Promoter-specific Modulation of Insulin-like Growth Factor II Genomic Imprinting by Inhibitors of DNA Methylation*

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The insulin-like growth factor II (IGF-II) gene is maternally imprinted in most normal tissues with only the paternal allele being transcribed. In several human tumors, however, IGF-II is expressed from both parental alleles. To explore the underlying mechanism of IGF-II imprinting, we have examined the effect of DNA demethylation in cultured human and mouse astrocyte cells. An increased expression of IGF-II was observed when these cells were treated with the DNA demethylating agents, 5-azacytidine or 2-deoxy-5-azacytidine. Allelic analysis indicated that, following DNA demethylation, the increment in IGF-II mRNA was primarily derived from the normally suppressed maternal allele. Examination of promoter usage revealed that only the most proximal promoter (mP3 in mouse and hP4 in human) responded to DNA demethylating agents, whereas the expression of IGF-II from the other promoters remained unchanged. The enhanced expression of IGF-II from these promoters suggests the presence of a methylation-response element in or near mP3 and hP4. This study indicates that DNA demethylating agents increase IGF-II expression primarily by stimulating the normally imprinted allele through the activation of the most proximal IGF-II promoter.

Insulin-like growth factor II (IGF-II) is an essential factor in embryonic and neonatal development, as demonstrated by the growth retardation in mice harboring the mutated paternal allele of IGF-II (1). Despite the importance of IGF-II during embryonic development, the physiologic role of IGF-II in postnatal life is still uncertain. In some tumors, IGF-II is overexpressed as compared with normal tissues (2, 3), and it has been suggested that IGF-II may act as an autocrine or paracrine factor, enhancing tumor growth. In a transgenic mouse model, IGF-II was shown to be a necessary second signal for oncogene-induced tumorigenesis of pancreatic islets (4).

In addition to regulation by multiple promoters and alternative RNA splicing, it was recently shown that IGF-II is also subject to genomic imprinting, such that only the paternal copy of IGF-II is transcriptionally active while the maternal copy of the gene is transcriptionally silent. This uniparental expression of IGF-II has been demonstrated in human (5, 6), mouse, (7, 8), and rat (9) tissues, except for human adult liver and human and murine CNS (7–12), where both parental alleles are actively transcribed. Additionally, there is loss of IGF-II imprinting in a number of human tumors (13–16). The loss of IGF-II imprinting (LOI) may lead to increased expression of the growth factor in human tumors. However, the molecular basis for the LOI in some malignancies remains uncertain.

DNA methylation is an epigenetic modification that may be associated with the imprinting of endogenously imprinted genes (11, 17–19) and with the variable expression of exogenous transgenic DNA (20, 21). In mice, differential parental DNA methylation has been identified in several imprinted genes, including IGF-II, H19, and IGF-II receptor (IGF-IIIR) (17, 18). Altered DNA methylation has been linked to abnormal expression of IGF-II and H19 in Wilms' tumors and Beckwith-Wiedemann Syndrome (22, 23). However, it remains to be clarified whether the alteration in DNA methylation in these tumors is an etiological event or an epiphenomenon in the development of genomic imprinting.

We have shown previously that the genomic imprinting of human IGF-II is promoter-specific (12), with IGF-II from the three most proximal promoters (hP2–hP4) being exclusively monoallelic and the hP1-derived IGF-II transcripts being biallelic. In Wilms' tumors that show LOI of IGF-II, all three imprinted promoters (hP2–hP4) drive IGF-II expression from both parental alleles (24, 37), suggesting that the reactivation of the three imprinted promoters causes the LOI of IGF-II in Wilms' tumors. In examining IGF-II imprinting in interspecific mouse (8), we have found that IGF-II is imprinted in all tissues except for CNS, where both parental alleles become transcriptionally active from all three mouse IGF-II promoters (mP1–mP3). Interestingly, the two parental alleles were not transcribed in equal abundance. Instead, in some CNS tissues the paternal allele is predominantly activated, whereas in others the maternal allele is primarily transcribed. Therefore, the loss of IGF-II imprinting in CNS may represent a deregulated process of IGF-II expression that is similar to that in human Wilms' tumors with the LOI of IGF-II.

