Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2159–2163, March 1995
Medical Sciences

Epigenetic changes encompassing the *IGF2/H19* locus associated with relaxation of *IGF2* imprinting and silencing of *H19* in Wilms tumor

(imprinting/epigenotype/nephroblastoma/gene methylation)

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Communicated by Alfred G. Knudson, Fox Chase Cancer Center, Philadelphia, PA, November 30, 1994

ABSTRACT **In most tissues *IGF2* is expressed from the paternal allele while *H19* is expressed from the maternal allele. We have previously shown that in some Wilms tumors the maternal *IGF2* imprint is relaxed such that the gene is expressed biallelically. We have now investigated this subset of tumors further and found that biallelic expression of *IGF2* was associated with undetectable or very low levels of *H19* expression. The relaxation of *IGF2* imprinting in Wilms tumors also involved a concomitant reversal in the patterns of DNA methylation of the maternally inherited *IGF2* and *H19* alleles. Furthermore, the only specific methylation changes that occurred in tumors with relaxation of *IGF2* imprinting were solely restricted to the maternal *IGF2* and *H19* alleles. These data suggest that there has been an acquisition of a paternal epigenotype in these tumors as the result of a pathologic disruption in the normal imprinting of the *IGF2* and *H19* genes.**

In humans, several genes are imprinted and monoallelically expressed (1, 2). Two of these genes, *IGF2* and *H19*, are oppositely imprinted such that *IGF2* is expressed from the paternal allele while *H19* is expressed only from the maternal allele (3–7). IGF2 is an embryonal growth factor; however, the function of H19 is currently not understood, and although *H19* RNA is abundantly transcribed in the embryo it does not appear to be translated (8). The expression patterns of *IGF2* and *H19* in differentiating human fetal tissues are similar (9, 10), and because of their close proximity at 11p15.5 (11), it has been proposed that these two genes may form an imprinted domain (1, 11–14).

Although the molecular basis of genomic imprinting is yet to be defined, allele-specific DNA methylation has been proposed as the most likely epigenetic mechanism (13, 15–17). Recently, we and others have found that in some Wilms tumors the transcriptionally silent maternal *IGF2* allele is activated such that *IGF2* expression occurs biallelically (3, 4). A similar biallelic pattern of *IGF2* expression is also seen normally in the choroid plexus and leptomeninges, but both *H19* alleles are silent (10). Conversely, in the methylase-deficient mouse, it has been shown that disruption of DNA methylation leads to the biallelic expression of *H19* and an absence of *IGF2* expression (14). Thus, DNA methylation appears to be essential to maintain the normal pattern of imprinting at the *IGF2/H19*, locus and it is possible that there is a common mechanism that regulates the reciprocal expression of these two genes. There is now evidence to suggest that *H19* is an unusual class of tumor suppressor gene (18), which is silenced in some Wilms tumors by DNA methylation (19, 20). We have now investigated the relationship between the expression of *IGF2* and *H19* and the patterns of DNA methylation at the *IGF2/H19* locus in three groups of Wilms tumors: those that show relaxation of imprinting and biallelically express *IGF2*, those that have a normal *IGF2* imprint and express *IGF2* monoallelically, and Wilms tumors that have lost heterozygosity at this locus.

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MATERIALS AND METHODS

Sample Preparation, DNA/RNA Extraction, and Northern Blotting. Tumor samples and adjacent normal kidney were obtained at nephrectomy from Wilms tumor patients. Fetal kidney samples were obtained from aborted fetuses (12–17 weeks of gestation). DNA and RNA were extracted as described (3).

Allelic Analysis of *IGF2* Expression. Allele-specific expression of *IGF2* was determined as described (3).

Blotting Analysis. Northern blots were prepared as described (21). For DNA methylation analyses, Southern blots were prepared by digesting DNA (5 μg) with 50 units of *Hpa* II, *Msp* I, or *Hha* I; electrophoresed in 1.5% agarose gels; and blotted to Hybond N^+ (Amersham). Filters were hybridized at 65°C with the following [^{32}P]dCTP-labeled DNA probes: IGF2 (22), H19 (8), insulin (23), HRAS (24), and β-globin (25).

