

Results

The Human H19 Gene Is Hypermethylated at the Silent Allele

The H19 gene is rich in CpG dinucleotides. On the basis of the published human sequence, the 829 bp of 5′ DNA preceding the transcriptional start site contains 5 *Hpa*II sites, 3 *Hha*I sites, and 46 CpG dinucleotides, while the 2,659-bp transcribed region contains 15 *Hpa*II sites, 9 *Hha*I sites, and 128 CpG dinucleotides (fig. 1). Several restriction site polymorphisms are found within the last exon of the human H19 gene (Zhang and Tycko 1992; and fig. 1). One of these, an *Rsa*I polymorphism, yields genomic DNA fragments of 1.7 kb and 1.3 kb in heterozygous individuals and is therefore useful in distinguishing alleles on Southern blots. To compare CpG methylation at transcriptionally active and inactive alleles, genomic DNAs from panels of fetal and adult organs were digested with *Rsa*I, either alone or together with a large excess of the methylation-sensitive restriction enzymes *Hha*I or *Hpa*II, and were subjected to Southern blotting with the H19-A probe. Completeness of digestion of unmethylated DNA under these conditions was ascertained by spiking control genomic DNA samples with a small amount of DNA from a genomic H19 phage clone (fig. 2*A* and *B*; +H19ϕ).

Blots of DNA from several fetal and adult organs, including kidney, adrenal gland, heart, lung, and liver, showed a selective loss of intensity in one allelic band on digestion with one or both of the methylation-sensitive enzymes, indicating allele-specific methylation (fig. 2*A*). In fetal kidney and adrenal gland, the hypomethylated allele was virtually completely digested by both *Hpa*II and *Hha*I, as indicated by the loss of one of the primary allelic bands and the generation of low-molecular-weight bands corresponding to the limit-digest frag-

