Genomic Deletion of an Imprint Maintenance Element Abolishes Imprinting of Both Insulin-like Growth Factor II and H19*

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Insulin-like growth factor II (Igf2) is maternally imprinted in normal tissues with only the paternal copy of the gene being transcribed, whereas the contiguous gene H19 is paternally imprinted. Dysregulation of IGFB2 imprinting is commonly observed in Wilms’ tumor and other human tumors. Previous work comparing promoter-specific imprinting of human and mouse Igf2 suggested the presence of a cis element upstream of Igf2 that regulates or maintains the imprinting of three downstream promoters. To explore the molecular mechanism of maintenance of genomic imprinting, we targeted the region between insulin 2 and Igf2, where the cis imprint maintenance element (IME) resides in mouse fibroblasts. In those clones in which the targeting vector was randomly integrated into the genome, mouse Igf2 remained imprinted. However, when the targeted region containing the IME was deleted by homologous recombination, whether from the paternal or maternal allele, activation of the imprinted maternal allele of Igf2 was observed. In addition, there was a loss of H19 imprinting when the IME was deleted. The requirement of IME from both parental alleles for the maintenance of genomic imprinting thus suggests the importance of a spatial structure of DNA around Igf2 and H19. Modifications in the IME, like abnormal methylation in Wilms’ tumors, may represent a novel mechanism for loss of genomic imprinting.

Genomic imprinting refers to a process whereby there is unequal transcription of the two parental alleles. Approximately 20 mammalian genes are known to be imprinted, and 5 of them, insulin-like growth factor II (Igf2)1 (1), H19 (2), Mash2 (3), insulin 2 (4), and KIP2 (5), are aggregated on the distal end of chromosome 7 in mouse. Although Igf2 and H19 are contiguous genes, Igf2 is exclusively expressed from the paternal allele, whereas H19 is only expressed from the maternal allele. In mouse, these two genes are coordinately regulated (6), with high expression in embryonic and neonatal tissues and decreased expression in adult tissues (7).

We (8) and others (9) have previously shown that the four promoters of human IGF2 show different patterns of imprinting, depending upon the tissues tested. The first promoter of human IGFB2 (hP1) is located just downstream of the insulin gene. Like insulin in the pancreas (4) and central nervous system (10), the IGFB2 transcripts from hP1 are derived from both parental chromosomes, showing no imprinting, in liver and chondrocytes. Three downstream promoters, hP2–hP4, on the other hand, are always imprinted. The differential imprinting of human IGFB2 promoters thus suggests the presence of an imprint maintenance element (IME) between hP1 and the other three promoters (8). Examination of promoter-specific imprinting of mouse Igf2 also lends support for the hypothesis that an IME lies upstream of three mouse promoters. Murine Igf2 contains three promoters (mP1–mP3) that are homologous to the three imprinted promoters (hP2–hP4) of human IGFB2. As expected, all Igf2 transcripts from these three promoters are expressed mono-allelically in all tissues tested (11), except for central nervous system, where all three promoters drive Igf2 expression from both parental alleles (1, 11).

DNA methylation studies also suggest the presence of an IME in the area upstream of Igf2. Genomic imprinting requires the two parental alleles to be differentially marked for the appropriate expression pattern. Methylation at CpG dinucleotides within or near the imprinted genes is a promising candidate for this epigenetic marking. A short stretch of genomic DNA ~3 kb upstream of mouse Igf2 has been found to be differentially methylated between the two parental alleles (12, 13). Dynamic changes in the methylation of CpG dinucleotides in this area parallel the establishment of genomic imprinting during development (12). In cultured MadI7 cells, acquired DNA methylation in the 5’ region of Igf2 is associated with reactivation of the maternal allele (6). It was thus proposed that differential methylation at these CpG sites may serve as an imprinting signal (12).

