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Absence of an Obvious Molecular Imprinting Mechanism in a Human Fetus with Monoallelic *IGF2R* Expression

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We have previously shown that, in contrast to its murine homologue, the human *IGF2R* gene is not imprinted. However, in a small number of individuals, partial or complete repression of the paternal allele has been observed and it has been speculated that in man, *IGF2R* imprinting is a polymorphic trait. We have confirmed monoallelic *IGF2R* expression in one fetus and investigated whether genomic imprinting was involved in the silencing of the paternal allele. Two CpG rich regions, known to be important for the imprinted expression of *Igf2r* in mice, were examined for sequence and methylation changes. A 17 bp deletion was identified within the intronic CpG island. This deletion was shown to be polymorphic and without consequence for the expression of the relevant *IGF2R* allele. Furthermore, in this fetus, methylation patterns of the intronic and promoter CpG islands were identical to that of normal controls, including hypomethylation of the paternal promoter region. In mice, this region is hypermethylated on the paternal allele which is silenced. The absence of paternal promoter methylation indicates that paternal silencing in this particular fetus is by a mechanism other than parental imprinting or, alternatively, that promoter methylation is not necessary for *IGF2R* imprinting.

Key Words: **IGF2R; imprinting; methylation.**

Genomic imprinting, a process leading to the parental specific expression of genes, is involved in various genetic disorders and in tumorigenesis. Therefore, it is important to identify genes subjected to genomic imprinting and to reveal the mechanisms by which a cell recognises and silences a specific parental allele. Accumulating evidence suggests that methylation is involved since all imprinted genes investigated so far contain regions with parental-specific methylation (1) and, in general, methylation of promoter regions is associated with gene silencing. Most genes that are imprinted in the mouse are imprinted in man as well. To date, the only known exceptions from this rule are the closely linked insulin-like growth factor type 2 receptor gene (*IGF2R*) and the *MAS* proto-oncogene. In mice, the *Mas* gene and the *Igf2r* gene are both imprinted with paternal- and maternal-specific expression respectively (2,3). We have recently shown that in contrast to the murine gene the human *MAS* gene is expressed from both parental alleles in first and second trimester fetuses (4). Furthermore, several independent studies have shown that in man, the *IGF2R* gene is biallelically expressed (5-7). In agreement with the biallelic *IGF2R* expression observed, the promoter-associated CpG island is hypomethylated on both parental alleles in man, whereas in mice the paternal allele is hypermethylated (8,9). In contrast, the possibility was raised that in man imprinting of the *IGF2R* gene is a polymorphic trait (10). In several fetuses and in 50% of sporadic Wilms Tumours (WT) studied, the paternal *IGF2R* allele was found to be partially or completely repressed (10,11). To verify these findings and to investigate the underlying mechanisms we reexamined one of the fetuses with complete suppression of the paternal *IGF2R* allele and could confirm a monoallelic expression pattern. Subsequently, detailed comparison of this fetus with controls in terms of mutation screening and methylation analysis did not show any obvious differences that could explain the monoallelic expression on the basis of parental imprinting.

MATERIALS AND METHODS

Fetal RNA and DNA. Isolation of fetal RNA and DNA samples was described previously (10).

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TABLE 1

Primer Sequences and Annealing Temperatures of the Overlapping Primersets Used for SSCP and Sequence Analysis of CpG1 (Promoter Associated CpG Island) and CpG2 (Intronic CpG Island), Accession Nos. X91875 and X91880, Respectively