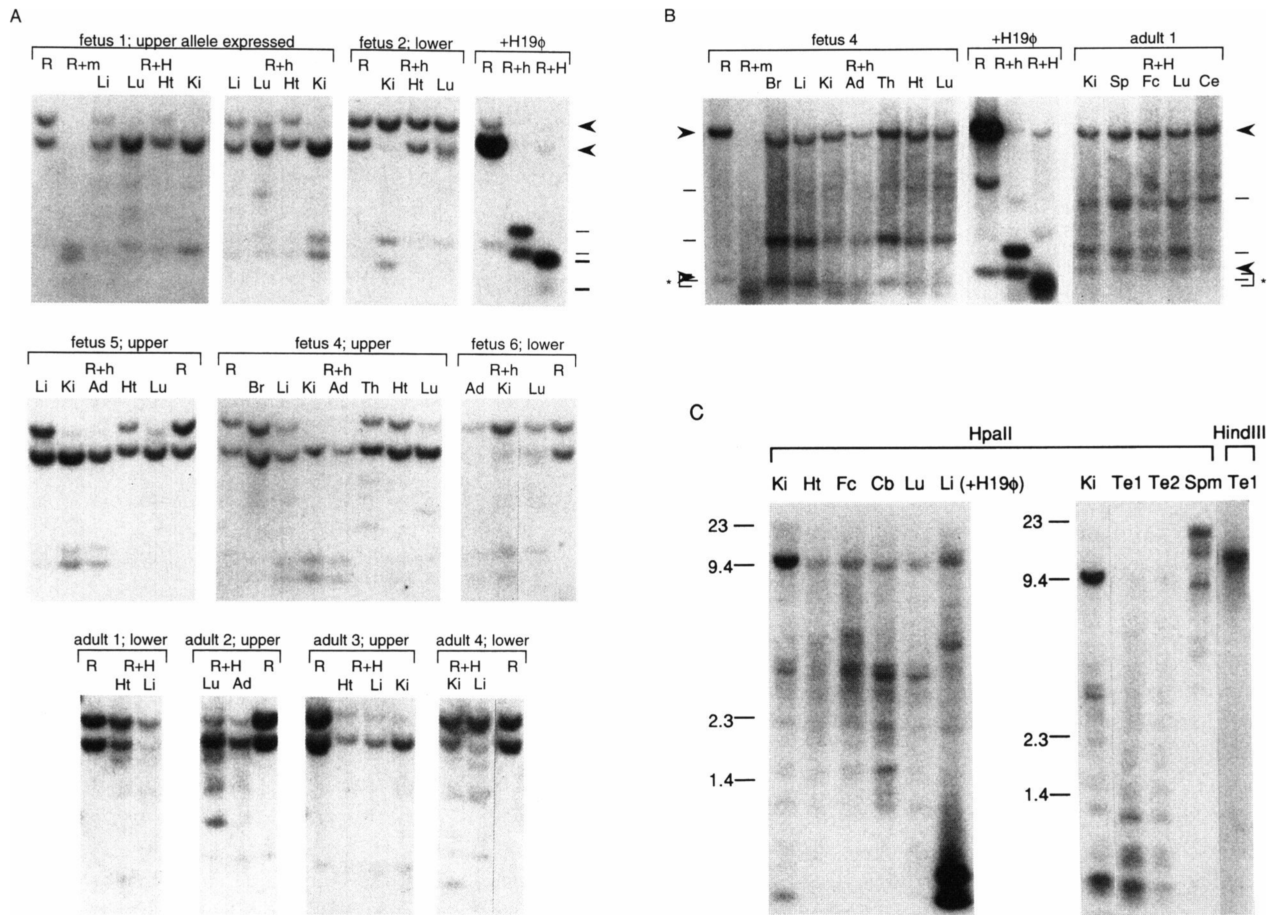

Figure 2 Southern blot analysis of CpG methylation in the human H19 gene. *A*, Genomic DNAs from fetal and adult organs, digested with the indicated restriction enzymes, subjected to Southern blotting, and hybridized with probe A. Heterozygosity for the *Rsa*I RFLP in each case is seen as the presence of two allelic bands of 1.7 kb and 1.3 kb (arrowheads) in samples digested with *Rsa*I alone; allele-specific methylation is indicated by the selective loss of either the upper band (fetuses 1, 4, and 5 and adults 2 and 3) or the lower band (fetuses 2 and 6 and adults 1 and 4). Loss of either of the primary allelic bands is accompanied by the appearance of low-molecular-weight bands (dashes), some of which correspond to the limit-digest fragments seen in the lanes spiked with H19 phage clone (+H19ϕ). The allele which was found to be transcribed (for analysis of fetuses 1, 2, and 4, see Zhang and Tycko 1992; for analysis of other cases, see fig. 3) is indicated above the corresponding case and, in each case, corresponds to the hypomethylated allele. *B*, Blots of DNA digested with *Rsa*I, *Rsa*I+*Hpa*II, or *Rsa*I+*Hha*I, rehybridized with probe B. Digestion with *Rsa*I alone yields bands at 2.1 kb and 0.4 kb (arrowheads). The 2.1-kb band is digested by, at most, 50% with *Hpa*II or *Hha*I, with the generation of lower-molecular-weight bands (dashes), some of which correspond to the limit-digest fragments seen in the parallel samples spiked with H19 phage clone DNA. In these samples there is complete digestion of the unmethylated H19 phage clone DNA (+H19ϕ). *C*, Genomic DNAs from adult and fetal organs, two benign cystic ovarian teratomas, and sperm, digested with *Hpa*II alone, as indicated. Southern blots were hybridized with probe A. The high-molecular-weight bands seen in the normal organ lanes are absent from the teratoma lanes. The control sample spiked with H19 phage shows complete digestion of unmethylated DNA. The presence of intact high-molecular-weight DNA in the teratoma samples was verified by ethidium-bromide staining (not shown) and by digestion with *Hin*dIII, as indicated, which yields a band at approximately 15 kb. The 9.5-kb band in the *Hpa*II digests also hybridized with probe B (not shown). R = *Rsa*I; h = *Hha*I; H = *Hpa*II; and m = *Msp*I. Li = liver; Ki = kidney; Ad = adrenal gland; Cb and Ce = cerebellum; Fc = frontal cortex; Lu = lung; Ht = heart; Br = brain; Te = teratoma; and Spm = sperm.

ments of the H19 phage clone. Most other organs also showed allele-specific methylation by this assay, but the degree of hypomethylation varied quantitatively between organs. In fetal and adult lung and heart there was reproducible, but less complete, digestion of the hypomethylated allele, probably reflecting tissue heterogeneity, and, in fetal and adult liver, allele-specific methylation was more clearly seen on *Hpa*II digestion than on digestion with *Hha*I. In fetal and adult forebrain (fig. 2*A*, fetus 4) allele-specific methylation was