In mouse embryo mutants that are deficient in the DNA methyltransferase gene (dnmt), allelic expression of Igf2 is altered such that the normally expressed paternal allele is repressed (14), suggesting the requirement of DNA methylation for the normal imprinting of Igf2 in the early embryo. By using neonatal astrocytes as a model, however, we found that DNA demethylation induced by 5-azacytidine increases expression of Igf2 by releasing the repressed allele (15). Taken together, these results suggest that the role of DNA methylation in Igf2 imprinting depends upon the developmental stage. Deficiency in DNA methylation prior to resetting of the imprint will suppress expression of Igf2 from the active allele, whereas DNA demethylation in cells derived from neonates will reactivate the imprinted allele, leading to loss of imprinting. When the wild-type dnmt was expressed in dnmt-deficient ES cells, normal methylation and expression of those imprinted genes were not efficiently restored unless the cells passed through the germ line (16). Thus, resetting and maintaining the imprint may represent two functionally separate processes that are

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1 The abbreviations used are: Igf2, insulin-like growth factor II; IME, imprint maintenance element; kb, kilobase pairs; PCR, polymerase chain reaction; bp, base pairs.
regulated by different mechanisms, although both are associated with DNA methylation. It is interesting to note that the high frequency of abnormal imprinting of IGF2 in many human tumors, especially in Wilms’s tumors (17, 18), is also related to the failure of maintaining the imprint.

Therefore, we utilized cultured skin fibroblasts as a model to study those factors that are involved in the maintenance of IGF2 imprinting. Because IGF2 imprinting in these fibroblasts has been established during embryonic life, this model will allow us to exclude those factors that participate in resetting of the imprint. In the knock-out mouse model, however, the targeting deletion occurs in stem cells, and therefore, this model may be more useful in studying those factors that participate in the development rather than the maintenance of the imprint. Using our fibroblast model, we demonstrate here that deletion of the IME by genomic targeting results in biallelic expression of both IGF2 and H19, suggesting that the loss and/or abnormal modification of IME, as in Wilms’s tumor, may directly or indirectly involved in alterations of the imprint.

MATERIALS AND METHODS

Targeting Vector—The goal of this experiment was to use homologous recombination to delete a DNA fragment that covers three HpaII sites that are differentially methylated between the parental alleles (12) and thus may contain the cis element that is required for the maintenance of IGF2 imprinting. A 2.5-kb BamHI fragment (19), containing the coding region of mouse insulin 2 (kindly provided by Dr. Chirgwin), was ligated into the BamHI site of pNTK vector (20). A 6-kb fragment of HindIII-SphI DNA (Fig. 1), covering the first four exons of mouse Igf2 (21, 22), was derived from a 15-kb clone of mouse Igf2 (kindly provided by Dr. Holthuizen) and was ligated to HindIII-SphI sites of pCAT2 vector (23). The 6-kb fragment of Igf2 DNA was then cut with HindIII-ClaI and was ligated into the pNcT in which 2.5-kb insulin DNA was introduced. Plasmid DNA of the targeting vector was purified by CsCl gradient centrifugation and was used for cell transfection by electroporation as below.

Mouse Skin Fibroblasts—Two mouse fibroblast cell lines that were prepared from newborn interspecific mice were used for genomic targeting. One fibroblast cell line (MSK02) was cultured from the skin of F1 interspecific mice derived from breeding M. musculus females with M. spretus males. The other cultured fibroblast cell line (MSK10) was derived from skin of the informative backcross between F1 females and M. musculus males. Fibroblasts were cultured as described previously (15). Fresh skin was removed from newborn mice and minced with scissors into small pieces and centrifuged 5 min at 150 × g. Fresh skin was removed from newborn mice and minced with scissors into small pieces and centrifuged 5 min at 150 × g. The tissue pellet was directly suspended in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 15% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin, and grown at 37 °C in 5% CO2. When confluent, the cells were trypsinized and subcultured into fresh plates. From those plates, cells were collected for targeting.

These two fibroblast cell lines contain a BsaAI polymorphism in exon 6 of IGF2 (Fig. 1), which allows each parental allele to be identified (11, 15). MSK02 cells derived from F1 mice expressed only the paternal M. spretus Igf2 allele (that can be digested by BsaAI), whereas MSK10 cells, derived from backcross mice, exclusively expressed the paternal M. musculus allele, which cannot be digested by BsaAI. These two fibroblast cell lines maintained the genomic imprinting of IGF2 under these culture conditions for at least 16 passages (data not shown). We used the third passage of fibroblasts for this genomic targeting study.