Gel Isolation PCR. DNA (10 μg) from tumors that expressed *IGF2* monoallelically was digested with *Hpa* II and electrophoresed in 1.5% agarose. The methylated DNA fraction at ≈3 kb (see Fig. 2*a*, lanes 8–12) was extracted from the gel and amplified by PCR with primers spanning the IGF2 *Apa* I or AC repeat polymorphisms (3, 4, 26). The PCR products were electrophoresed in 6% polyacrylamide gels after *Apa* I digestion to detect the *Apa* I polymorphism or directly to detect the AC repeat polymorphism.

RESULTS

***IGF2* and *H19* Expression in Wilms Tumors.** Northern blots of a series of Wilms tumors showed that *IGF2* was expressed at high levels in all 23 cases examined (Fig. 1*a*), as has been demonstrated (27, 28). Unlike *IGF2*, *H19* RNA was detected in only 5/23 tumors, and all of these tumors expressed *IGF2* monoallelically (Fig. 1*b*, lanes 8–12). In contrast to this group of tumors, the expression of *H19* was undetectable or very low in 11 tumors that had undergone loss of heterozygosity (LOH) at 11p15 (Fig. 1*b*, lanes 13–23). These data are consistent with LOH studies in Wilms tumors that show a preferential loss of the maternal 11p allele (29–32), from which *H19* is normally expressed (7). In a third group of tumors *IGF2* was expressed biallelically and the levels of *H19* RNA were also very low or undetectable (Fig. 1*b*, lanes 1–7). These data suggest that in these tumors an epigenetic change on the maternal chromosome has led to the activation of the maternal *IGF2* allele and silencing of the maternal *H19* allele.

Abbreviation: LOH, loss of heterozygosity.

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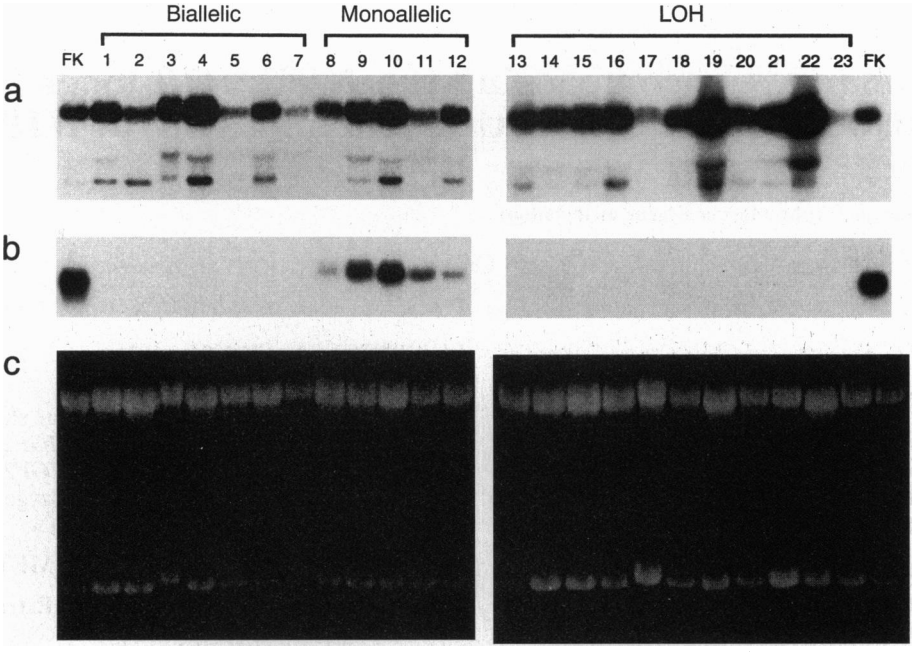