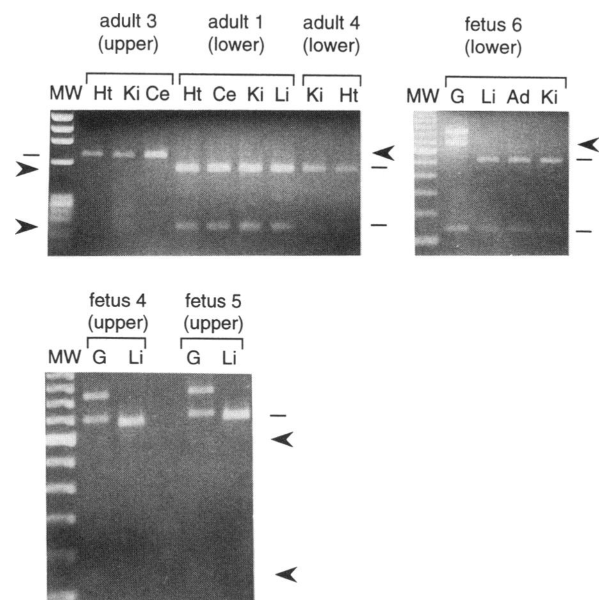

Figure 3 Allelic representation in H19 cDNAs. RNA was subjected to reverse transcription, and cDNAs from the indicated fetal and adult organs were amplified by PCR and digested with *Rsa*I. Dashes indicate the bands derived from the expressed alleles, and arrowheads indicate the predicted positions of the absent bands which would have resulted from expression of the silent allele. The expressed alleles (upper or lower) are indicated above each case. PCR products from genomic DNA (G) yield the expected two allelic bands. The presence of two small introns causes these to run at a higher position in the gel than the corresponding cDNA fragments. The results of similar analyses for fetuses 1 and 2 are shown elsewhere (Zhang and Tycko 1992). Organs are abbreviated as in fig. 2. Molecular-weight markers (MW) are ϕX *Hae*III digest (*upper left*) or 100-bp ladder (*upper right* and *lower left*).

not detected by this assay but was subsequently detected by a different assay examining the 5′ end of the gene (see below). Consistent with genomic imprinting, while different cases showed different hypomethylated alleles, within a single case all organs (with the exception of cerebellum in one case; see below) showed the same hypomethylated allele.

We have previously shown that H19 RNA is monoallelically expressed in several fetal organs, including kidney, adrenal gland, heart, liver, lung, thymus, meninges, and brain (Zhang and Tycko 1992). In both the previous report and the current study, for each case we analyzed the allelic representation in H19 RNA by carrying out PCR of oligo-dT-primed cDNA from the appropriate organs, with nested primers spanning the last two intron/exon junctions (fig. 1), and by digesting the PCR products with enzymes detecting exonic restriction site polymorphisms (Zhang and Tycko 1992; and figs. 3 and 6). To date, we have examined six fetuses and four adults for allelic DNA methylation and, in parallel, allelic RNA expression in various organs. In each case the hypomethylated allele has corresponded to the single allele represented in the cDNA PCR product (for examples, see fig. 2*A* compared with fig. 3; fig. 2*A* compared with Zhang and Tycko 1992; and fig. 6*A* compared with fig. 6*B*). Consistent with the *Rsa*I polymorphism being a neutral allelic marker, in our series the upper *Rsa*I allele has been expressed in five cases and the lower allele in five cases. While different cases have shown expression of different *Rsa*I alleles, in 9 of the 10 cases, within a given case all organs have shown expression of the same allele. The isolated exception to this has been one case (adult 4) in which we have observed a nearly complete switch in allelic expression in the cerebellum, with silencing of the otherwise active allele, and an apparent erasure of imprinting, with biallelic expression, in the lung (see below).

The Southern analyses with probe A indicated that allele-specific methylation in the 3′ half of the human H19 gene correlates, in each organ examined, with monoallelic RNA expression. To examine the 5′ half of the gene the blots were stripped and rehybridized with probe B. In this case, while no RFLPs were present, digestion with the methylation-sensitive enzymes reduced the intensity of the 2.1-kb band seen with *Rsa*I alone by about 50%, with generation of a series of lower-molecular-weight bands, some of which corresponded to limit-digest fragments (fig. 2*B*). Since results with control samples spiked with H19 phage indicated complete digestion of unmethylated DNA by both *Hpa*II and *Hha*I, the persistence of about 50% of the signal intensity in the 2.1-kb band suggested the presence of allele-specific methylation within this *Rsa*I fragment, extending from the middle of the first exon to about 1.5 kb upstream of the transcriptional start site, with one H19 allele fully methylated at every *Hpa*II and *Hha*I site within this region.

Proof of allele-specific methylation in this region was obtained from a gel-isolation/PCR protocol. First, genomic DNA was digested with *Hpa*II or *Hha*I alone, was blotted, and hybridized sequentially with probes A and B, with intervening stripping of the blots. While each probe detected a different series of low-molecular-weight bands, both hybridized to fairly intense and uniform high-molecular-weight bands of about 9.5 kb (*Hpa*II digestions) and about 14 kb (*Hha*I digestions) in several organs (for results with *Hpa*II digestion, see fig. 2*C*). Taken together with the previous *Rsa*I+*Hpa*II analysis with probe B, the 9.5-kb *Hpa*II fragment must represent a fully *Hpa*II-methylated fragment spanning the entire H19 gene and at least 1.5 kb of its 5′ flanking

sequences. To establish which allele(s) was present in the high-molecular-weight fragments, *Hpa*II-and *Hha*I-digested DNAs from various organs of three fetuses and two adults, in which the expressed allele had been previously assigned, were fractionated by agarose gel electrophoresis and gel-isolated in the high-molecular-weight size ranges—9–10 kb for *Hpa*II and 12–15 kb for *Hha*I—were subjected to PCR with the A-region H19 primers, and the products were digested with *Rsa*I. The PCR-product digestion patterns in each case showed virtually exclusive representation of the transcriptionally silent allele in the high-molecular-weight DNA (for results for fetal cases, see fig. 4). We can conclude that a major population of cells in a number of different organs contains an inactive H19 allele that is hypermethylated (fully methylated at *Hpa*II and *Hha*I sites) over a large span, including both the immediate 5′ flanking region and the entire transcribed region.