We suggest that the biallelic expression of IGF-II in the CNS may provide a useful model for studying the underlying mechanism of the LOI of IGF-II in Wilms' tumor. Based on this assumption, we prepared primary cultures of astrocyte cells from newborn mice and human embryos. We examined the effects of DNA demethylating agents on IGF-II imprinting, hypothesizing that DNA methylation governs the genomic imprinting of IGF-II. Our results indicate that DNA demethyla-
IGF-II Imprinting and DNA Methylation Inhibitor

MATERIALS AND METHODS

Mouse Brain Cells (MBR01)—The interspecific F2 mice from which cells were derived have been described previously (8). Briefly, Mus musculus male mice that carry a BsaA1 site in exon 6 of mouse IGF-II were mated with M. spreatus female mice that do not contain the BsaA1 site. The heterozygous F1 mice were informative for BsaA1 polymorphism in IGF-II exon 6 (8); therefore, each IGF-II parental allele can be easily distinguished by BsaA1 digestion.

F2 mice were sacrificed at 2 days of age. The cerebral cortex was removed under sterile conditions and placed into phosphate-buffered saline. The tissues were minced with scissors into small pieces and centrifuged 5 min at 150 g. The tissue pellet was then suspended in complete DMEM (Life Technologies, Inc.), supplemented with 15% fetal bovine serum and 100 units/ml of penicillin and 100 µg/ml of streptomycin and grown at 37 °C with 5% CO2. The medium was replaced with fresh medium 24 h after plating. When confluent, the cells were trypsinized and subcultured into fresh plates. From those plates, cells were collected and were frozen for future use.

Human Brain Cells—Fresh human brain tissues collected from three separate embryos (days 45–67) were obtained from the Central Laboratory for Human Embryonic Tissue, University of Washington, Seattle, and were shipped in phosphate-buffered saline to our laboratory. Upon arrival, the tissues were treated and cultured using the same method described above. Among three brain tissues received, two (HBR440 and HBR441) were heterozygous and thus informative for the IGF-II BsaA1 polymorphism (25) and were, therefore, used for the imprinting study.

Immunostaining of Glial Fibrillary Acidic Protein (GFAP)—GFAP is a major fibrillary component of astrocytes and is absent in fibroblasts and neurons (26, 27). Thus, GFAP staining was used to characterize the origin of the cultured brain cells used in this study. Human and mouse brain cells were grown overnight in 4-chamber slide flasks (Scientific Glass Co., San Francisco, CA), and were fixed in 0.1% paraformaldehyde. The fixed cells were stained for GFAP (28). The positive GFAP staining of these three brain cells indicated that they were derived from an astrocyte cell population.

Treatment of Cells with Demethylating Agents—Astrocyte cells at passages 5–8 were seeded in 80-cm2 flasks at a density of 2 × 10⁴ cells/ml and were treated with two cytidine analogs, 5-aza-cytidine (5-aza-C) or 2-deoxy-5-aza-cytidine (2-deoxy-5-aza-C) that are known to block DNA methylation (29, 30). The cells were randomly assigned into treatment and control groups. In treatment flasks, the culture medium was replaced with medium containing DNA demethylating agents, 5-aza-C (2 µm) or 2-deoxy-5-aza-C (1 µm), 24 h after seeding. For control flasks, media was supplied with DNA supplemented with the same volume of phosphate-buffered saline as used in the treatment flasks. Twenty-four hours later, the medium containing the DNA demethylating agents was removed, and the cells were washed twice with phosphate-buffered saline. The cells were allowed to grow in DMEM with fetal bovine serum until they became confluent and were then harvested.

Analogs of cytidine, 5-aza-C and 2-deoxy-5-aza-C, only block DNA methylation in newly replicated DNA molecules (29, 31). To obtain complete DNA demethylation, we treated cells for several continuous passages. When harvested, one portion of the cells were suspended in DMEM and treated again with 5-aza-C or 2-deoxy-5-aza-C for another three to four passages. The remainder of the cells were lysed and total nucleic acids (TNAs) were prepared using TNA extraction solution containing 4 mM guanidinium thiocyanate, 25 mM sodium citrate, 1% 2-mercaptoethanol, and 0.5% Sarkosyl. The homogenate was extracted with phenol/chloroform and then precipitated with 2-propanol. The TNA pellet was washed with ethanol and dissolved in distilled water for PCR and methylation analysis.