Genomic Targeting—The targeting vector was linearized at a unique MluI site and then transfected into the two mouse fibroblast cell lines by electroporation at 960 microfarads and 220 V. The transfected cells were double-selected by G418 (200 μg/ml) and ganciclovir (100 μg/ml) 24 h after electroporation. Antibiotic-resistant cell clones were collected for DNA and RNA analysis. Total nucleic acids, prepared as described previously (15) from the cell clones, were directly screened by PCR using primers in the targeted region. The oligonucleotide primers used for screening targeting clones were as follows: number 3302 (5′-primer), GGGCAACACGTC-ATGCTCCCTGTAG; and number 3303 (3′-primer), CTGGTCCTGCT-CAAGAGGAGGTCA. The 5′-primer (#3302) used in the PCR reaction was end-labeled with [γ-32P]ATP. The cDNAs were amplified for 30 cycles at 95 °C for 30 s, 65 °C for 40 s, and 72 °C for 20 s, followed by a 5-min extension at 72 °C. The amplified PCR products were diluted and digested with the BsaAI restriction enzyme for examining the allelic expression of IGF2 (11).

The allelic expression of H19 was assessed using PCR primers covering H19 exons 8 and 9 under the same PCR conditions as used for IGF2 imprinting. PCR primers were as follows: number 4025 (5′-primer), TAATGGATTGCAGCTGTGGTGAGGT; and number 4026 (3′-primer), TTGACTCGTTCCTGGAGGT. The 5′-primer (no. 4025) was end-labeled with [γ-32P]ATP. PCR DNA were digested with FokI, a morphic site found only in M. spretus (12), to distinguish allelic expression of H19.

Promoter-specific Expression of Igf2—The promoter-specific RNA transcripts were amplified from cDNA samples by using each promoter-specific primer and a common γ-32P-end-labeled reverse primer (11). The oligonucleotide primers used for promoter amplification were as follows: 1) no. 1871 (mp1-specific 5′-primer), ACCGGGCATTGCGCGATGC/TTCC; 2) no. 1872 (mp2-specific 5′-primer), CGTGCGCATTGGCGCCTC/TGGCCT; and 3) no. 1873 (mp3-specific 5′-primer), TCTCTCTCTCT/TCTCTAGCCCCACGGC; and no. 2384 (the common 3′-primer), TGTAAGAACTGCGACCGCCTAGTATC. The PCR conditions were the same as those used for allelic expression of Igf2, except for the longer extension time (72 °C for 1 min). For the dominant promoter, mp2, a single PCR was used to amplify the full-length cDNA. However, nested PCR was used to amplify the less-abundantly utilized promoters (mp1 and mp3). The amplified products (2.5 μl) were digested with 0.5 units of MboI, which specifically digests the M. spretus allele of H19 (24), and then separated on a 4.5% polyacrylamide gel.

RESULTS

Genomic Targeting of IME—The DNA fragment deleted between the mouse insulin 2 gene and Igf2 (Fig. 1) contains a short stretch of CpG dinucleotide sequences, which are differentially methylated on each parental chromosome (12). These CpG dinucleotides were assumed to function as an imprint maintenance element to guide the allelic expression of three downstream imprinted promoters. After double selection with G418 and ganciclovir (25), a total of 64 single resistant colonies were obtained. We then used the DraI polymorphism site (12), which is located within the deleted region, to distinguish those clones that showed homologous recombination from those in which the targeting vector is randomly integrated into the genome (Fig. 2). In the homologously targeted clones, only one of the parental alleles is detected by the sensitive PCR method. The other parental allele in the targeted region is not amplified because it was deleted by genomic targeting (Fig. 2B, lanes 9–12). In those clones in which the vector was randomly integrated, however, the two intact parental alleles are amplified (Fig. 2B, lanes 5–8). Furthermore, by using this DraI polymorphism, it is possible to distinguish which parental allele has been targeted.

