Monoallelic Expression of Human *PEG1/MEST* Is Paralleled by Parent-Specific Methylation in Fetuses

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We have isolated the human *PEG1/MEST* gene and have investigated its imprinting status and parental-specific methylation. FISH mapping assigned the gene to chromosome 7q32, and homologous sequences were identified on the short arm of human chromosomes 3 and 5. Through the use of a newly identified intragenic polymorphism, expression analysis revealed that *PEG1/MEST* is monoallelically transcribed in all fetal tissues examined. In two informative cases, expression was shown to be confined to the paternally derived allele. In contrast to the monoallelic expression observed in fetal tissues, biallelic expression was evident in adult blood lymphocytes. Biallelic expression in blood is supported by the demonstration of *PEG1/MEST* transcripts in a lymphoblastoid cell line with maternal uniparental disomy 7. The human *PEG1/MEST* gene spans a genomic region of approximately 13 kb. Sequence analysis of the 5' region of *PEG1/MEST* revealed the existence of a 620-bp-long CpG island that extends from the putative promoter region into intron 1. We demonstrate that this CpG island is methylated in a parent-of-origin-specific manner. All *MspI/HpaII* sites were unmethylated on the active paternal allele but methylated on the inactive maternal one. © 1997 Academic Press

INTRODUCTION

Imprinting is a process by which some genes are silenced in a parent-of-origin-dependent manner, resulting in monoallelic expression in the offspring. The mechanisms underlying genomic imprinting and by which parental alleles are distinguished are largely unknown. All imprinted genes so far examined in more detail contain DNA sequences methylated in a parental-specific manner, suggesting that this epigenetic process plays an important role in regulating imprinted gene expression.

Recently, the mouse *PegI* gene has been identified in a systematic screen using subtraction hybridization between cDNAs from parthenogenetic and similar stage-matched normal control mouse embryos (Kaneko-Ishino et al., 1995). *PegI* is expressed from the paternally derived allele only. Subsequent homology search revealed that *PegI* is identical to a previously identified “mesoderm-specific” cDNA (*Mest*), which maps to the proximal part of chromosome 6, band B1, a region homologous to the long arm of human chromosome 7 (Sado et al., 1993). The *PegI/Mest* gene codes for an enzyme that shows significant similarity to the α/β-hydrolase fold family; the precise function is as yet unknown.

Indirect evidence for the existence of at least one maternally imprinted gene on the long arm of human chromosome 7 came from the study of patients with maternal uniparental disomy 7 (mUPD7; Kotzot et al., 1995). These patients are characterized by intrauterine and postnatal growth retardation, indicating that the lacking paternal contributions for chromosome 7 may account for growth retardation.

Since imprinting is generally conserved between mouse and humans, with the exception of *IGF2R* and *MAS*, which are oppositely imprinted in the mouse but equally expressed from both parental alleles in humans (Barlow et al., 1991; Kalscheuer et al., 1993; Ogawa et al., 1993; Villar and Pedersen, 1994; Riesewijk et al., 1996a), human *PEG1/MEST* was suggested as the first candidate imprinted gene located on chromosome 7. In the present study, we have isolated and characterized the human *PEG1/MEST* gene, mapped it to chromosome 7q32, and found homologous sequences on the short arm of human chromosomes 3 and 5. We determined the imprinting status of *PEG1/MEST* by making use of a newly identified intragenic single nucleotide deletion/insertion polymorphism. Sequencing of RT-PCR products revealed monoallelic expression in all
fetal tissues examined. In two informative cases, expression was found to be confined to the paternally derived allele, as in the mouse. Southern blot analysis of the 5’ Cpg island of PEG1/MEST demonstrated parent-of-origin-specific methylation.

**MATERIALS AND METHODS**

**Library screening.** Arrayed human fetal cdNA libraries (lung and liver), a chromosome 7-specific cosmid library, and a human PAC library were screened with a mouse PEG1/MEST RT-PCR product of 993 bp amplified with primer set Peglf and Peglr (GAGATCGCT-TGGCGAGGT, 258–276; AGGAGTTGATGAAGCCTA, 1250–1231; Accession No. D16262). DNA of the positive clones was prepared according to standard procedures (Sambrook et al., 1989).