DNA Methylation by 5-Aza-C—To correlate DNA demethylation by 5-aza-C with IGF-II expression, we quantitated the level of DNA methylation in MBR01 cells using the method by Bestor et al. (32). TNA samples (600 ng) were digested overnight with 0.2 unit of restriction enzyme (Boehringer Mannheim). The digested DNAs were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Life Technologies, Inc., Gaithersburg, MD) and digested with nuclease P1 (U. S. Biochemical Corp.). The 5’-terminal cytosine residues were separated on silica thin layer chromatography sheets (J. B. Baker, Phillipsburg, NJ) with isobutyric acid:water:ammonium hydroxide (66:33:1) as the developing reagent.

cDNA Synthesis—For cDNA analysis, genomic DNA was first removed from the TNA samples with DNase I, and cDNA was synthesized with RNA reverse transcriptase. In a typical reaction mixture, aliquots of 2.0 µl of TNA (200–300 µg/ml), under the evaporation barrier of 10 µl of water (M, Research), were treated with 1.0 or 0.4 unit of DNase I (Stratagene, La Jolla, CA) in 25 µl Tris (pH 8.0), 25 µM NaCl, 5 µM MgCl₂, and 0.15 unit of RNase inhibitor (5 Prime → 3 Prime, Boulder, CO) at 37 °C for 45 min, followed by enzyme denaturing at 80 °C for 3 min. After DNA digestion, RNAs were reverse-transcribed into cDNAs with murine leukemia reverse transcriptase (Life Technologies, Inc.) in the presence of random hexamers at 37 °C for 25 min, followed by 5 cycles (50 °C, 20 s and 37 °C, 5 min) (8, 12).

PCR Quantitation—For an accurate quantitation of IGF-II expression among treatment groups, we used PCR to amplify IGF-II and β-actin in the same PCR reaction. The β-actin PCR products were used as an internal control to adjust for the variation among samples.

The cDNA samples were amplified in a 2-µl reaction mixture in the presence of 50 µM dNTPs, 0.2 µM of 5’-32P]-labeled primers, 0.25 µl of Taq DNA polymerase (Perkin Elmer Cetus), and 0.25 µl of cDNA (0.1 µg) in 25 µl of PCR buffer (10 mM Tris-HCl, pH 8.4; 50 mM KCl; 2.0 mM MgCl₂; 0.01% gelatin; 10% glycerol). The PCR products were electrophoresed on 5% polyacrylamide-urea gel and were exposed to the screen of the PhosphorImager scanner (Molecular Dynamics, Sunnyvale, CA). After PhosphorImager scanning, the image density was calculated against the density of internal β-actin PCR control.

For the quantitation of human IGF-II expression, primers spanning IGF-II introns 8 and 9 were used so that the PCR products from the genomic DNA and RNA could be distinguished on 5% polyacrylamide gels. To confirm this pair of primers, we determined the possibility of DNA contamination during the cDNA synthesis can be eliminated. For the PCR quantitation of mouse IGF-II expression, the primers covering the BsaA1 polymorphic site were used (8).

The oligonucleotide primers used for IGF-II quantitation (Fig. 1) include: human IGF-II: 3038 (5’-primer), TCAGCCCTTCCGGAGCGG (ATACTGTGCG, and 2384 (3’-primer), TTGGAGTACCCCTGCACCAGGGATAC; mouse IGF-II: 3302 (5’-primer), GGCGAAAACCTAGTC- TCCCCGTAT and 3303 (3’-primer), CTGCTCGCTGCTCAAGAGG- GTGCTA; and β-actin: 774 (5’-primer), GGAATTTCAACAGTGGAGG- GTGAAGGG and 775 (3’-primer), GGAAGTTTCAAGGTTCTCCGGC- CACA.

IGF-II RNA Quantitation by Ribonuclease Protection Assay (RPA)—We also used the RPA method to confirm the increased IGF-II expression by 5-aza-C treatment. The RPA quantitation of IGF-II was performed in selected MBR01 RNA samples using RPA II kit (Ambion Inc., Austin, TX). The RPA probe for IGF-II was modified by MAXIscript T7 kit (Ambion Inc.) from M. spreatus liver (8). The control probe of β-actin was in vitro transcribed from actin plasmid DNA provided by the kit. The protected RNA fragments of IGF-II and β-actin were separated on 5% polyacrylamide-urea gel and analyzed by PhosphorImager scanning.