FIG. 1. *IGF2* (*a*) and *H19* (*b*) expression in Wilms tumor. Total RNA from 7 Wilms tumor cases with biallelic *IGF2* expression (lanes 1–7), 5 with monoallelic *IGF2* expression (lanes 8–12), and 11 with 11p LOH (lanes 13–23) and a fetal kidney (lanes FK) were analyzed by Northern blotting with the probe for *IGF2* (*a*) or *H19* (*b*). Photography of the gel stained by ethidium bromide (*c*) demonstrates 28S and 18S ribosomal bands for estimating the loading amount of RNA. A similar RNA loading pattern was obtained when the filter was probed with glyceraldehyde phosphate dehydrogenase (data not shown).

Methylation of *IGF2* in Wilms Tumors with Normal *IGF2* Imprinting. Methylation of *IGF2* was examined with the methylation-sensitive restriction enzyme *Hpa* II or *Hha* I. When Southern blots were hybridized with an *IGF2* cDNA probe that includes exons 3, 7, 8, and 9 (22), tumors that expressed *IGF2* monoallelically showed a unique pattern of fragments of ≈3 kb, which were resistant to both *Hpa* II (Fig. 2*a*, lanes 8–12, arrows) and *Hha* I (Fig. 2*b*, lanes 8–12, arrows) digestion. Similar methylation patterns were obtained for human fetal kidney (data not shown). Since this high molecular weight DNA fraction was not seen in tumors that had undergone maternal LOH (Fig. 2 *a* and *b*, lanes 13–16), these methylated fragments were presumed to be derived from the maternal *IGF2* allele.

To determine whether there was allele-specific methylation of *IGF2*, the *Hpa* II-resistant fragments in Fig. 2*a* (lanes 8–12) were isolated from an agarose gel and amplified by PCR using primers to detect the *Apa* I or AC repeat polymorphism. As shown in Fig. 3*a* (lanes 2, 4, and 6) and Fig. 3*b* (lanes 1 and 3), the *Hpa* II-resistant fragments were derived from one allele only. This finding contrasts with RNA PCR data, which showed that transcription occurred from the other *IGF2* allele—that is, the allele that was not methylated (Fig. 3*a*, lanes 3, 5, and 7; Fig. 3*b*, lanes 2 and 4). Because *IGF2* is transcribed from the paternal allele (3–5, 10), we conclude that the nonexpressed maternal *IGF2* allele in these tumors was extensively methylated. This conclusion was formally verified in one case in which parental DNA was available. As shown in Fig. 3*b*, the methylated *Hpa* II-resistant fragments were derived from the upper allele (lane 3), while the expressed allele (lane 4) originated from the lower allele. A comparison of these data with the parental genotypes (lanes 6 and 7) demonstrated that *IGF2* was expressed from the unmethylated paternal allele. In contrast to DNA methylation studies in the paternally disomic mouse (33), these studies show that in human kidney and Wilms tumors with paternal *IGF2* expression the active paternal allele is unmethylated.

Methylation of *IGF2* in Wilms Tumors with Relaxed *IGF2* Imprinting. In seven Wilms tumors with biallelic *IGF2* expression (Fig. 2*a*, lanes 1–7), *Hpa* II digestion resulted in a limited digest pattern (two fragments of 1.2 and 0.8 kbp) corresponding to that seen with *Msp* I digestion (Fig. 2*a*, lanes 17–19). In contrast to the tumors that expressed *IGF2* monoallelically, in these tumors the maternally derived *Hpa* II-resistant fragments were absent (Fig. 2*a*, lanes 8–12). The same pattern was observed with *Hha* I digestion (Fig. 3*b*, lanes 1–7). These data indicate that there was extensive hypomethylation of both *IGF2* alleles in tumors in which relaxation of *IGF2* imprinting had occurred. Similarly, in tumors with maternal 11p LOH (Fig. 2 *a* and *b*, lanes 13–16) and another seven tumors (data not shown), the absence of *Hpa* II- and *Hha* I-resistant fragments indicates that the retained *IGF2* paternal allele was extensively hypomethylated.