**RT-PCR and sequence analysis.** All reverse transcription reactions were performed exactly as described previously (Kalscheuer et al., 1993).

For PEG1/MEST polymorphism and expression analysis, reverse transcribed cdNAs and genomic cdNAs were amplified with primer set 4 and 10 (TTGGCTATTAGAAATTCAGA, 1473–1493; GACATCGCTTCTGTTGAG, 1716–1699; Accession No. Y11534) under the following conditions: initial denaturation for 3 min at 95°C, 1 min at 94°C, 1 min at 57°C, and 1.5 min at 72°C for 30 cycles; elongation for 7 min at 72°C. Single-strand conformational polymorphism (SSCP) analysis was performed under standard conditions (Orita et al., 1989). For each RT-PCR experiment, a control reaction without addition of reverse transcriptase was performed in parallel. Prior to sequencing, PCR and RT-PCR products were cut out of the agarose gel and purified using the Quiaquick gel extraction kit (Qiagen). Sequencing, PCR and RT-PCR products were analyzed on an ABI 377 automated sequencer.

**Northern blot analysis.** The Clontech multiple tissue Northern blot was hybridized with the PEG1/MEST cdNA clone (ICRFp507M19178Q11) according to the GeneScreen Plus protocol. Final wash was in 0.1X SSPE at room temperature for 15 min.

**Southern blot and methylation analysis.** DNAs were digested with the appropriate restriction enzymes, and the fragments were separated by agarose gel electrophoresis, transferred to GeneScreen Plus membranes, and hybridized under standard conditions. Methylation analysis of genomic DNAs from tissues and blood digested with (HindIII + MspI) or (HindIII + HpaII) was performed exactly as described previously (Riesewijk et al., 1996b). Uniparental diomy was confirmed for all cases included in our study (J. Kere et al., unpublished; Höglund et al., 1994; Koziot et al., 1995).

**RESULTS**

**Sequence, Homology Analysis, and Mapping of the Human PEG1/MEST Gene**

A cdNA clone (ICRFp507M19178Q11) of the human PEG1/MEST gene was isolated from a fetal cdNA library, and Northern blot hybridization showed that the insert represents the entire mRNAs (not shown). Sequencing of the 2470-bp insert showed that our clone is identical to the PEG1/MEST cdNA described by Nishita et al. (1996; Accession No. D78611), except for two thymidine residues in the 5’ untranslated region (T88 and T98), which were absent in our cdNA sequence, and four base changes in the open reading frame (A591→G, G593→A, G634→A, and A726→T). These changes result in the substitution of two nonhomologous amino acids into two residues identical to those of the mouse protein. Sequence comparison of the human PEG1/MEST cdNA and its mouse counterpart with the program BESTFIT displayed 84% homology for the entire sequence and 91% for the coding region, and amino acid sequence comparison with the program BLASTN demonstrated 98% identity.

To characterize the human PEG1/MEST gene in more detail, cosmids were isolated (ICRFc113M1711Q4, K246Q4, and G0353Q4), and restriction digestion with EcoRI or HindIII, followed by Southern hybridization with cosmid G0353Q4, demonstrated that they mostly overlapped. FISH mapping of cosmid G0353Q4 assigned the human PEG1/MEST gene to chromosome 7q32, thereby confirming the results of Nishita et al. (1996).

Subsequently, EcoRI-digested genomic and cosmid DNAs were subjected to Southern blot hybridization with PEG1/MEST cdNA. Surprisingly, eight EcoRI fragments (8.7, 6.2, 4.0, 2.65, 2.3, 1.7, 1.05, and 0.6 kb) were detected in genomic DNA while cosmid DNA lacked three of them (6.2, 2.65, and 2.3 kb) (not shown). Attempts were therefore made to isolate PAC clones from this region. Screening of a PAC library with the same PEG1/MEST cdNA revealed 10 individual PAC clones, and Southern hybridization of EcoRI-digested PAC DNA with the PEG1/MEST cdNA resulted in hybridization patterns, allowing us to subgroup the PAC clones into three categories. One subgroup of PAC clones (LLNLp704G0495Q13, H10144Q13, and M10128Q13) showed positive EcoRI fragments previously found in all cosmids, indicating that these harbor the PEG1/MEST gene. These results were confirmed by FISH mapping of DOP-PCR products of 1 of these PAC clones to chromosome 7q32. Subcloning and sequencing...
of EcoRI-digested cosmid fragments that hybridized to PCR products generated from the 5' and 3' ends of the human PEG1/MEST cDNA demonstrated that the cosmids contain the complete human PEG1/MEST gene, spanning a genomic region of approximately 13 kb. The presence of additional EcoRI fragments in genomic DNA that were absent in the PEG1/MEST cosmid and PAC clones pointed to the existence of PEG1/MEST-related sequences elsewhere in the human genome. This is in line with the finding that two groups of PAC clones that had been identified with PEG1/MEST as a probe could not be accomodated within the PEG1/MEST cosmid and PAC contig. FISH mapping of MEST-related sequences elsewhere in the human genome. The presence of additional the human PCR products generated from the 5' and 3' ends of PEG1/MEST in adult blood lymphocytes. Interestingly, Monoallelic and Biallelic Expression of the Human PEG1/MEST Gene