Determination of IGF-II Imprinting—Allelic expression of IGF-II in MBR01 cells was examined as described previously (8). In brief, the cDNA samples prepared from MBR01 cells were amplified with 1-µl aliquots of end-labeled primers and digested by BsaA1 (8). To ensure the complete digestion by BsaA1, a 380-base pair control PCR DNA covering the same BsaA1 site was added to the BsaA1 digestion reaction (8). Allelic expression of IGF-II was evaluated only if the control DNA was completely digested.

The imprinting status of human IGF-II was examined using an Alu polymerase chain reaction (PCR) method in exon 9 (25). DNA-DNA hybridization assays were amplified with PCR primers end-labeled with [γ-32P]ATP. The PCR products were spiked with an Alu control DNA (300 base pairs), digested with 0.2 unit of Alu in an 8-µl reaction mixture, and then separated on 5% polyacrylamide-urea gel.

The oligonucleotide primers used for examining IGF-II imprinting (Fig. 1) include: human IGF-II: 3039 (5’-primer), TGTTGCTA; mouse IGF-II: 3302 (5’-primer), CTGCTCGCTGCTCAAGAGG-GTGCTA; mouse IGF-II: the same primer set for mouse IGF-II and β-actin PCR products were used as an internal control to adjust for the variation among samples. The cDNA samples were amplified in a 2-µl reaction mixture in the presence of 50 µM dNTPs, 0.2 µM of 5’-32P]-labeled primers, 0.25 µl of Taq DNA polymerase (Perkin Elmer Cetus), and 0.25 µl of cDNA (0.1 µg) in 25 µl of PCR buffer (10 mM Tris-HCl, pH 8.4; 50 mM KCl; 2.0 mM MgCl₂; 0.01% gelatin; 10% glycerol). The PCR products were electrophoresed on 5% polyacrylamide-urea gel and were exposed to the screen of the PhosphorImager scanner (Molecular Dynamics, Sunnyvale, CA). After PhosphorImager scanning, the image density was calculated against the density of internal β-actin PCR control.
Promoter Usage—The method for examining IGF-II promoter usage has previously been described and validated (8). For a quantitative comparison of promoter usage, we used a multiplex PCR in which cDNAs were amplified with three promoter-specific 5'-primers and a common 3'-primer end-labeled with [γ-32P]ATP. In the PCR reaction, three promoter-specific primers compete with each other for the end-labeled primer, depending upon the relative abundance of the promoter-derived transcripts (8).

The PCR conditions were the same as those used to assess IGF-II imprinting. For convenience, we designed oligonucleotide primers that were complementary to sequences shared by both human and mouse IGF-II (Fig. 1). These oligonucleotide primers were: 3490 (hP1-specific 5'-primer), CAGTCCTGAGGTGAGCTGCTGTGGC; 1871 (hP2- and mP1-specific 5'-primer), ACCGGGCATTGCCCCCAGTC(T)CC; 1872 (hP3- and mP2-specific 5'-primer), CGTGCACATTGCCCGC(T)CC(T)GCCACT; 1873 (hP4- and mP3-specific 5'-primer), TCTCCTCC(T)TCCT(A)GCCCCAGCC; and 1605 (the shared 3'-primer), CAGCAATGCGACCAA(G)AGGCGGAGCC.

RESULTS

Human and Mouse Brain Astrocyte Cells—We cultured cells from fresh brain tissues of three human embryos (days 45–67) and a newborn F1 mouse in DMEM with 15% fetal bovine serum. Under these culture conditions, the neurons died quickly after the first passage and were replaced by fibroblast-like cells. These fibroblast-like cells grew confluent in the hormone-free medium and became homogenous after several passages. Positive immunostaining for GFAP, an astrocyte-specific protein (26, 27), identified these cultured cells as astrocytes (data not shown).