Methylation of *H19* Alleles in Wilms Tumor. The methylation status of the *H19* alleles was also examined. The Southern blot that had previously been probed with an *IGF2* cDNA probe (Fig. 2*a*) was stripped and reprobed with an *H19* cDNA probe. As shown in Fig. 2*c* (lanes 8–12, arrows), low molecular weight fragments were present in DNA samples from tumors that expressed *IGF2* monoallelically. Since these low molecular weight *H19* fragments were not present in the DNA of tumors that had undergone maternal 11p LOH (Fig. 2*c*, lanes 13–16), this provides evidence that the inactive paternal allele of the *H19* gene was methylated. A similar pattern of methylation was found in tumors in which *H19* expression was absent and relaxation of *IGF2* imprinting had occurred. As shown in Fig. 2*c* (lanes 1–7), both alleles of the inactive *H19* gene were extensively methylated. These data are consistent with previous work, which showed that the nonexpressed paternal *H19* allele is methylated (34).

Methylation of Other 11p15 Genes in Wilms Tumor. Because altered methylation of some 11p genes has been previously observed in human tumors and cell lines (35), we determined whether these changes were due to a generalized alteration in DNA methylation at 11p15 or confined to the *IGF2/H19* locus. As shown in Fig. 4, after reprobing the Southern membranes used in Fig. 2 with a series of chromosome 11p probes, including insulin (Fig. 4*a*), HRAS (Fig. 4*b*),