To isolate and characterize the 5' end of the human PEG1/MEST gene, a PCR product of 219 bp (primer set 15 and 16), comprising part of the promoter region and exon 1, was hybridized to EcoRI-digested cosmid DNA. A positive fragment of 8.7 kb was identified and subcloned following digestion with HindIII. Partial sequencing of a 4.3-kb HindIII clone revealed a high G+C content of 72% in the 5' region of the gene and a high frequency of CpG dinucleotides, indicating the presence of a 620-bp-long CpG island, which includes the promoter region and exon 1 and extends into intron 1 (Fig. 1). This region contains four SP1 binding sequences (GGCGGG) but lacks TATA and CCAAT boxes. Sequence analysis revealed the presence of an imperfect direct repeat of 20 bp arranged in tandem (Fig. 1A).

Methylation Analysis of the Human PEG1/MEST Gene

To determine the methylation status of the MspI/HpaII sites in the CpG island of the human PEG1/MEST gene, we performed Southern hybridizations of (HindIII + MspI)- and (HindIII + HpaII)-digested genomic DNAs from adult intestine, cerebellum, stomach, liver, lung, and blood lymphocytes with the 4.3-kb HindIII fragment, which encompasses the CpG island, exon 1, and part of intron 1 (Fig. 3A). (HindIII + MspI)-digested DNA showed positive fragments of 2.3, 0.65, 0.45, 0.3, and 0.23 kb. Because of their small size, a few fragments could not be detected. (HindIII + HpaII)-digested DNA resulted in a similar banding pattern with two additional fragments of 4.3 and 2 kb. The presence of the 4.3-kb fragment, which was observed in all DNA samples, indicates that part of the genomic DNA is completely methylated in this region and therefore undigested by HpaII. The fragment of 2 kb, which contains the complete CpG island, was prominent in lung and blood, much fainter in stomach and liver, and nearly undetectable in cerebellum. Obviously, the degree of methylation of this particular MspI/HpaII recognition site (Fig. 3A, arrow) varies between tissues while all other MspI/HpaII sites in the CpG island and its proximity are completely methylated and therefore resistant to digestion (Fig. 3C). Compared to the MspI fragments, the HpaII fragments of 0.65, 0.45, 0.3, and 0.23 kb were much fainter. Quantification of the intensities by the program ImageQuant showed that radioactivity was approximately 50% lower, indicating that 50% of these restriction sites are methylated and resistant to HpaII digestion and 50% are unmethylated and therefore digested.

Southern hybridization of (HindIII + MspI)- and (HindIII + HpaII)-digested fetal DNA derived from brain, liver, chorionic villi, placenta, and control DNA from adult blood lymphocytes was performed with a NarI probe of 545 bp, encompassing part of the CpG island (Fig. 1, position 15–561). The hybridization pattern demonstrated that in fetal tissues too, 50% of the MspI/HpaII sites are unmethylated and 50% are methylated (not shown).