DNA Methylation by 5-Aza-C—The ability of demethylating agents to affect the extent of DNA methylation was examined in MBR01 cells using MspI and HpaII restriction enzymes (32). MspI will cut cytosine residues regardless of their methylation status, whereas HpaII will only cut unmethylated cytosine residues. The amount of 5'-terminal cytosine residues created by the methylation-insensitive enzyme MspI was not significantly different between control and treated cells (Fig. 2, lane 1 versus lane 3), nor was there a difference in the methylation status of DNA in control cells at different passages. In cells treated with 5-aza-C, however, genomic DNA became largely demethylated, as demonstrated by increased density seen in those lanes where the DNA was digested with methylation-sensitive HpaII as compared with control cells (Fig. 2, lane 4 versus lane 2). It is interesting to note that treatment of MBR01 cells with 5-aza-C for one passage resulted in the demethylation of most of the genomic DNA (Fig. 2, lane 4 versus lane 3). When astrocyte cells were treated with 5-aza-C for three additional passages, virtually all CpG dinucleotides were demethylated, as shown by the equal density between MspI- and HpaII-digested samples.

IGF-II Expression in Cells Treated with DNA Demethylating Agents—To quantitate IGF-II reliably and accurately, we first used PCR to amplify a liver cDNA derived from a newborn mouse. The liver cDNA was diluted in a series of 5-fold dilutions and then amplified with IGF-II primers. One set of the diluted cDNAs was amplified by PCR for 26 cycles and the other for 30 cycles. As expected, the PCR amplification reached a plateau at high concentrations of the cDNA templates (1:50 dilution). At the lower concentrations of cDNAs, the measurement of the IGF-II PCR products, as assessed by density scanning, was linear across a wide range (Fig. 3). All assays were performed in the linear range of the curve, using 30 cycles of PCR.

Low levels of IGF-II mRNA were observed in MBR01 cells. However, when cells were treated for one passage with the DNA demethylating reagent 5-aza-C, a 100–200% increase in IGF-II expression was observed (Fig. 4A). When these treated cells were seeded and retreated with 5-aza-C for three more passages, the expression of IGF-II increased dramatically with each passage (Fig. 4A). The increased expression of IGF-II with passages was highly correlated with DNA demethylation by 5-aza-C (Fig. 2).

The increased expression of IGF-II after DNA demethylation was also confirmed by RPA quantitation in several selected samples from which we had isolated a relatively large quantity of TNA (Fig. 4B). As in PCR quantitation, IGF-II was transcribed at very low or undetectable levels in untreated control cells. However, a substantial increment in IGF-II expression was observed after DNA demethylation with 5-aza-C.

We also treated two human astrocyte cells (HBR440 and HBR441) with 5-aza-C. As in the mouse cells, the treatment of human cells with the DNA demethylating reagent resulted in the increased expression of IGF-II, especially in those cells exposed for several passages (Fig. 4, C and D). Both human astrocyte cell preparations responded to DNA demethylation with a similar increase in IGF-II expression.
A second DNA demethylating reagent, 2-deoxy-5-aza-C, is more specific for DNA demethylation and has less toxicity to host cells than 5-aza-C (33). We treated the same two human cell systems with 2-deoxy-5-aza-C at a concentration of 1 μM for two sequential passages. As in the treatment with 5-aza-C, 2-deoxy-5-aza-C significantly increased IGF-II expression (Fig. 5).

The Effect of DNA Demethylation on IGF-II Imprinting—We then asked whether the increased amount of IGF-II seen after treatment with the demethylating agents was derived from the expressed paternal allele or from the imprinted maternal allele. By clarifying this question, we hope to further explore the biochemical role of DNA methylation in the regulation of IGF-II imprinting.

Using the Apal polymorphism in human IGF-II (25) and the BsaA1 polymorphism in mouse IGF-II (8), we were able to distinguish the two parental alleles. As seen in Fig. 6, both human cell preparations showed biallelic expression of IGF-II, indicating the loss of imprinting. However, the two parental alleles were not expressed equally in these two cells. Prior to DNA demethylation, human HB441 cells primarily expressed the a allele of IGF-II (Fig. 6A), whereas HB440 cells predominantly transcribed the b allele of IGF-II. After treatment with DNA demethylating agents, these two cells showed increased IGF-II expression from both parental alleles (Fig. 6B). It was interesting to note that the DNA demethylating treatment increased the expression of the less dominant allele (presumably the imprinted allele) to levels that are comparable to those of the more abundantly expressed allele.