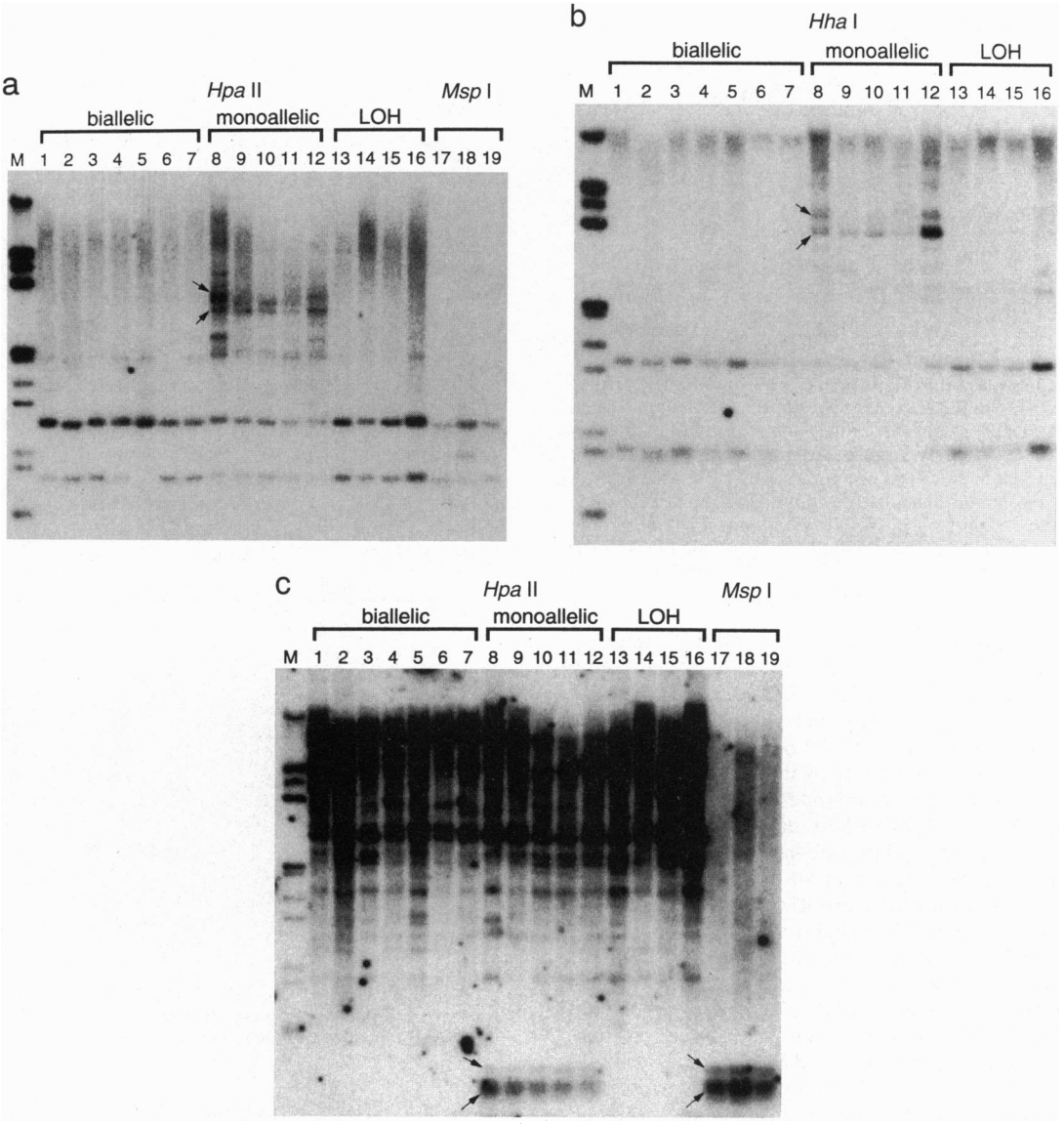

FIG. 2. *IGF2* (*a* and *b*) and *H19* (*c*) methylation in Wilms tumor. Genomic DNA from seven Wilms tumors with biallelic *IGF2* expression (lanes 1–7), five with monoallelic *IGF2* expression (lanes 8–12), and four with 11p LOH (lanes 13–16) were digested with *Hpa* II (*a* and *c*) or *Hha* I (*b*). Tumors with biallelic *IGF2* expression (lane 17), monoallelic *IGF2* expression (lane 18), and LOH (lane 19) were digested with *Msp* I (*a* and *c*) and then analyzed by Southern blotting with probes for *IGF2* (*a* and *b*) or *H19* (*c*). Molecular size was indicated as λ *Hin*dIII/*Eco*RI-digested fragments in lane M.

and β-globin (data not shown), it is apparent that there was no consistent difference in methylation between tumors with or without relaxation of imprinting. These data indicate that in Wilms tumors with relaxed imprinting of *IGF2*, the changes in DNA methylation—i.e., the gain of methylation at *H19* and the loss of methylation at *IGF2*—are confined to the *IGF2/H19* locus.

DISCUSSION

A major finding of this paper is that relaxation of *IGF2* imprinting in Wilms tumors is linked to specific alterations in the methylation patterns of both the *IGF2* and *H19* genes. These methylation changes affect the maternally inherited *IGF2* and *H19* genes such that there is a gain of methylation at *H19* and a loss of methylation at *IGF2*. Concomitant with these methylation changes is a reversal in the normal pattern of gene expression such that the maternally inherited *H19* and *IGF2* alleles are silenced and activated, respectively.

Recent studies have shown that relaxation of *IGF2* imprinting in Wilms tumor is associated with methylation of the *H19* gene (19, 20), while here we report that methylation changes encompass the entire *IGF2/H19* domain. More importantly, we have addressed the question of the specificity of methylation of these imprinted genes in Wilms tumor. DNA methylation changes at the *IGF2* and *H19* gene loci could reflect one of the two following situations: (*i*) the changes in DNA methylation were specific for the *IGF2/H19* locus, or (*ii*) the methylation changes were simply a by-product of a generalized alteration in DNA methylation, which is characteristic of many tumors (36–38). We have shown that in all Wilms tumors, 11p15 genes were hypermethylated, regardless of the specificity of the methylation pattern at the *IGF2/H19* locus. The specificity of the methylation changes of the *IGF2* and *H19* maternal alleles in tumors in which relaxation of *IGF2* imprinting had occurred therefore provides evidence consistent with an epigenetic alteration at this locus being central to the tumorigenesis pathway.