Inhibition of H19 Promoter Activity by CpG Methylation

If CpG methylation in fact plays a role in silencing expression of the imprinted H19 allele, it might be predicted to shut down transcription from the H19 promoter in functional assays. A tissue-specific H19 promoter region upstream of the transcriptional start site has been previously defined in the mouse (Yoo-Warren et al. 1988). For functional assays of the effect of CpG methylation, we placed an 832-bp fragment of the human H19 gene, corresponding to the region immediately 5′ of the start site (fig. 1), upstream of the CAT reporter gene. To ascertain cell type–specific promoter activity with this reporter construct, transient transfections were carried out in Hep3B (hepatocellular carcinoma) and G401 (WT) cells. Hep3B cells express the endogenous H19 gene (Brannan et al. 1990), while G401 cells do not (Y. Zhang and B. Tycko, unpublished observations). CAT expression was seen only in the Hep3B cells (fig. 5*A*). When the construct was methylated in vitro with either universal CpG or *Hpa*II methylases prior to transfection, CAT activity was dramatically reduced; however, premethylation with *Hha*I methylase did not reduce promoter activity (fig. 5*B*). This indicates that, at least in the context of this simple construct, the H19 promoter is susceptible to silencing by methylation at critical CpG dinucleotides, including those within *Hpa*II sites which are methylated at the silent allele of the endogenous gene in normal tissues.

Hypomethylation of H19 DNA in Gynogenetic Teratomas

Benign cystic teratomas of the ovary are gynogenetic, i.e., derive parthenogenetically from an unfertilized oocyte (Linder et al. 1975). These tumor-like lesions consist entirely of well-differentiated mature tissues, and their genomes may therefore reflect the imprinting state of maternal alleles in normal tissues. When DNA from two teratomas was digested with *Hpa*II or *Hha*I and subjected to Southern analysis with probe A, the bulk of the signal was seen in low-molecular-weight bands (for results with *Hpa*II, see fig. 2*C*). In contrast, H19 DNA from normal somatic organs showed the expected biphasic pattern of bands (with prominent high-molecular-weight 9.5-kb *Hpa*II fragments and a series

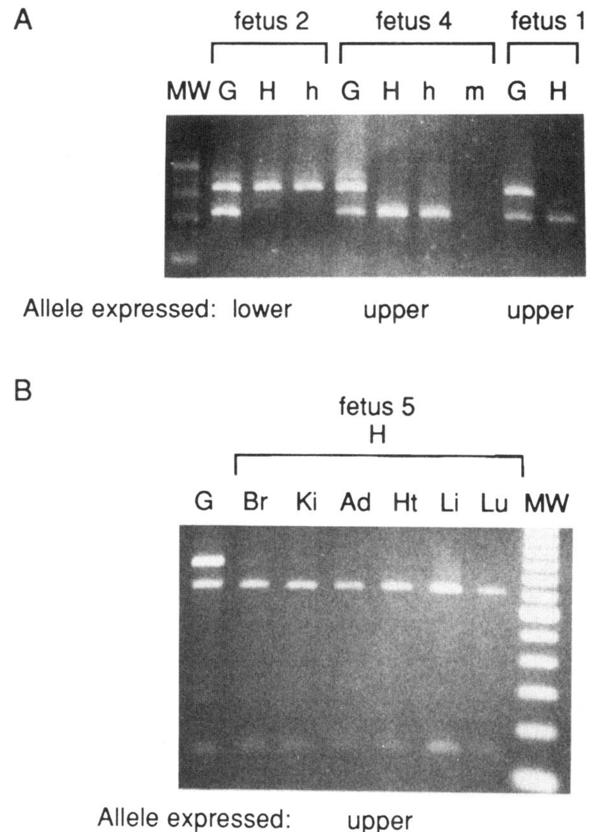

Figure 4 Allelic representation in hypermethylated H19 DNA determined by gel isolation/PCR. *A*, Genomic DNAs from fetal kidneys, digested with *Hpa*II or *Hha*I alone and subjected to agarose gel electrophoresis. DNA fractions in the 9–10-kb (*Hpa*II) and 12–15-kb (*Hha*I) range were isolated from the gel, and the DNA was amplified by PCR with the H19 A-region primers. PCR products were digested with *Rsa*I. In each case the hypermethylated DNA contains only the transcriptionally inactive allele. Both alleles are present, as expected, in PCR products from unfractionated genomic DNA (G). *B*, Genomic DNAs from a panel of organs from fetus 5, which were fractionated after *Hpa*II digestion and analyzed according to the above protocol. Restriction enzymes used for genomic digestions are as in fig. 2. Molecular-weight markers (MW) are ϕx *Hae*III digest (panel *A*) and 100-bp ladder (panel *B*).