In mouse MBR01 cells, treatment with 5-aza-C increased IGF-II expression from the imprinted maternal allele (allele a) to a far greater extent than it did from the expressed paternal allele (allele b; Fig. 6C). We first used [α-32P]dCTP-labeled PCR to amplify cDNA samples. After BsaA1 digestion, the PhosphorImager scanning of PCR products showed a greater increase in the maternally derived a allele of IGF-II than in the paternally derived b allele. We then used the IGF-II primers end-labeled with [γ-32P]ATP for PCR amplification. This method also demonstrated a greater increase in the a allele (maternal) than in the b allele (paternal). Finally, to exclude the possibility of the formation of heteroduplexes that resist restriction enzyme digestion, we used the primer extension method to examine allelic expression in these cells. The cDNAs were first amplified with PCR for 40 cycles without [32P]deoxynucleotide triphosphate and then extended for one cycle with [γ-32P]ATP end-labeled primer. The one-cycle-extended PCR products were free of heteroduplexes and thus were sensitive to BsaA1 enzyme digestion. Again, we found more expression of IGF-II from the previously imprinted allele than from the expressed allele (data not shown).

We also examined IGF-II imprinting in the two human cells treated with 2-deoxy-5-aza-C. These studies also showed the increased expression of both alleles of human IGF-II in the treated cells (Fig. 7).

DNA Demethylation and IGF-II Promoter Usage—The expression of both human and mouse IGF-II are driven by multiple promoters. In the region of these promoters, the distribution of CpG dinucleotides varies substantially. In particular, two homologous promoters (hP3 and mP2) are rich in CpG dinucleotides.

We, therefore, asked whether there are differences in promoter-driven IGF-II expression in response to DNA demethylating agents. Because mP2 and hP3 contain more HpaII sites than other promoters, we expected that they would be more responsive to the treatment of 5-aza-C. Surprisingly, when we examined promoter usage in MBR01 cells treated with 5-aza-C, we found that the increased IGF-II expression after DNA demethylation was exclusively derived from mP3, a promoter
that contains only two HpaII sites, whereas the expression of IGF-II from mP2, which harbors 17 HpaII sites, did not change (Fig. 8A). We also examined promoter usage in the two human astrocyte cell systems treated with 5-aza-C (Fig. 8, B and C). These two human cells demonstrated results similar to those seen in MBR01 cells, i.e. only the most proximal promoter (hP4) of human IGF-II responded to the 5-aza-C treatment, whereas the IGF-II transcripts derived from hP3 were unchanged. Similar results were also obtained when these cells were treated with 2-deoxy-5-aza-C (data not shown). Thus, these results suggest the presence of a DNA methylation-response element (MRE) within or near the most proximal promoter of IGF-II (mP3 and hP4). This MRE sequence is likely to be conserved between human and mouse IGF-II genes.

Fig. 4. IGF-II expression in mouse and human astrocyte cells treated with 5-aza-C. The mouse MBR01 cells were incubated with (+) or without (−) 5-aza-C (2.0 μM) for four continuous passages. The cDNAs were prepared and amplified by PCR with IGF-II and β-actin primers (A). The increased IGF-II expression was also validated by RPA method in selected samples (B), which had a relatively large quantity of total RNA. The human HBR441 (C) and HBR440 (D) cells were incubated with (+) or without (−) 5-aza-C (2.0 μM) for three passages.
DISCUSSION

The genomic imprinting of IGF-II is an epigenetic, reversible process (1, 7, 8). The mechanism for this allele-specific expression is poorly understood. Differential DNA methylation has been proposed as the best candidate for epigenetic marking on the parental alleles. Studies in mice have demonstrated a dynamic pattern of DNA methylation during embryogenesis (18). More direct and convincing evidence stems from studies that examined mice that were DNA methyltransferase-deficient (34). When the expression of the methylation enzyme was interrupted by gene targeting, there was no expression of IGF-II from the paternal allele, indicating the importance of DNA methylation in maintaining IGF-II expression and imprinting. In this communication, we cultured astrocyte cells from mouse neonates and human embryos in which IGF-II imprinting had been established previously. By using this cell system, we confirmed the importance of DNA methylation in the regulation of allelic expression of IGF-II in both human and mouse central astrocytes.