PEG1/MEST Methylation Is Allele-Specific

Our finding of CpG island methylation in the promoter region of an imprinted gene raises the possibility...
FIG. 1. Sequence and CpG plot of the human PEG1/MEST promoter region, exon 1 (capital letters), and part of intron 1. (A) Sequence of the 5' region of PEG1/MEST. A tandemly arranged imperfect direct repeat of 20 bp is marked by boxes, and consensus sites for SP1-binding sites are underlined. The putative transcription start, determined from the cDNA sequence, is marked by an arrow, exon I is written in capital letters, and the 620-bp-long CpG island is marked by a box. (B) The CpG plot shows the G+C density (broken line) and CpG content (solid line) per 100 bp for this sequence. The data are expressed as the observed number of CpGs per 100 bp over the expected number of CpGs per 100 bp. The diagram reveals that this region contains a CpG island of 620 bp that is more than 72% G+C.
FIG. 2. Expression analysis of PEG1/MEST. Arrows point to the newly identified deletion/insertion polymorphism resulting in 7 or 8 thymidine residues. (A) Sequence analysis of PCR and RT-PCR products of three heterozygous individuals (F6, F7, and Fr3) show that PEG1/MEST is monoallelically expressed. (B) Sequence analysis of maternal DNA displaying 8 thymidines, paternal DNA displaying 7 thymidines, heterozygous fetal DNA, and corresponding monoallelic RT-PCR product displaying 7 thymidines, which indicates that PEG1/MEST is expressed from the paternal allele only. (C) Sequence analysis of blood DNA from two heterozygous individuals (Blood 4 and 6) and corresponding adult blood lymphocyte RT-PCR products. The presence of 7 and 8 thymidine residues in both RNAs indicates cell-type specific biallelic expression of PEG1/MEST.
PARENT-SPECIFIC METHYLATION OF THE IMPRINTED PEG1/MEST GENE

FIG. 3. Methylation analysis of the 5' end of PEG1/MEST. (A) Restriction map of the 4.3-kb HindIII probe showing the positions of the restriction sites analyzed (H, HindIII; M, MspI/HpaII) and the CpG island (box). The arrow refers to the tissue-specific partially methylated HpaII site. (B) Allele-specific methylation of the 4.3-kb HindIII fragment. The horizontal line represents the chromosome; the paternal methylation pattern is depicted above and the maternal pattern below the lines. Unmethylated CpGs are shown as open circles, methylated CpGs as black circles. The partially methylated MspI/HpaII site of the maternal allele is depicted as dotted circle. (C) Southern blot analysis of the promoter region using the 4.3-kb HindIII fragment as probe and genomic DNA from adult brain, stomach, liver, lung, and blood lymphocytes (the upper part shows a short exposure time and the lower part a longer exposure time of the same blot). A 4.3-kb fragment is seen after digestion with HindIII and is reduced to 2.3, 0.65, 0.45, 0.3, and 0.23 kb following digestion with MspI. Because of their small size, a few fragments could not be detected. The 4.3-kb HindIII fragment was digested to approximately 50% by the methylation-sensitive isochizomer HpaII, indicating partial or monoparental methylation at these sites. In contrast to the MspI digestions, a fragment of 2 kb, prominent in lung and blood, less intense in stomach and liver, and nearly undetectable in cerebellum DNA, is present, indicating tissue-specific methylation of one particular MspI/HpaII site (arrow). (D) Southern blot analysis of HindIII, (HindIII + MspI)-, or (HindIII + HpaII)-digested genomic DNA from peripheral blood leukocytes of patients with mUPD7 and pUPD7 and a normal control, probed with the 4.3-kb HindIII fragment. MspI digests of control and patient DNA show the same banding pattern. Following HpaII digestion, fragments of 0.65, 0.45, 0.3, and 0.23 kb are absent in mUPD7 DNA but present in pUPD7 and control DNA. Absence of these fragments in maternal DNA and the dosage effect of paternal DNA in comparison to control DNA indicates specific methylation of the maternal allele.

of allele-specific DNA methylation on the silenced maternally derived allele. To study this, blood DNA from three patients with mUPD7 and one patient with pUPD7 was digested with (HindIII + MspI) or (HindIII + HpaII) under the same conditions. Following Southern blot analysis with the 4.3-kb HindIII probe, MspI-digested DNAs showed the same banding pattern as control blood DNAs (Fig. 3D). HpaII-digested mUPD7 DNAs showed fragments of 4.3 and 2 kb. In contrast to the control DNAs, the smaller fragments of 0.65, 0.45, and 0.3 kb were absent in all mUPD7 DNAs and of identical intensity compared with the respective MspI fragments in pUPD7 DNA. Our methylation analysis clearly demonstrates that the maternal MspI/HpaII
sites of this CpG island are completely methylated and therefore not digested by HpaII, whereas the respective paternal sites are unmethylated.