	Product size	Primer number	Forward primer 5′ → 3′	Primer number	Reverse primer 5′ → 3′	Anneal temp
CpG1	332	I6-70	CCGCTGCCACTAGGCTGTGC	I6-19	CCGCTCACGTGACTCCCTTGTT	60°C
	264	I6-71	AGGCACCTGGAGCCTGCGCC	I6-09	AGGGCAAAGGCGGAGGTGGAGA	72°C*
	292	I6-01	AGTCGAGCCGCGCTCACCT	I6-02	CTGCACAGCTCGGGGAACG	60°C
	243	I6-14	GCTCTCTGCTCCTGCTGCA	I6-73	ACGCAGGCAACTTTCCCTCG	56°C
	312	I6-72	CGTTCCCCGAGCTGTGCAG	I6-74	CCGCTGGCGAAACTCTGATG	56°C
	286	I6-24	GCGTCGGGAAAGTTGGAGG	I6-75	ACACTGGACGACACCCCCTG	56°C
CpG2	320	I6-56	AGGTGTGTTCTGATTGGATC	I6-59	CCAAGCTCCAGGTTCACG	55°C
	320	I6-60	GGCTCTGCCGGCGAGTCTTA	I6-61	GGAGTGACCCAGCCGCACGG	66°C
	218	I6-62	CCGGCTCTACCGGCGAGGGA	I6-64	GGATGCGGTGTTTCCTGGCA	62°C
	252	I6-63	TGTGAACAGGTTACGAGGTT	I6-57	CGGGGAGGCGCAAGAGGC	59°C
	275	I6-65	TCTCGCGCTTCCCCAGGACC	I6-33	GGGCATGCAGGGTACAGGGA	64°C
	301	I6-43	CGCGCCCCATACCTCCCCAC	I6-30	GAGGCGCATGAGGGCTGGAG	63°C
	322	I6-76	CCCTGCATACCCCGTGAGCC	I6-67	AACACCCAGGGTCTTTTCTA	56°C
	250	I6-68	CTTTTGGCTGAGCCGACTGG	I6-69	AGGTTAGTCTATGGATTAACAC	62°C

Note. PCR conditions were initial denaturation 5 min at 98°C followed by 5 min at 95°C in which the enzyme was added; 30 cycles consisting of 1 min at 95°C, 2 min at Ta and 2 min at 72°C; final elongation for 7 min at 72°C.

* For this primerset a two step PCR was applied consisting of 1 min at 95°C and 2 min at 72°C for 30 cycles.

RT-PCR. Reverse transcriptase reactions were performed exactly as previously described (5). For PCR amplification of the *IGF2R* polymorphism the primers A2 and S2 (10) were used whereas for the *MAS* polymorphism primerset I6-91/92 was used as previously described (4).

Southern blotting and hybridisations. Genomic DNA (7μg) was digested with either *Hin*dIII or *Eco*RI according to the manufacturers' recommendations, separated electrophoretically on an 0.8% agarose gel and transferred onto a Gene Screen plus membrane. Southern hybridisation was performed under standard conditions o/n at 65°C with a 8.5 kb EcoRI fragment covering the promoter region of *IGF2R* (9) or a probe encompassing the second CpG island of *IGF2R* (probe 2 (9)). Final washing was performed in 0.1× SSC/ 0.1% SDS at 65°C for 20 min.

SSCP and sequence analysis. SSCP analysis was performed under standard conditions (12). Amplification for 35 cycles was accomplished in the presence of [α^{32}P]dCTP (>3000 Ci/mmol ICN). The products were fractionated on a 5% nondenaturing polyacrylamide gel containing no, or 10% glycerol at 4°C or at RT. The dried gels were exposed to Kodak X-ray films. For sequence analysis the PCR products were phosphorylated using T4 polynucleotide kinase (Gibco BRL) and ligated into the *Sma*I-digested plasmid vector pT7T3 (Pharmacia).

At least seven individual clones were sequenced using the T7 sequencing kit (Pharmacia). The primers and PCR conditions used for SSCP and sequence analysis are listed in Table 1.

Methylation analysis. The sodium bisulphite genomic sequencing technique of Frommer et al. (13) was employed exactly as outlined in (9). In short, 10 μg genomic DNA was digested with *Hind*III and alkaline denatured. Treatment with sodium bisulphite was performed as described by Clark *et al.* (14).