Data suggesting that *IGF2* is methylated in an allele-specific manner has previously been reported in one kidney sample;

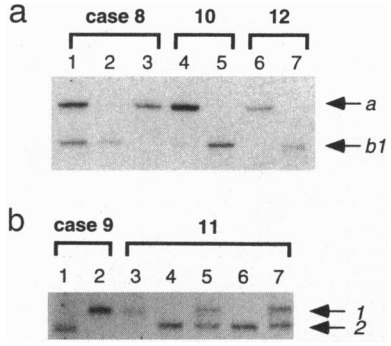

FIG. 3. Correlation between expression and undermethylation of *IGF2* (*a* and *b*) and parental origin of the methylated alleles (*b*) in Wilms tumors with monoallelic expression of *IGF2*. (*a*) Genomic DNA of case 8 (lane 1), DNA isolated from the 3-kb region in Fig. 2, lanes 8, 10, and 12 (lanes 2, 4, and 6), and the corresponding cDNA of cases 8, 10, and 12 (lanes 3, 5, and 7) were amplified by PCR with a set of primers for *Apa* I polymorphism. (*b*) Genomic DNA of case 11 (lane 5), DNA from the 3-kb region in Fig. 2, lanes 9 and 11 (lanes 1 and 3), cDNA of cases 9 and 11 (lanes 2 and 4), and genomic DNA of the father (lane 6) and mother (lane 7) of case 11 were amplified with a set of primers for AC repeat polymorphism.

however, the parental origin of methylation and *IGF2* imprinting status were not determined (39). Recently, it was found that a region in exon 9 of the human *IGF2* gene is specifically methylated on the paternal allele in peripheral blood and in a variety of tissues from patients with the Beckwith–Wiedemann syndrome (40), but we have been unable to demonstrate a similar pattern of methylation at this site in normal kidney and Wilms tumors (M.J.S., unpublished data). The epigenetic modification of the *IGF2* locus that we have detected in Wilms tumors and kidney is not present in all tissues and therefore provides an example of a tissue-specific methylation imprint that is unrelated to gamete-of-origin imprinting. In contrast to these findings, it has been reported that in mouse embryos there is a region of methylation upstream from the paternal *IGF2* allele, although exons of both alleles are largely unmethylated (33). The differences in methylation seen in these mouse tissues and in human kidney and Wilms tumors may be due to variations in tissue-specific methylation imprinting.

IGF2 is expressed monoallelically in most tissues; however, biallelic expression is found in the choroid plexus and leptomeninges where there is also no expression of *H19* (10, 41, 42). This finding, together with the close proximity of *IGF2* and *H19*, has led to the suggestion that *IGF2* and *H19* form a coordinately regulated imprinted domain, which may be controlled by a bidirectionally acting *H19* enhancer (1, 11–14). The question arises as to the mechanism of the epigenetic changes that lead to relaxation of *IGF2* imprinting. Parallels with the *XIST* gene may provide clues to understanding the events leading to the epigenetic changes at the *IGF2/H19* locus. For example, *H19* and *XIST* both act in cis, neither of the RNA products appears to be translated, and both genes lie within imprinted regions of the genome (43, 44). By analogy with *XIST* and chromosome X inactivation, the function of *H19* may be required to initiate events that lead to inactivation of the *IGF2/H19* locus. Accordingly, data shown here which demonstrate that methylation of the human *IGF2* gene occurs in a tissue-specific fashion and equates to gene silencing are consistent with *IGF2* methylation being secondary to the events that initiate the allele-specific repression of *IGF2* transcription.