In summary, our methylation analysis of the 5’ CpG island of the human PEG1/MEST gene demonstrates parent-of-origin-specific methylation in fetal and adult DNAs with the paternal allele being unmethylated and the maternal allele being methylated.

**DISCUSSION**

In this study we report that the human PEG1/MEST gene is imprinted and contains a CpG island that is methylated in a parent-of-origin-specific manner, with the active paternal allele being unmethylated and the inactive maternal allele being methylated.

The imprinted mouse Peg1/Mest gene was identified in a systematic screen using subtraction hybridization with cDNAs from parthenogenetic and control embryos (Kaneko-Ishino et al., 1995). Subsequent homology search revealed that Peg1 was identical to the previously identified mesoderm-specific cDNA Mest (Sado et al., 1993). During our studies, the Peg1/Mest homologous human cDNA (PEG1/MEST) was isolated and mapped to chromosome 7q32. Expression analysis of a hydatidiform mole, which is mostly paternal in origin, and dermoid cysts, which are mostly maternal in origin, demonstrated that PEG1/MEST is abundantly expressed in moles but scarcely in dermoid cysts (Nishita et al., 1996). This observation is in good agreement with our results which provide clear evidence that the human PEG1/MEST gene is imprinted. In our approach to isolate the human PEG1/MEST gene we have identified homologous sequences on the short arm of human chromosomes 3 and 5. At present it is unknown whether these cross-hybridizing sequences are expressed and if so, whether they are subject to imprinting, too.

A newly identified single nucleotide insertion/deletion polymorphism in the 3’ UTR of the human PEG1/MEST gene enabled us to study allelic expression in a series of fetal tissues and adult blood lymphocytes. Monoallelic expression of PEG1/MEST was observed in all fetal tissues examined, including brain, skeletal muscle, kidney, adrenal, tongue, heart, skin, and placenta. In two informative cases, we could show that PEG1/MEST expression is confined to the paternally derived allele. In contrast to the monoallelic expression observed in fetal tissues, biallelic expression was evident in adult blood lymphocytes of all three heterozygous individuals. In addition, we have demonstrated the presence of PEG1/MEST transcripts in a lymphoblastoid cell line of a patient with mUPD7, which is in keeping with our finding that this gene is expressed in adult blood lymphocytes. The imprinting status of human PEG1/MEST in fetal blood lymphocytes is currently unknown, and it remains to be elucidated whether in humans the imprint is lost at some time during development or whether imprinting is not established in these cells. Interestingly, it has been shown recently that in contrast to the human PEG1/MEST gene, the mouse gene is only expressed from the paternal allele in both fetal and adult blood (M. Reule and R. Fundele, Berlin, pers. comm., March 1997). On the basis of this observation, it is most likely that in humans, PEG1/MEST imprinting in blood lymphocytes is lost during development. It is becoming increasingly apparent that imprinted genes do not necessarily repress one parental locus in all tissues, and our results indicate that this is also true for the human PEG1/MEST gene. Tissue-specific escape or relaxation of imprinting was previously observed for the IGF2 gene in adult liver (Kalscheuer et al., 1993), fetal choroid plexus, and leptomeninges (Ohlsson et al., 1994) and for H19 in placenta. Human H19 is biallelically expressed in the placenta at an early stage, which contrasts with consistent monoallelic expression in the mouse placenta (Jinno et al., 1995; Tremblay et al., 1995).