Modified DNAs were desalted and concentrated using GeneClean (Bio 101), ethanol precipitated and resuspended in 110 μl 100 mM Tris-HCl (pH8.0), 1 mM EDTA. Nested amplification was performed under the following conditions: initial denaturation for 5 min at 95°C followed by 35 cycles consisting of 1 min at 94°C, 2 min at 63°C and 3 min at 72°C and a final elongation step for 6 min at 72°C. Primer sequences are given in (9). The PCR fragments were cloned and individual clones were sequenced as described above.

RESULTS

Monoallelic expression of the human IGF2R gene. RNA from fetus 9224, which was previously shown to repress the paternal allele of the *IGF2R* gene completely (10), was reexamined under different experimental conditions in an RT-PCR assay. Complete monoallelic expression of the human *IGF2R* gene was observed (Fig. 1).

Southern analysis of the promoter region and an intronic CpG island. To test for gross aberrations in the promoter region and an intronic CpG island (CpG2) known to be important for the imprinted expression of *Igf2r* in mice (8), Southern blots of *Eco*RI or *Hin*dIII digested DNA of fetus 9224 were hybridised with

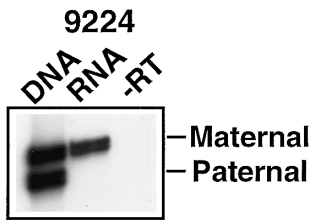

FIG. 1. Expression analysis of the *IGF2R* gene of fetus 9224. Total RNA from fetus 9224 was used as template for reverse transcription. Amplification of cDNA and genomic DNA was performed in the presence of [α^{32}P]dCTP using primers flanking a CAC deletion polymorphism in the 3′UTR (10). Monoallelic maternal specific expression is demonstrated by the presence of the maternal allele only after RT-PCR (origin of parental alleles as determined previously (10)). Contamination with genomic DNA was excluded by an RT-PCR reaction without the addition of reverse transcriptase (-RT).

probes from the corresponding regions. The detected banding pattern was identical to that of two control DNAs which rules out gross deletion or insertion in the DNA of fetus 9224 (data not shown). To exclude smaller aberrations including point mutations, we performed an extensive sequence and methylation analysis of both regions in more detail.

Sequence and methylation analysis of the IGF2R promoter region. To identify mutations in the promoter region of fetus 9224 we performed SSCP analysis with overlapping primersets covering the promoter associated CpG island. No shifted bands were found and subsequent sequence analysis of PCR products from this region did not reveal any mutation. Since it is known that in mice the silenced paternal allele is heavily methylated at the promoter region we employed the genomic sequencing technique of Frommer et al. (13) to determine the methylation status in fetus 9224. No methylation was found for any of the 138 CpGs present in the promoter region (Fig. 2). This unmethylated pattern has also been found in random human controls and in patients with paternal or maternal uniparental disomy for chromosome 6 (9).

A 17 bp deletion in the intronic CpG island. Both the human and the mouse *IGF2r* genes contain a second CpG island (CpG2) located in intron 2, which is methylated on maternally derived alleles only (8,9,15). This CpG island contains several direct repeats, a characteristic of CpG-rich regions associated with imprinted genes (16). We performed an SSCP analysis using overlapping primersets covering the entire CpG2 island to identify mutations in this region. With primers I6-76 and I6-67 a shifted band was observed and sequence analysis revealed the deletion of 17 bp exactly corresponding with one direct repeat unit within the differentially methylated region. RT-PCR analysis of unspliced nuclear RNA showed that the deletion is present on the silent paternally derived allele (Fig. 3).