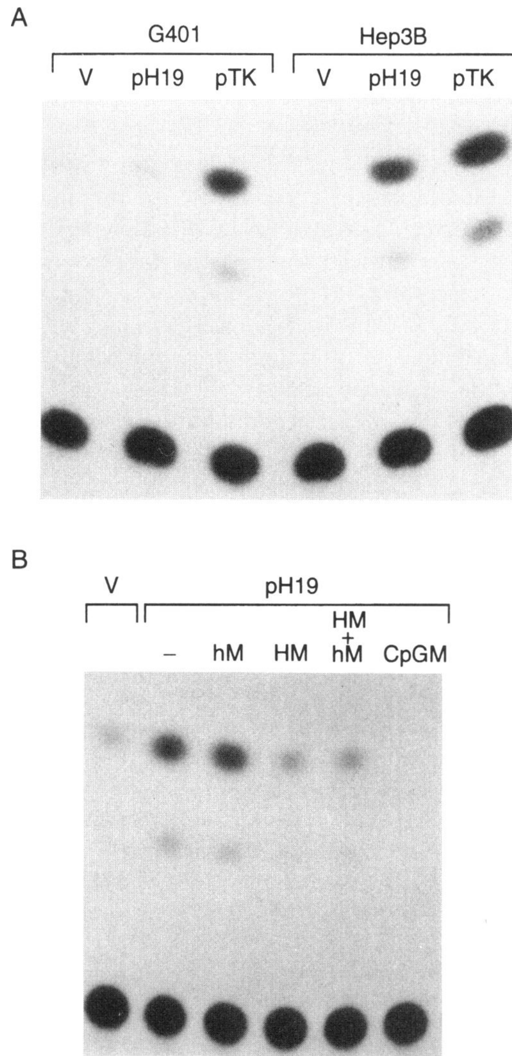

Figure 5 Effect of CpG methylation on activity of the minimal H19 promoter. *A*, CAT constructs, transfected into G401 cells (which do not express endogenous H19) and Hep3B cells (which express high levels of endogenous H19). Acetylated ^{14}C-chloramphenicol was measured by thin-layer chromatography. With the H19 promoter construct, CAT activity above background is present in the Hep3B cells but not in the G401 cells. Both cell lines express the TK promoter construct. *B*, H19 promoter CAT, which was premethylated with either *Hpa*II, *Hha*I, *Hpa*II+*Hha*I, or universal CpG methylases, as indicated, and transfected into Hep3B cells. CAT activity is significantly reduced (70% reduction from unmethylated control, as measured by scintillation counting) by methylation of the *Hpa*II sites in the promoter but not by methylation of the *Hha*I sites. v = vector control; pH19 = H19 promoter construct; and pTK = thymidine kinase promoter construct.

of low-molecular-weight bands), and male germ line (sperm) was even more highly methylated, yielding only very-high-molecular-weight (9–23-kb) bands. In view of the correlation of allelic hypomethylation with expression in normal tissues, the hypomethylation observed in the gynogenetic teratomas suggested that the expressed H19 allele in normal tissues might be maternal. A complementary line of evidence in this regard is a previous report of lack of expression of H19 RNA in androgenetic trophoblastic tumors (Rachmilewitz et al. 1992*b*)

Expression of the Maternal H19 Allele and Loss in WT

To directly investigate the parental derivation of the expressed H19 allele, it was necessary to collect expressing tissues and matched parental samples. Since we did not wish to trace parents of aborted fetuses and since the mature kidney continues to express detectable H19 RNA, a convenient source of cases was pediatric patients with WT undergoing complete nephrectomies. Our interest in such cases was additionally motivated by the previous circumstantial evidence for involvement of an imprinted 11p15 tumor-suppressor gene(s) in the pathogenesis of WT (Schroeder et al. 1987; Williams et al. 1989; Pal et al. 1990). One of three cases of WT in which we obtained tumor and normal kidney tissue from the patient and peripheral blood from the parents proved to be sufficiently informative. Southern blots of normal kidney and tumor DNAs from this case digested with *Rsa*I, *Rsa*I+*Hha*I, or *Rsa*I+*Hpa*II and hybridized with probe A were informative in two respects. First, the tumor showed LOH at the H19 locus, as indicated by the absence of the lower allelic band on digestion with *Rsa*I alone (fig. 6*A*). Second, the normal kidney showed clear hypomethylation of the lower *Rsa*I allele, reflected in the loss of the corresponding band on digestion with either *Hha*I (fig. 6*A*) or *Hpa*II (not shown). These results suggested that the normal kidney cells in this case were expressing the lower *Rsa*I H19 allele. To prove this, RNA from the kidney was subjected to RT-PCR analysis, which showed exclusive representation of H19 transcripts derived from the lower allele (fig. 6*B*).