IGF-II expression in all three astrocyte cell systems responded to DNA demethylating agents. After treatment with 5-aza-C or 2-deoxy-5-aza-C, the expression of IGF-II increased (Figs. 4 and 5). To ensure complete DNA demethylation, we treated cells sequentially for several passages to “dilute” the remaining methylated CpG carrying over from old cells (Fig. 2). After cells were treated with demethylating agents for one passage, only a small increment in IGF-II expression was detected. Presumably, some important CpG dinucleotides remained methylated in these cells. However, when the cells were reseeded and treated again with DNA demethylating agents for several more sequential passages, a dramatic increase in IGF-II expression was observed (Fig. 4). The increased IGF-II expression with each treated passage is not surprising because more and more CpG dinucleotides become demethylated after continuous treatment with 5-aza-C (Fig. 2). This effect of cell passage can be demonstrated in all three cell systems, with both 5-aza-C and 2-deoxy-5-aza-C. In a pilot experiment, we also have applied different doses of 5-aza-C to MBR01 cells, ranging from 0.5–10.0 μM. The cells showed increasing IGF-II expression in a dose-dependent manner (data not shown). Taken together, these results indicate that DNA demethylation enhanced the expression of IGF-II in these brain-derived cells.

In agreement with our results, Eversole-Cire et al. (35) examined IGF-II expression in cells that were cultured from a disomic mouse that possessed a duplicated maternal (and absent paternal) chromosome 7; they found that the treatment of cells with 2-deoxy-5-aza-C increased IGF-II expression by 2–4-fold from the imprinted allele. Interestingly, the treatment of cells with other cytidine analogs, for example 1-β-D-arabinofuranosylcytosine and deoxycytidine, failed to alter IGF-II expression, suggesting a specific effect of DNA demethylation at the 5' position of CpG on IGF-II expression. Jaenisch et al. (36) also provided evidence that 5-aza-C treatment activated silent retroviral transgenes in postnatal mice, indicating that DNA demethylation affects the expression of a previously suppressed gene in vivo as well.

We then determined if DNA demethylation increased expression from the expressed paternal allele or from the release of the imprinted maternal allele. In untreated MBR01 cells,
IGF-II was transcribed at very low levels and was primarily derived from the paternal allele. However, after treatment with 5-aza-C, both parental alleles were transcribed at high levels. Surprisingly, most of the increase in IGF-II expression originated from the maternal allele, which is normally suppressed (Fig. 6C). The results from two human astrocyte cell systems also indicated that after treatment with 5-aza-C and 2-deoxy-5-aza-C, the less abundantly expressed allele was activated to
or approximately to the level of the normally expressed allele (Figs. 6 and 7). These data indicate that DNA demethylation increases IGF-II expression primarily by releasing the suppression of the imprinted allele.

Our data suggest that the maternal allele of mouse IGF-II is normally hypermethylated and thus is transcriptionally less active. Treatment of cells with 5-aza-C will remove the methylation restriction and thus enhance the expression of IGF-II from the imprinted allele. This association between DNA methylation and gene expression has been demonstrated in the cases of IGF-II (23) and H19 (22, 23) in Wilms' tumors. Comparison of DNA methylation of the IGF-II gene indicated that the IGF-II locus was hypermethylated in tumors with maintenance of IGF-II imprinting, whereas it was hypomethylated in the tumors with loss of IGF-II imprinting (23). The altered methylation of IGF-II occurred on the maternal allele, concomitant with the relaxation of imprinting of IGF-II, suggesting that the loss of DNA methylation on the normally unexpressed maternal allele may be related to the reactivation of the imprinted IGF-II allele in these tumors. In Wilms' tumors with LOI, there is no expression of H19. Similarly, examination of the methylation status indicated that the inactivated maternal allele of H19 became heavily methylated (22, 23). Thus, hypermethylation of a specific allele is probably an important event involved in the suppression of allelic gene expression.