That *H19* may be the primary imprinted gene, the activity of which is required to suppress *IGF2* transcription in cis, has previously been suggested to explain the patterns of *H19* and *IGF2* expression in methylase-deficient mice (14). Experiments reported here are consistent with the notion that

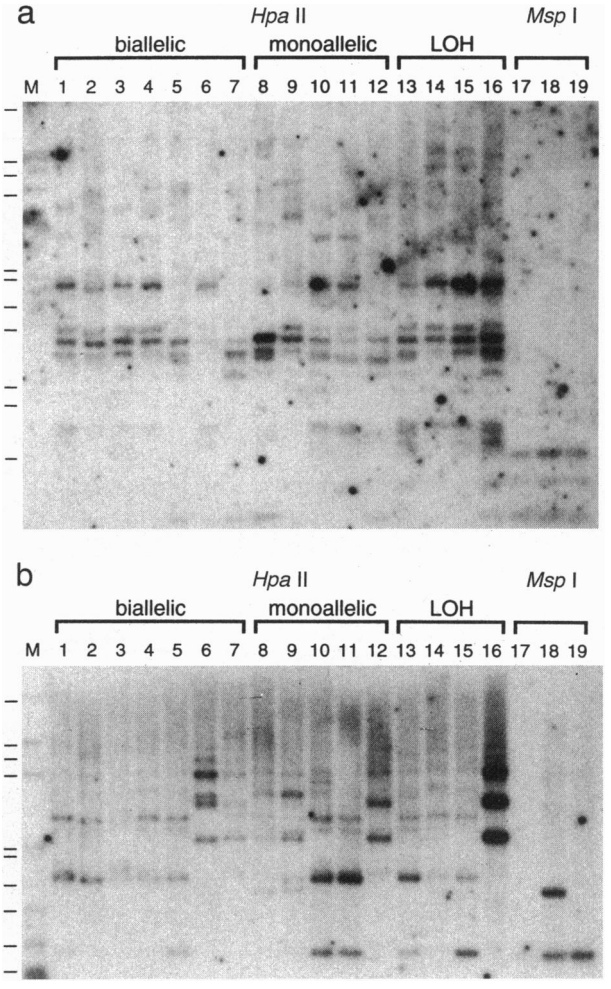

FIG. 4. Insulin (*a*) and HRAS (*b*) methylation in Wilms tumor. Southern filters of Fig. 2 were stripped and reprobed with insulin (*a*) and HRAS (*b*) DNA probes. DNA samples correspond to the same lanes in Fig. 2.

relaxation of *IGF2* imprinting in Wilms tumor may be initiated by a pathologic methylation and silencing of the maternal *H19* allele, which in turn leads to loss of methylation and activation of the maternal *IGF2* allele as a secondary event. In support of this model, we have shown that in all Wilms tumors examined, genes within 11p15 were consistently methylated, suggesting that relaxation of *IGF2* imprinting could have arisen by an aberrant *H19* methylation. This model makes several predictions. For example, the constitutional relaxation of *IGF2* imprinting observed in some children with somatic overgrowth and predisposition to Wilms tumor (45, 46) may sometimes be associated with aberrant methylation and silencing of the actively transcribed maternal *H19* allele. In support of this possibility, we have recently shown that in one patient with gigantism and Wilms tumor, both *H19* alleles were constitutionally methylated and *IGF2* was transcribed biallelically (T.T., unpublished data). Similarities to this situation may be forthcoming from *H19* null mice. It will be intriguing to determine whether the germ-line inactivation of the maternal *H19* gene in mice can lead to a growth-enhanced phenotype similar to the Beckwith–Wiedemann syndrome.

We thank Michael Eccles for helpful comments on the manuscript, Shirley Tilghman for providing the *H19* cDNA probe, and Luciano Dallapozza, Childrens Medical Research Institute, Sydney, for providing tumor material. M.J.S. is a recipient of an Air New Zealand Cancer Research Repatriation Fellowship. This work was supported by

the Lottery Grants Board, Health Research Council, and the Cancer Society of New Zealand.

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