Several previous studies of LOH at 11p15 marker loci in WTs have shown a highly selective (95%) loss of maternal alleles (Schroeder et al. 1987; Williams et al. 1989; Pal et al. 1990), and, in view of this strong statistical bias, the loss of the expressed H19 allele in our case suggested that the active H19 allele might be maternal. This "direction" of H19 parental imprinting would match that previously observed in mice (Bartolomei et al. 1991). To ascertain whether the expressed allele was in fact inherited from the mother we first compared *Rsa*I, *Alu*I (Zhang and Tycko 1992), and *Avi*II (Y. Zhang and B. Tycko, unpublished observations) poly-

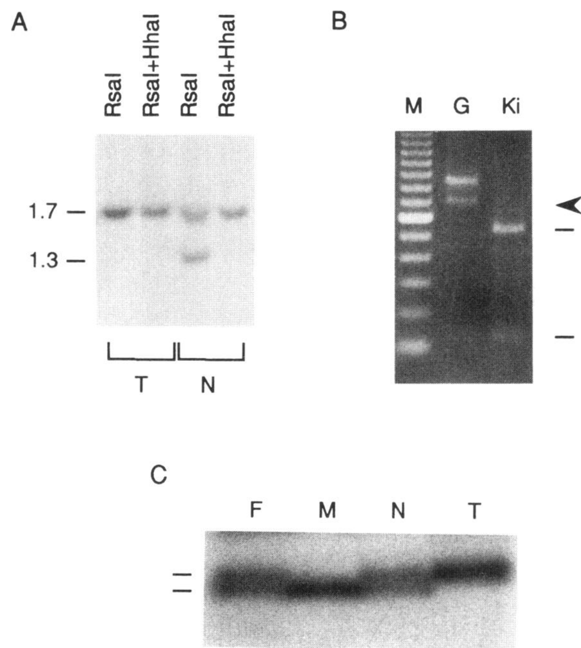

Figure 6 Determination of the parental origin of the active human H19 allele. *A*, *Rsa*I- and *Rsa*I+*Cfo*I-digested DNAs from WT tissue and normal kidney of patient, subjected to Southern analysis with H19 probe A. The lower allele is hypomethylated in normal kidney and lost in tumor tissue. *B*, RNA from the kidney, subjected to RT-PCR followed by *Rsa*I digestion. Predicted positions of bands corresponding to each allele are indicated as in fig. 3. There is monoallelic expression of the lower allele. G = genomic DNA; and Ki = kidney cDNA. *C*, IGF2 CA-repeat region amplified by PCR. Radiolabeled PCR product was cut with *Nsp*I and separated by denaturing acrylamide gel electrophoresis. Both the patient (N) and her father (F) are heterozygous for a length polymorphism in the 110-bp *Nsp*I fragment. The mother (M) only carries the lower allele. The tumor (T) has lost the lower IGF2 allele, which is maternal in origin.

morphic sites at the H19 locus in DNAs from the patient and her parents. Unfortunately, all three individuals were heterozygous at all three sites. We next searched for polymorphisms at a closely linked locus, IGF2, which has been shown by physical mapping to lie within less than 200 kb of H19 (Zemel et al. 1992). A region in the IGF2 last exon, which contains a highly polymorphic simple sequence (CA) repeat, was amplified by PCR, and uniformly radiolabeled products from kidney, tumor, and parental samples were digested to smaller fragments with *Nsp*I and were examined on a sizing gel. A length polymorphism in one of the *Nsp*I fragments clearly indicated that the tumor had lost the maternal IGF2 allele (fig. 6C). In view of the assignment of the lost allele as the active one, we can conclude that (barring a highly improbable meiotic recombination event in the less than 200 kb of DNA separating IGF2 and H19) the expressed H19 allele in this case was maternal in origin.

Somatic Allele Switching

In our analysis of H19 RNA expression, we encountered one interesting exception to the rule that all organs in a given individual show the same expressed allele. In adult 4 there was a nearly complete switch of allelic expression in cerebellum relative to heart, kidney, and liver, while in the lung of this individual there was biallelic expression (fig. 7*A*). In our series this case is unique—repeated analyses of cDNAs from the cerebellums of the three other adults and two of the fetuses have shown no evidence of allele switching, and no other lung has shown biallelic expression. While we do not yet understand the mechanism underlying allele switching in this case, it is intriguing that, among the organs studied, all cerebellar DNAs, regardless of the presence or absence of allele switching, have shown a unique pattern of biallelic demethylation of one or more *Hpa*II sites at the 3′ border of the last H19 exon (fig. 7*B*).