To further investigate the relevance of this deletion for the monoallelic expression of *IGF2R*, we analysed 103 unrelated individuals by PCR and denaturing polyacrylamide gel electrophoresis. Three individuals carrying the same deletion were identified and in two cases we could demonstrate a paternal inheritance of the deletion. To study the expression pattern of the *IGF2R* gene in these individuals RT-PCR analysis on RNA isolated from lymphoblastoid cell lines was performed. Both individuals expressed the *IGF2R* gene biallelically, indicating that the deletion by itself does not prevent the expression of the relevant *IGF2R* allele (Fig 3). However, this deletion might still alter the maternal specific methylation of the intronic CpG island. Therefore we analysed the methylation pattern of CpG2 for fetus 9224 by applying the bisulphite genomic sequencing method. Both maternal and paternal methylation patterns characterised by hyper- and hypo-

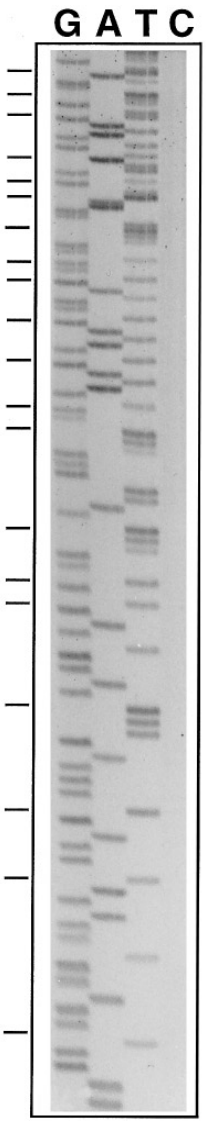

FIG. 2. Methylation analysis of the promoter associated CpG island (CpG1). Following sodium-bisulfite modification of fetal genomic DNA nested PCR was performed and cloned PCR products were sequenced. Part of the CpG1 sequence, amplified with primer set I6-48/49 followed by I6-49/50 (for sequences and PCR conditions see (9)), is shown. The bars indicate the position of CpG dinucleotides. As can be seen by the absence of signal in the C lane all CpG dinucleotides are unmethylated and therefore converted into TpG dinucleotides.

methylation, respectively, were identified. The 17 bp deletion was found on the hypomethylated (paternal) allele (data not shown).

Biallelic expression of the MAS proto-oncogene. In mice, the *Mas* gene is imprinted with exclusive expression of the paternal allele whereas in man this gene is biallelically expressed (3,4). The *IGF2R* and *MAS* genes are closely linked, with a distance of 65 kb in mice (17) and at most several hundred kb in man, and it is suggested that they are part of an imprinting cluster which