By screening all the resistant clones, we noted that all deletion-positive clones lost the M. musculus allele, whether it was paternally or maternally derived. None of the identified clones showed deletion of the M. spretus allele. This specificity was probably due to the fact that the targeted vector (Fig. 1) was constructed from a DNA fragment derived from M. musculus.
Fig. 1. Deletion of the IME of Igf2 by homologous recombination. A, human IG2, which contains three imprinted promoters (hP2–hP4) and the nonimprinted hP1. B, mouse Igf2, which encompasses only three imprinted promoters (mP1–mP3). Two pseudoexons, α1 and α2 (21), which are homologous to exons 2 and 3 of human IG2, are also shown. Comparison of these two Igf2 genes suggests the presence of an IME for Igf2 in the area between insulin 2 and Igf2 in mouse (11), and between hP1 and three imprinted promoters (hP2–hP4) in human (8). C, the targeting vector. TK, thymidine kinase. D, the predicted structure of the deleted area of Igf2 and insulin. As a result of homologous recombination, a 13-kb fragment of DNA between mouse insulin 2 and Igf2 was replaced by a neomycin gene (neo). All three downstream promoters (mP1–mP3) were maintained in their intact structure. The BsaAI polymorphic site (bold) (11) was used to distinguish the allelic expression of Igf2.

and is therefore identical to the M. musculus allele. As a result, the latter allele had a higher likelihood of being targeted by homologous recombination (25, 26). The M. spretus allele contains several known and potentially many other unknown polymorphisms in the targeted region (12, 13), and it thus was much less likely to be targeted.

Loss of Igf2 Imprinting by Targeting—The influence of deletion of the IME on genomic imprinting of Igf2 (Fig. 3) was examined in fibroblast clones by methods described elsewhere (11, 15). As predicted, both MSK02 and MSK10 cells collected prior to genomic targeting showed absolute mono-allelic expression of Igf2 (Fig. 3, A and B, lane 4), with a typical maternal imprinting pattern (11, 15), indicating that the imprinting trait has been faithfully maintained in these fibroblasts.

The cells that contained the targeted vector by random integration into the genome also expressed only the paternal allele (Fig. 3A, lanes 5–8 and Fig. 3B, lanes 5–7), indicating that DNA integration of the targeting vector into random sites of the mouse genome does not interfere with the regulation of Igf2 imprinting. In those cells in which the IME was deleted by homologous recombination, however, the Igf2 maternal allele, which was originally suppressed, became expressed at variable levels (Fig. 3A, lanes 9 and 10 and Fig. 3B, lanes 8–11). Biallelic expression of Igf2 was observed in cells in which the IME had been deleted from the maternal chromosome (Fig. 3A) as well as from the paternal chromosome (Fig. 3B). As a result, all targeted clones showed loss of genomic imprinting, expressing Igf2 from both parental alleles.

Igf2 Promoter Usage—We then asked whether all three Igf2 downstream promoters exhibited absence of imprinting after targeted deletion of the IME. Full-length RNA transcripts of each Igf2 promoter were amplified by PCR using promoter-specific primer sets (11, 15) and were subsequently subjected to MboI digestion (24), allowing each parental allele to be distinguished (Fig. 4A). In MSK02 cells, Igf2 promoter mP2 was the predominant promoter; similar results have previously been observed in astrocytes (15) and in the F1 generation skin (11) from which MSK02 was cultured. After digestion with MboI, the clones that were targeted by homologous recombination showed biallelic expression of Igf2. As expected, there was a maintenance of Igf2 imprinting from each of the three promoters in those clones that exhibited random integration of the targeting vector in the genome (Fig. 4B). As compared with mP2, the other two promoters (mP1 and mP3) of Igf2 were less abundantly expressed. In cells targeted by homologous recombination, Igf2 was biallelically expressed from each of the three promoters. Thus, genomic imprinting from each of the three downstream promoters of Igf2 was lost when the upstream IME was deleted.