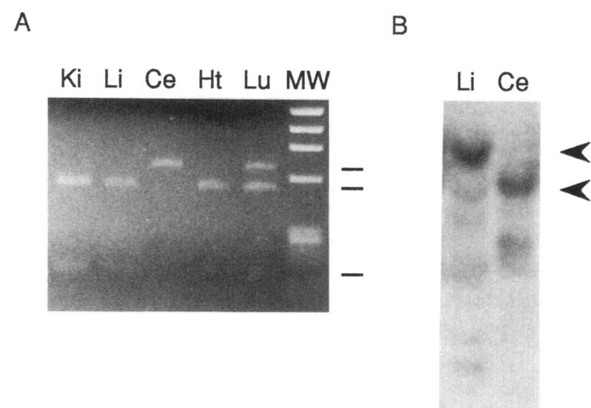

Figure 7 Somatic allele switching in the cerebellum of adult 4. *A*, RNA from the indicated organs, subjected to RT-PCR followed by *Rsa*I digestion. There is allele switching in cerebellum and biallelic expression in lung. Allele switching was confirmed by direct sequencing of the RT-PCR products (not shown). *B*, Southern analysis of genomic DNAs from adult 4, after digestion with *Rsa*I+*Hpa*II and hybridization with probe A. While the liver DNA shows hypomethylation of the lower allele, there is demethylation of the imprinted (upper) allele in cerebellum at one or more sites at the 3′ border of the last exon. This is indicated by loss of the upper band and generation of a prominent band which reproducibly appears slightly above the expected position of the lower allele (lower arrow). The lower allele in the cerebellum is also demethylated and is digested to lower-molecular-weight fragments. Cerebellar DNAs from five other individuals, without evidence of allele switching, have shown a similar pattern of biallelic demethylation (not shown).

Discussion

While there is increasing evidence for genomic imprinting of an important subset of genes in mice and humans, our understanding of the mechanism and consequences of this phenomenon remains largely theoretical. Imprinting in mammals was originally identified in studies of gynogenetic and androgenetic mouse embryos and mice inheriting unbalanced complements of maternal and paternal chromosomes (Searle and Beechey 1978; McGrath and Solter 1984; Surani et al. 1984; Cattanach and Kirk 1985), but it was first demonstrated at defined loci in transgenic mice (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). At least two of these lines in which RNA expression was examined showed a correlation of monoallelic silencing of RNA expression with hypermethylation of CpG dinucleotides in the transgene. These observations led to the proposal that allele-specific DNA methylation might account for the imprinted state. Although distinguishing cause from effect has been notoriously difficult, a priori considerations, most importantly the well-documented effects of DNA methylation on gene activity (Cedar 1988; Bird 1992) and the presence of differential methylation of DNA in male versus female gametes (Monk et al. 1987; Sanford et al. 1987; Howlett and Reik 1991; Kafri et al. 1992), have supported this notion. The simplest methylation model for imprinting would posit that, because of a critical positioning and/or density of CpGs unique to each imprinted gene, gametic methylation differences are preserved at these loci in early somatic development, resulting ultimately in allele-restricted gene expression. In this scheme the imprinting-controlling elements would be predicted to be local, i.e., inseparable from the primary sequence of the imprinted gene itself.

An alternative model for the imprinting mechanism postulates the existence of extended subchromosomal "imprinting domains" (Zemel et al. 1992). By analogy with the phenomenon of X-chromosome inactivation, in which gene silencing spreads over large distances from the X-inactivation center, accompanied and perhaps caused by progressive spreading of CpG methylation along the DNA (Riggs and Pfeifer 1992), putative imprinting-controlling elements in the "domain" model would in most cases lie outside a given imprinted gene. One variation of this model—in which the controlling element(s) consist of segments of DNA which, because of some aspect of their sequence, are particularly refractory to demethylation in early development and nucleate the spread of methylation to adjacent sequences (Toth et al. 1989)—is also consistent with a role for methylation in both the initiation and effector phases of imprinting.

Deciding among these possibilities will ultimately require functional tests of candidate imprinting-controlling sequences in cell-culture or whole-animal reconstitution experiments. However, to lay the groundwork for such studies and to begin to examine the imprinting effector mechanism, it is important to document the methylation status of endogenous imprinted genes in somatic tissues. Here we have described extensive allele-specific CpG methylation at one such gene, human H19. The silent H19 allele in several fetal and adult organs is fully methylated at numerous CpG-containing restriction sites over a span including the entire gene and at least 1.5 kb of its 5′ flanking DNA, which includes a functionally defined minimal promoter region. In contrast, the active allele is unmethylated at many, though not all, of these sites.

These findings are therefore similar to the earlier observations in transgenic mice. A further potentially useful comparison can be drawn with recent findings regarding the relationship of CpG methylation at the human fragile X syndrome (FMR) locus and transmission of the fragile X phenotype. In this disease it appears likely that methylation of the CpG-containing FMR triplet repeat (Bell et al. 1991; Oberlé et al. 1991; Verkerk et al. 1991) occurs during X inactivation, and it has been suggested that the presence of a physically expanded triplet repeat in female carriers blocks reactivation of the FMR gene, possibly by conferring a local resistance to demethylation during oogenesis (Laird 1987, 1991). Lack of transmission of the fragile X karyotype to female offspring of those rare affected males who reproduce suggests that the abnormal methylation state can be erased in spermatogenesis, a feature perhaps analogous to the parental germ-line–specific erasure of genomic imprinting at autosomal loci (Laird 1991).