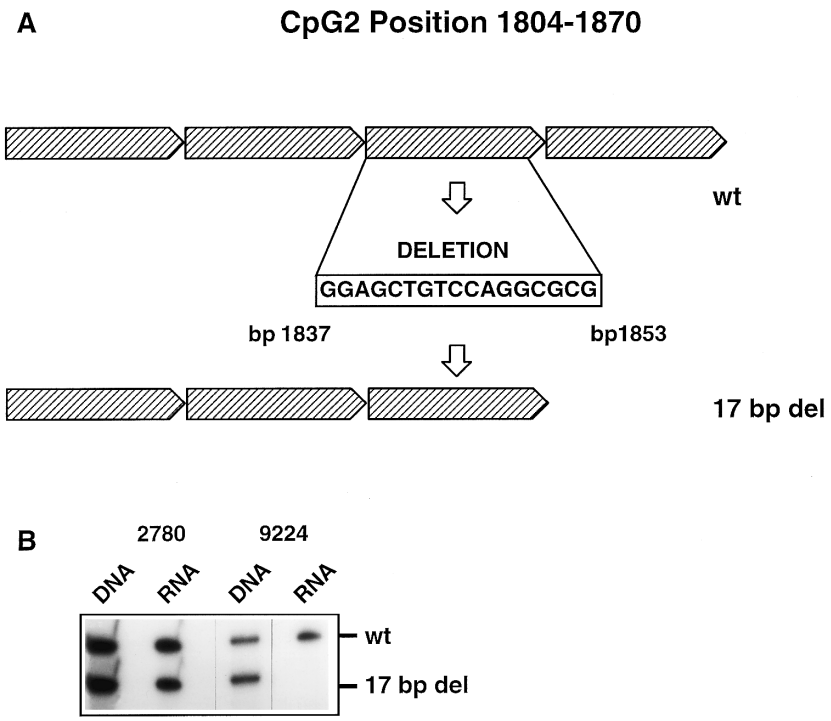

FIG. 3. A 17 bp deletion of one direct repeat unit within the differentially methylated CpG2 region. (A) Schematic representation of the 3′ part of CpG2 normally containing 4 direct repeats. The bp numbers mentioned refer to the position of these repeats within the published sequence, accession number X91880. Fetus 9224 has a deletion of 17 bp corresponding to repeat unit 3 as confirmed by sequence analysis. (B) RT-PCR of the 17 bp deletion polymorphism. DNA and RNA from fetus 9224 and a heterozygous control individual (2780) was amplified with primer I6-66 5′→3′ GCGCCTCCAGCTGCGCAT and I6-67 (see table 1), at 59°C. RNA from fetus 9224 contains the wildtype (wt) allele only. Since only the maternal IGF2R allele of fetus 9224 is expressed, the deletion has to be present on the silenced paternal allele. This was confirmed by methylation analysis which revealed the presence of the deletion on the hypomethylated (paternal) allele and not on the hypermethylated (maternal) allele (data not shown). RNA from control 2780 contains both parental alleles indicating that the deletion is not sufficient to silence the relevant IGF2R allele.

is regulated by a common cis-acting imprinting centre (IC). We have previously speculated that this IC might be defunct or lost in man resulting in biallelic expression of both genes (4). If in fetus 9224, the monoallelic expression of *IGF2R* results from genomic imprinting, the apparent functional IC could influence the expression of the neighbouring *MAS* gene as well. However, using the C900T polymorphism (4), for which fetus 9224 is heterozygous, we could demonstrate that the *MAS* gene is biallelically expressed (data not shown).

DISCUSSION

It has been suggested that imprinting of the human *IGF2R* gene might behave as a polymorphic trait (10,11). One fetus (9224, number 12.5 (10)) completely repressed the paternal allele resulting in monoallelic, maternal-specific expression of the *IGF2R* gene. Using a different RT-PCR protocol we reanalysed RNA from this fetus and could confirm the monoallelic *IGF2R* expression described. Subsequently we have analysed two regions known to be important for the maternal specific expression of *Igf2r* in mice but no indication for the involvement of genomic imprinting in the silencing of the paternal allele was found.

Imprinted expression of the *Igf2r* gene in mice is associated with the parental-specific methylation of two CpG islands, one surrounding the promoter region and one located in intron 2 (CpG2). These two CpG islands and their positions are conserved within the human *IGF2R* gene. In mice and humans, CpG2 is hypermethylated on the active maternal allele. The promoter-associated CpG island, however, is hypermethylated on the repressed paternal allele in mice, whereas in man this CpG island is hypomethylated on both parental alleles, in agreement with the biallelic expression found (8,9,15). Paternal-specific methylation of the promoter region in mice is acquired after fertilisation and silencing of the paternal allele as a consequence of genomic imprinting (8). If the repression of the paternal allele of fetus 9224 is the result of genomic imprinting one would expect hypermethylation of the promoter region. Using the sodium bisulphite genomic sequencing method (13) we have demonstrated that all the 138 CpGs in this region from fetus 9224 are unmethylated. This clearly shows that repression of the

paternal allele is not achieved by methylation of the promoter region.