Genomic Imprinting of H19—We were curious whether the deletion of the Igf2 IME would affect the local DNA structure and thus influence the imprinting of H19. The cDNA samples were amplified by PCR using the same PCR conditions as used for Igf2 and were subjected to digestion by FokI, which identifies a unique polymorphic site in M. spretus H19 (12). As seen in Fig. 5, H19 also showed loss of genomic imprinting when the IME was deleted in MSK02 fibroblasts (lanes 9 and 10). In those clones with random integration of the targeting vector, H19 was monoaallelically expressed from the maternal allele (lanes 5–8), indicating the maintenance of genomic imprinting. Similar results were also seen in targeted MSK10 fibroblast clones, although the expression levels of H19 were very low (data not shown). Taken together, these results suggest that although the IME is located upstream of Igf2, it may also participate in the distal regulation of H19 genomic imprinting.

DISCUSSION

Using genomic targeting, we have demonstrated that deletion of an IME located upstream of Igf2 interferes with the maintenance of imprinting of both Igf2 and H19. All three Igf2 promoters showed the loss of genomic imprinting in the targeted cell clones. In those clones in which the targeting vector

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was integrated into the genome in a random fashion, there was a normal imprinting pattern identical to that seen in normal control fibroblasts. These results suggest that the IME is essential for the maintenance of normal imprinting of the downstream \textit{Igf2} gene. Loss of genomic imprinting of all \textit{Igf2} promoters is also seen in mouse central nervous system (11), Wilms' tumors (27), and rhabdomyosarcoma (28), where all imprinted promoters of \textit{Igf2} (mP1–mP3 in mouse and hP2-hP4 in human) become biallelically expressed. Thus, loss of the IME as achieved by targeted deletion in this study or abnormal modification of the IME, such as changes in DNA methylation, may represent a mechanism for the dysregulation of genomic imprinting of \textit{Igf2} seen in Wilms' tumor (17, 18, 27) and many other human tumors (29, 30).

DNA methylation has been suggested to be an important molecular mechanism for initiating and/or maintaining genomic imprinting. Parental DNA –3 kb upstream of \textit{Igf2} promoter is differentially methylated, with hypermethylation observed in the expressed paternal DNA and hypomethylation in the imprinted maternal DNA (12, 13). Reduction of \textit{Igf2} expression during passage of cells was paralleled by the demethylation of the 5' region of \textit{Igf2} (31). Mouse embryos that lack DNA methyltransferase activity by homologous targeting (14) were found to have abnormal imprinting. The paternal allele of the \textit{IGF2} gene, which is normally expressed, became transcriptionally silent due to the global demethylation of the genome because of the deleted DNA methyltransferase. In both human and mouse astrocyte cells, DNA demethylation induced by treatment with 5-azacytidine and 2-deoxy-5-azacytidine, two potent DNA methylation inhibitors, also modulated the allelic expression of \textit{Igf2} (15), leading to biallelic \textit{Igf2} expression.

\textit{H19} is located downstream of \textit{Igf2}. However, as opposed to \textit{Igf2}, only the maternal allele of \textit{H19} is usually transcribed. When the region of chromosome 7 containing \textit{H19} and its enhancers is deleted by homologous recombination, there was an increase in expression and loss of genomic imprinting of \textit{Igf2} (32), suggesting the requirement of a spatial structure of genomic DNA around \textit{Igf2} and \textit{H19}. It was hypothesized that both \textit{H19} and \textit{Igf2} may compete for two shared enhancers (33). Due to the paternal imprinting, untreated MSK02 cells as well as those clones that had the randomly integrated vector exclusively expressed the \textit{M. musculus} allele. Two MSK02 clones that underwent homologous recombination, however, showed biallelic expression of \textit{H19}. In four targeted MSK10 clones, which were cultured from backcross mice, \textit{H19} was transcribed at very low levels. However, using nested PCR (11), we were still able to observe either loss of imprinting or allelic switching in some clones (data not shown).