While very few other studies of endogenous imprinted loci have been reported, one interesting exception to CpG hypermethylation being correlated with silencing of the imprinted allele was recently described for murine IGF2, a gene which, at least in mice, is oppositely imprinted from H19 (Sasaki et al. 1992). In this case it appears that, in the promoter region, the repressed allele is unmethylated at numerous assayable CpGs and shows an overall methylation pattern not obviously different from the active allele. However, in addition to the opposite directions of imprinting of H19 and IGF2, the finding of a low, but detectable, level of transcription from the maternal IGF2 allele in embryos in that study, together with observations of

consistent high-level expression of the maternal IGF2 allele in choroid plexus and meninges of fetuses (DeChiara et al. 1991; Sasaki et al. 1992), suggests that the mode of repression at the IGF2 locus may not be entirely analogous to that at H19, in which, while rare individuals can show somatic allele switching, there is no evidence of consistent incomplete repression.

Based largely on studies of X-linked genes, there is an increasing consensus that CpG methylation can play a primary role in silencing gene expression (Cedar 1988; Bird 1992; Riggs and Pfeifer 1992). However, the pathway by which silencing is brought about remains controversial. Two possibilities are direct repression by effects of methylated CpGs on binding of sequence-specific transcription factors (Dynan 1989) or, alternatively, indirect repression resulting from the occupation of heavily methylated DNA regions by non–sequence-specific binding proteins (i.e., MeCPs) with an affinity for stretches of closely spaced methylated CpGs (Meehan et al. 1989; Boyes and Bird 1991; Bird 1992). Our finding that there is nearly complete repression of H19 promoter activity by methylation of as few as 5 of 46 CpGs when *Hpa*II methylase is used and that no repression is obtained after methylation of 3 of 46 CpGs when *Hha*I methylase is used provides a preliminary indication that methylation of a small number of specific sites may be sufficient for repression. This in turn suggests that a direct mechanism may be acting and that a search for methylation-sensitive transcription factors interacting with the H19 promoter might be productive.

Our observation of the potential for tissue-specific somatic allele switching is also interesting in this context since, if small changes in CpG methylation can strongly influence transcription and if particular embryonic or fetal cells are poised at a critical threshold level of DNA methylation, this may explain the susceptibility of particular tissues to switching. To explain switching, as opposed to simple erasure of imprinting, it must also be postulated that there are positive and negative feedback effects which, once allele-specific RNA expression is established, lead to increasing methylation of the inactive allele and decreasing methylation of the active allele. Precedents for such effects have been observed in tissue culture systems (Toth et al. 1989). The occurrence of switching in only a subset of individuals may then be analogous to strain-specific differences in imprinting behavior in mice (Sapienza et al. 1989; Forejt and Gregorova 1992) and, while this remains to be investigated, may reflect genetically determined differences in CpG-methylase activity.

The second aspect of our study was the examination of the direction of imprinting of human H19. The answer to this question has potential implications for the role of H19 in human embryonal tumorigenesis. The expression pattern of this gene in human development, in particular the lack of expression in undifferentiated kidney blastemal cells and the onset of high-level expression in differentiating fetal tubular epithelial cells (T. Shields and B. Tycko, unpublished observations), together with data on humans and mice indicating a general correlation of H19 expression with cellular differentiation (Pachnis et al. 1988; Wiles 1988; Poirier et al. 1991; Rachmilewitz et al. 1992*a*), suggests the possibility of a differentiating and/or growth-regulating activity of H19 in the precursor tissues of embryonal tumors, including WT. Since WTs and ERs are known to preferentially delete maternal alleles of 11p15 markers (Schroeder et al. 1987; Scrable et al. 1989; Williams et al. 1989; Pal et al. 1990), we have suggested that a strong first criterion for identifying candidate WT and ER suppressor genes in this chromosomal region is monoallelic expression from the maternal allele (Zhang and Tycko 1992). H19 was shown elsewhere (Bartolomei et al. 1991) to be maternally expressed in mice, and data showing lack of expression of H19 RNA in human androgenetic tumors (Rachmilewitz et al. 1992*b*) have suggested that this might be the case in humans as well. Our analysis of H19 expression in normal human kidney in the present study directly demonstrates the evolutionary conservation of this direction of imprinting. H19 therefore remains a candidate WT and ER suppressor gene. Direct functional tests of growth-regulatory activity of human H19 will clearly be of great interest. A suggestion that such experiments might be fruitful is already provided by evidence from the murine system, in which H19-overexpressing transgenics die in utero at a late fetal stage (Brunkow and Tilghman 1991).

Note added in proof.—Ohlsson et al. (in press) have recently shown that human IGF2 is imprinted and that the direction of imprinting at this locus is conserved between mice and humans.

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