The maternal-specific methylation of CpG2 in the mouse is inherited directly from the oocyte and is believed to represent the primary imprinting signal (8). In both mice and humans, CpG2 contains several direct repeats, which is a characteristic of parental-specific methylated CpG-rich regions associated with imprinted genes (8,9,16). Promoter-associated CpG islands of non-imprinted genes do not contain these direct repeats. Remarkably, one repeat unit of the direct repeats found in CpG2, is deleted on the paternal allele in fetus 9224. It is tempting to speculate that this deletion renders the paternal allele inactive. However, the biallelic expression of the *IGF2R* gene in two cell lines containing the same, paternally inherited, deletion, suggests that the deletion itself is not sufficient to induce monoallelic expression, nor does this deletion change the methylation status of CpG2 since methylation analysis revealed the presence of a hypomethylated paternal allele, containing the 17 bp deletion, and a hypermethylated maternal allele, as is normally found.

Examination of the two regions were imprinting signals are thought to reside, did not reveal any differences between fetus 9224 and non-imprinted controls. Therefore, if paternal expression is due to genomic imprinting, the imprinting mechanism functions quite different than that in mice, and imprinting signals might reside outside the areas investigated. One would have to assume that promoter methylation is not required for monoallelic expression in the human, and that only the methylation imprint at the intronic CpG island is needed. However this methylation imprint, present in all humans studied, is not sufficient for monoallelic expression. Furthermore, the conserved presence of the two CpG islands and the maternal specific methylation of the intronic CpG island suggests similar mechanisms in humans and mice. These discrepancies lead us to propose that repression of the paternal allele of fetus 9224, is not the result of genomic imprinting but is due to an unidentified mutation which renders the paternal allele inactive. Gross aberrations in the promoter region or CpG2 could be excluded by Southern blotting and hybridisation with corresponding probes. SSCP and sequence analysis of the promoter region did not reveal any mutation. However, we have analysed only 1220 bp surrounding the transcription start site and the presence of an inactivating mutation elsewhere cannot be excluded. This mutation could influence transcription initiation, RNA stability, or lead to an abnormal early termination of transcription. The lack of methylation of the paternal promoter-associated CpG island is not in agreement with silencing through promoter methylation imprinting, a potential silencing mechanism in the mouse. Furthermore, biallelic *MAS* expression argues against the possibility that monoallelic *IGF2R* expression is due to an active IC affecting the entire imprinting domain. Very recently, Miller *et al.* showed tissue-specific monoallelic expression of the *MAS* gene in adult breast samples which contrasts to the biallelic expression of *MAS* in fetal tissues (18). It would be interesting to study whether in breast tissue, the human *IGF2R* gene is imprinted as well arguing an active IC in this particular tissue.

Biallelic expression of the *IGF2R* gene in man is well documented. In total 20 heterozygous fetuses were investigated (5,6,10,11). Of these, only two fetuses expressed the *IGF2R* gene monoallelically (10) but the studies described here failed to find an imprinting related molecular mechanism that would explain the silencing. Thus no molecular basis for polymorphic *IGF2R* imprinting was found in the areas examined. Recently, Wutz *et al.* demonstrated that in mice, the second CpG island is in fact the promoter of an antisense RNA molecule. This molecule is transcribed from the paternal allele only and deletion of the intronic CpG island prevents the expression of this RNA molecule leading to loss of imprinting (19). Absence or inactivating mutations of this RNA in humans might explain the biallelic *IGF2R* expression observed. At present, we cannot rule out the possibility that fetus 9224 has a sequence variant that permits expression of a functional form of this RNA.

It has been suggested that *IGF2R* can function as a tumour suppressor gene and deletions of the *IGF2R* chromosomal region as well as mutations within the gene have been found in numerous tumours (20-23). Moreover, in 50% of informative Wilms Tumours (WT) the paternal *IGF2R* allele was partially repressed (11). It would be of interest to see whether this polymorphic, partial, repression is due to hypermethylation of the promoter region, and hence to genomic imprinting, or whether, as in the case studied here, other mechanisms are involved too.