Our interspecific cell culture provides a unique system for studying genomic imprinting. Although it would be interesting to replicate our data in an in vivo model, this fibroblast model provides different information from a knock-out mouse. In our model, the deletion of the IME occurs after the imprinting pattern has already been established and maintained through numerous cell passages, whereas in a traditional knock-out mouse, the targeting deletion occurs before the establishment of genomic imprinting. Thus, our model allows us to examine those factors that are involved in the maintenance of \textit{Igf2} imprinting which was established during embryonic life. Various factors may play ontogenetically different roles in control-
referred to as the Igf2 Imprinting Element. A, MSK02 cells cultured from F1 newborn mouse derived from M. spreptus male and M. musculus female. The IME has been deleted from the maternal chromosome. Prior to genomic deletion (lane 4), or in those cell clones that have random integration of the targeting vector (lanes 5–8), only one parental allele of Igf2 was transcribed, indicating the maintenance of normal imprinting. In two targeted cell clones (lanes 9 and 10), however, both parental alleles of Igf2 were actively expressed, indicating loss of imprinting of Igf2 after deletion of the IME. Lane 1 is a 100-bp DNA ladder. B, MSK10 cells cultured from a backcross mouse derived from F1 female and M. musculus male. The IME has been deleted from the paternal chromosome. MSK10 cells normally express Igf2 from the paternal M. musculus allele, which was not digested by BsaI (11). In these cells, the maternal M. spreptus allele of Igf2 was not expressed (lanes 4–7). In four clones (lanes 8–11) in which the IME from the paternal M. musculus allele was deleted, Igf2 was biallelically transcribed. Lane 1 is a 100-bp DNA ladder.

The IME, which is located upstream of the neo gene, was not digested by BsaI in these cells, whereas biallelic expression of Igf2 was observed when the IME was deleted (lanes 9 and 10). Lane 1 is a 100-bp DNA ladder.

In our study, however, we did not observe the adoption of expression of the neo gene because as a selective marker, neo was always expressed, regardless of its paternal or maternal position in targeting. Neither did we see the effect of the inserted neo on the relative expression of the downstream Igf2 promoters. As previously reported in astocytes (15) and in F1 skin (11), the three Igf2 promoters (mP1–mP3) are differentially transcribed (Fig. 4), with the majority of Igf2 mRNA transcripts originating from mP2. It should be emphasized that after targeting, those clones undergoing homologous recombination still maintained the same pattern of promoter usage as those randomly integrated or normal control clones, where Igf2 promoter (mP2) was dominantly transcribed, and the first promoter (mP1), which is located immediately downstream of neo, was utilized at very low level. This finding suggests that neo did not artifactually induce the transcription of genes downstream from its insertion.

It is not obvious how the IME, which is located upstream of Igf2, can affect the genomic imprinting of H19 which is ~100 kb distant from Igf2. Experiments in knock-out mice have demonstrated that parental deletion of H19 and two H19 enhancers dramatically affect the allelic expression of both Igf2 and H19 (32). However, loss of Igf2 imprinting in these mice could not be rescued by introducing the transgene containing H19 and H19 enhancers (34). These results suggest that the intact chromosomal structure around Igf2 and H19 is crucial
Igf2 Imprinting Element

for the regulation of Igf2 and H19 imprinting. One possible explanation for this phenomenon may be that Igf2 and H19 are organized in separate topological domains (35) that are anchored together in specific regions via interactions between trans factors and cis-regulating elements. IME may be one of the cis-anchoring elements. Imprinting factor(s), when binding to the IME, may also interact with other trans factors, like enhancer-binding factors and transcription factors, thus twist-ting together the chromatin domains where Igf2 and H19 reside. As in the “kiss” model (36), it may be possible that both parental alleles join together by interacting with these trans factors and create a spatial structure that is required for the proper regulation of genomic imprinting. With this chromatin structure, genomic imprinting of Igf2 and H19 can be tightly regulated by trans factors, depending upon the methylation status of IME and other cis elements. Deletion of either parental IME or alterations in DNA methylation in the IME or in the promoter region of H19, as seen in Wilms’ tumors (37–39), or disturbances of local spatial DNA structure as accomplished by targeting in this study, may break this parental communication and thus disturb the normal maintenance of imprinting of Igf2 and H19.

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FIG. 5. Loss of imprinting of H19 in targeted MSK02 clones. Lane 1 is a 100-bp DNA ladder.
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