The human PEG1/MEST gene spans a genomic region of approximately 13 kb. Sequence analysis of the 5’ end revealed the presence of a CpG island, spanning the promoter region, exon 1, and part of intron 1. Three imperfect direct repeats of 20 bp, arranged in tandem, have been identified upstream from this CpG island. Direct repeats have been found in most imprinted genes analyzed to date and are also evolutionarily conserved (Neumann et al., 1995), thus, the human PEG1/MEST gene shares this probably important feature of imprinted DNA sequences, too. Our methylation studies of the 5’ region of PEG1/MEST, including the CpG island, revealed that it is methylated in a parent-of-origin-specific manner with hypomethylation of the paternal allele and hypermethylation of the maternal allele in all tissues examined. We suggest that the observed DNA methylation is involved in the silencing of the maternally derived PEG1/MEST allele. Interestingly, however, this parent-of-origin-specific methylation does not parallel the PEG1/MEST expression pattern in all tissues examined. In fetal tissues, PEG1/MEST is monoallelically expressed from the paternally derived allele. Despite the biallelic expression of PEG1/MEST in adult blood lymphocytes, specific DNA methylation of the maternally derived allele was identical to that of adult and fetal tissues. From our results, it is most likely that methylation at all HpaII sites in the putative promoter region is not sufficient to completely silence the maternally derived PEG1/MEST allele in blood lymphocytes. We cannot determine from the present data whether there are specific CpGs, which are unmethylated on this allele in blood lymphocytes, or whether lymphocyte-specific transcription factors allowing transcription of the methylated maternally derived allele are involved. On the other hand, an alternate promoter that is regulated differently and that escapes imprinting in blood lymphocytes could be re-
sponsible. A well-known example of regulation by imprinting in development by alternate promoter usage is the IGF2 gene. The P1 promoter is located more than 20 kb upstream of the 5' CpG island and appears to escape imprinting in several adult tissues (Vu and Hoffman, 1994; Ekström et al., 1995).

In summary, the results presented here show that the human PEG1/MEST gene is imprinted and that it contains a CpG island that is differentially methylated on maternally and paternally derived chromosomes. Our finding of a paternally expressed gene on human chromosome 7q32 is interesting in two respects. First, there is strong evidence for the existence of at least one maternally imprinted gene on the long arm of chromosome 7 that controls intrauterine and postnatal growth. Maternal UPD for human chromosome 7 was found in 3 patients of short stature (Spence et al., 1989; Voss et al., 1989; Spotila et al., 1992), and mUPD for the long arm of chromosome 7 and pUPD7 for the short arm were found in 1 patient with short stature (Eggerding et al., 1994). In addition, a systematic study of patients with either Silver–Russell syndrome or primordial growth retardation revealed mUPD7 in 4 of the 35 patients investigated (Kotzot et al., 1995). These findings contrast with normal growth observed in 1 known patient with pUPD7 (Höglund et al., 1994). Hitherto it is unclear whether PEG1/MEST plays a role in growth or another yet unidentified paternally expressed imprinted gene of chromosome 7 is involved. In the mouse, maternal duplication of the proximal region of chromosome 6 is lethal in embryogenesis, possibly because of deficient Peg1/Mest expression. In contrast, paternal duplication of the proximal region of chromosome 6 is viable, suggesting that the excess gene dosage for Peg1/Mest, or of any other imprinted gene in this region, has no detectable influence on development (Cattanach and Beechey, 1990; Beechey and Cattanach, 1995; Kaneko-Ishino et al., 1995).

Second, Pérez Jurado et al. (1996) determined the parental origin of a deletion found in patients with Williams syndrome, a neurodevelopmental disorder involving growth retardation. A significant correlation between more severe growth retardation of postnatal onset among patients with maternal deletion of part of chromosomal band 7q11.23 compared to those with paternal deletion was observed. The authors suggested that a yet unidentified paternally imprinted gene might be involved. A single patient with paternal isodisomy for chromosome 7 has been reported to date (Höglund et al., 1994). As this patient has normal stature, it is likely that, as in the mouse, paternal disomy for chromosome 7 has no phenotypic effect on growth. The existence of a paternally imprinted gene on human chromosome 7 remains to be elucidated.

There is good evidence that imprinted genes are clustered in chromosomal regions. Human chromosome 11 harbors several imprinted genes; some are expressed from the same parental allele, others from the opposite allele. Similarly, the Prader–Willi syndrome/Angelman syndrome region of human chromosome 15 contains a cluster of imprinted genes, but in contrast to chromosome 11, all yet identified monoallelically expressed transcripts are exclusively paternal in origin. In addition, the region is predicted to contain at least one other gene that is only expressed from the maternal allele. In this respect, it is tempting to speculate that on human chromosome 7 too, imprinted genes may be clustered. A systematic search for imprinted genes in the vicinity of PEG1/MEST is in progress.

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