However, contrary to these results, a different methylation pattern has been observed in mouse IGF-II (18). Four of the HpaII sites upstream of IGF-II are differentially methylated in an allele-specific pattern, but with the paternally derived allele being hypermethylated and the maternally derived allele left unmodified. It is not obvious why different results were obtained from the normal mouse tissues studied by Brandeis et al. (18) and from our astrocytes. However, it is likely that astrocyte cells used in the present study, which have very leaky IGF-II imprinting, may behave similarly to Wilms' tumors but differently from those normal tissues reported previously (18).

It is unclear why, in mouse IGF-II, the imprinted maternal allele was transcribed to a greater degree than the normally expressed parental allele after DNA demethylation (Fig. 6C). Although we have no adequate explanation for this phenomenon, it is possible that the global DNA demethylation with 5-aza-C may have extensive effects upon the host cells. For example, many other trans factors in the cell may also be regulated by DNA methylation. The treatment with 5-aza-C could increase the expression of these trans factors. Among these factors, some may be involved in the transcriptional regulation of IGF-II and may bind preferentially to the maternal allele. It is also possible that both parental alleles are marked differentially by modifications other than methylation. Consequently, removal of restriction from DNA methylation by 5-aza-C treatment will not affect the differential transcription from two parental alleles.

Of particular interest is the finding that the IGF-II promoters respond to DNA demethylation in a differential fashion. Both human and mouse IGF-II contain multiple promoters. In most tissues, the dominant promoters are hP3 and hP4 in human (2) and mP2 and mP3 in mouse (8). In this study, the mouse astrocytes (MBR01) predominantly transcribed IGF-II from mP2 prior to 5-aza-C treatment. However, after treatment with the DNA demethylating reagent, the transcription of

Fig. 7. IGF-II imprinting in HBR441 (A) and HBR440 (B) cells incubated with (+) or without (−) 2-deoxy-5-aza-C.
IGF-II from mP3 increased substantially, especially in those cells continuously treated with 5-aza-C (Fig. 8). The pattern of increased IGF-II expression from mP3 accounted for the entire increment of IGF-II (Fig. 4A). The expression of IGF-II from mP2, in contrast, was unchanged between treated and control groups. This was also true for cells in every passage tested. In agreement with the results from these mouse cells, two human astrocyte cell systems also showed that only the promoter (hP4), which is equivalent to the mouse IGF-II mP3, responded to DNA demethylation. The mP2 equivalent, hP3, was transcribed at a relatively constant level before and after treatment.

Sequence comparison for these promoters indicate that the promoters (mP2 and hP3), which did not respond to the demethylating agents, contain more CpG dinucleotides than the promoters (mP3 and hP4) that did respond. The promoter regions of many housekeeping genes are characterized by clusters of CpG dinucleotide islands that are usually unmethylated. It is possible that mP2 and hP3, as dominant promoters in many tissues, may be equipped in a pattern similar to those promoters in housekeeping genes. In other words, they are usually unmethylated and thus are not affected by the treatment of 5-aza-C or 2-deoxy-5-aza-C. The DNA demethylation-responding promoters (mP3 and hP4), on the other hand, are located within a short stretch of DNA sequence of the most proximal promoter of IGF-II (hP4 and mP3), on the other hand, increased after treatment with the inhibitors of DNA methylation.

**Fig. 8.** The altered promoter usage in MBR01 (A), HBR441 (B), and HBR440 (C) cells treated with 5-aza-C. The cDNA samples were amplified by a multiplex PCR consisting of multiple promoter-specific 5' -primers and a common 3' -primer end-labeled with [γ-32P]ATP. The PCR products will reflect the relative abundance of the RNA transcripts derived from each promoter (Ref. 8). Note the consistent expression of IGF-II from two equivalent promoters: the human IGF-II hP3 and the mouse IGF-II mP2. The expression of IGF-II from the most proximal promoter of IGF-II (hP4 and mP3), on the other hand, increased after treatment with the inhibitors of DNA methylation.
maternal suppression of IGF-II. The paternal MRE is likely to be hypomethylated and thus would be less responsive to 5-aza-C treatment. Alternatively, treatment of astrocyte cells with the inhibitors of DNA methylation may enhance the expression of some transactivators, which may selectively bind to and thus activate the most proximal promoter of IGF-II. The identification of these putative MREs and transactivators may help to delineate the molecular function of DNA methylation in the regulation of genomic imprinting of IGF-II.

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