REFERENCES

1. Razin, A., and Cedar, H. (1994) *Cell* **77,** 473–476.
2. Barlow, D. P., Stöger, R., Herrmann, B. G., Saito, K., and Schweifer, N. (1991) *Nature* **349,** 84–87.
3. Villar, A., and Pedersen, R. A. (1994) *Nature Genet.* **8,** 373–379.
4. Riesewijk, A. M., Schepens, M. T., Mariman, E. M., Ropers, H. H., and Kalscheuer, V. M. (1996) *Genomics* **35,** 380–382.
5. Kalscheuer, V. M., Mariman, E. C., Schepens, M. T., Rehder, H., and Ropers, H.-H. (1993) *Nature Genet.* **5,** 74–78.
6. Ogawa, O., McNoe, A., Eccles, M. R., Morison, I. M., and Reeve, A. E. (1993) *Hum. Mol. Genet.* **2,** 2163–2165.
7. Treacy, E., Polychronakos, C., Vekemans, M., Eydoux, P., Blaichman, S., Scarpelli, H., Ross, M., Xu, Y., and Der Kaloustian, V. M. (1996) *J. Med. Genet.* **33,** 42–46.
8. Stöger, R., Kubicka, P., Liu, C.-G., Kafri, T., Razin, A., Cedar, H., and Barlow, D. P. (1993) *Cell* **73,** 61–71.
9. Riesewijk, A. M., Schepens, M. T., Welch, T. R., van den Berg-Loonen, E. M., Mariman, E. C. M., Ropers, H.-H., and Kalscheuer, V. M. (1996) *Genomics* **31,** 158–166.

10. Xu, Y., Goodyer, C. G., Deal, C., and Polychronakos, C. (1993) *Biochem. Biophys. Res. Comm.* **197,** 747–754.
11. Xu, Y. Q., Grundy, P., and Polychronakos, C. (1997) *Oncogene* **14,** 1041–1046.
12. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2766–2770.
13. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., and Paul, C. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1827–1831.
14. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) *Nucl. Acids Res.* **22,** 2990–2997.
15. Smrzka, O. W., Fae, I., Stöger, R., Kurzbauer, R., Fischer, G. F., Henn, T., Weith, A., and Barlow, D. P. (1995) *Hum. Mol. Genet.* **4,** 1945–1952.
16. Neumann, B., Kubicka, P., and Barlow, D. P. (1995) *Nature Genet.* **9,** 12–13.
17. Schweifer, N., Valk, P. J. M., Delwel, R., Cox, R., Francis, F., Meier-Ewert, S., Lehrach, H., and Barlow, D. P. (1997) *Genomics* **43,** 285–297.
18. Miller, N., McCann, A. H., O'Connell, D., Pedersen, I. S., Spiers, V., Gorey, T., and Dervan, P. A. (1997) *Genomics* **46,** 509–512.
19. Wutz, A., Smrzka, O. W., Schweifer, N., Schellanders, K., Wagner, E. F., and Barlow, D. P. (1997) *Nature* **389,** 745–749.
20. De Souza, A. T., Hankins, G. R., Washington, M. K., Orton, T. C., and Jirtle, R. L. (1995) *Nature Genet.* **11,** 447–449.
21. Ouyang, H., Shiwaku, H. O., Hagiwara, H., Miura, K., Abe, T., Kato, Y., Ohtani, H., Shibba, K., Souza, R. F., Meltzer, S. J., and Horii, A. (1997) *Cancer Research* **57,** 1851–1854.
22. Hankins, G. R., De Souza, A. T., Bentley, R. C., Patel, M. R., Marks, J. R., Iglehart, J. D., and Jirtle, R. L. (1996) *Oncogene* **12,** 2003–2009.
23. De Souza, A. T., Hankins, G. R., Washington, M. K., Fine, R. L., Orton, T. C., and Jirtle, R. L. (1995) *Oncogene* **10,** 1725–1729.