Identification and characterization of an imprinted antisense RNA (MESTIT1) in the human MEST locus on chromosome 7q32

Kazuhiko Nakabayashi1,‡, Louise Bentley2,†, Megan P. Hitchins2, Kohzoh Mitsuya3, Makiko Meguro3, Sachi Minagawa1,4, John S. Bamforth5, Philip Stanier2, Michael Preece2, Rosanna Weksberg1, Mitsuo Oshimura3, Gudrun E. Moore2 and Stephen W. Scherer1,*

1Department of Genetics, The Hospital for Sick Children, and Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada, 2Department of Fetal and Maternal Medicine, Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College, London, UK, 3Department of Molecular and Cell Genetics, Faculty of Medicine, Tottori University, Yonago, Tottori, Japan, 4Kihara Institute for Biological Research, Graduate School of Integrated Science, Yokohama City University, Yokohama, Japan and 5Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada

Received April 5, 2002; Revised and Accepted May 24, 2002 DDBJ/EMBL/GenBank accession nos:

Imprinted gene(s) on human chromosome 7 are thought to be involved in Russell–Silver syndrome (RSS), based on the fact that ~10% of patients have maternal uniparental disomy of chromosome 7. However, involvement of the known imprinted genes (GRB10 at 7p12, PEG10 at 7q21.3 and MEST at 7q32) in RSS has yet to be established. To screen for new imprinted genes, we are initially using somatic cell hybrids containing a paternal or maternal human chromosome 7. Transcripts located between D7S530 and D7S649 (a 1.5Mb interval encompassing MEST) were subjected to RT–PCR analysis using somatic cell hybrids. One transcript named MESTIT1 (for MEST intronic transcript 1) reproducibly showed paternal-specific expression. Upon further analysis, we found MESTIT1 to be (1) paternally (and not maternally) expressed in all fetal tissues and fibroblasts examined, (2) to be located in an intron of one of the two isoforms of MEST but transcribed in the opposite direction, (3) to be composed of at least two exons without any significant open reading frame, and (4) to exist as a 4.2kb transcript in many fetal and adult tissues. We could also identify two isoforms of the mouse Mesi gene as observed in humans, but it is still unknown if a murine ortholog of MESTIT1 exists. We also examined the imprinting status of MEST isoforms as a first step in assessing whether MESTIT1 might influence the allelic expression pattern of the sense transcript. MEST isoform 1 was determined to be exclusively expressed from the paternal allele in all fetal tissues and cell lines examined, whereas MEST isoform 2 was only preferentially expressed from the paternal allele in a tissue/cell-type-specific manner. Our results suggest that MESTIT1 is a paternally expressed non-coding RNA that may be involved in the regulation of MEST expression during development. MESTIT1 (also known as PEG1-AS) is now the third independent transcript (with MEST and COPG2IT1) identified at human chromosome 7q32 demonstrating paternal chromosome-specific expression.

INTRODUCTION

Genomic imprinting is an important mechanism of gene regulation which can lead to the non-equivalent representation of the expressed maternal and paternal genomes (1). This phenomenon can influence mammalian development, growth and behavior (2). Imprinted gene(s) on human chromosome 7 have been postulated to be involved in Russell–Silver syndrome (RSS), based on the fact that up to 10% of patients have maternal uniparental disomy of chromosome 7 (mUPD7) (3–6). Three imprinted loci, GRB10 at 7p12 (7–10), PEG10 at 7q21 (11) and MEST at 7q32 (12), have been identified on...
The results provide evidence for the involvement of an impaired placentophagia, a distinctive mammalian behavior expressed in human and mouse fetal tissues (12, 17, 19, 7q31). RSS has yet to be established (8, 10, 13) on human chromosome 7. However, involvement of these loci in human imprinted genes (20). Although the deduced amino acid sequence of the gamma 2, and is located distal to MEST, with the two genes having overlapping 3′ UTRs in a tail-to-tail orientation (22, 23). Initially, COPG2 was shown to be imprinted (22). However, other studies could not reproduce this result, but instead indicated that the observation was due to overlap with the MEST gene (23). Subsequently, mouse Copg2 was shown to be maternally expressed in adult brain and embryos (24). Moreover, COPG2IT1 (COPG2 intronic transcript 1) is a transcript located within intron 20 of COPG2 that is paternally expressed in all fetal tissues examined (23). Copg2as2 (Mit1/Lb9), located within intron 20 of mouse Copg2, was also shown to be paternally expressed (24). The mRNA sequences for COPG2IT1 and Copg2as2 are both transcribed in the opposite direction to the COPG2/Copg2 genes, and while the complete transcription units are not yet known, they appear to represent non-coding RNAs.

The accumulating evidence for multiple transcripts at the MEST/COPG2 locus led us to search for additional imprinted transcripts. Our initial approach was to screen 29 transcripts located between D7S530 and D7649 (encompassing an approximately 1.5 Mb region flanking MEST/COPG2) (25) by RT–PCR analysis using somatic cell hybrids. Since the imprinting status of at least some imprinted genes has been shown to be maintained in somatic cell hybrids (26, 27), such cell lines have been used widely for systematic screening of human imprinted genes (28–30).

In our present study we have used established and characterized somatic cell hybrid lines containing a paternal or maternal human chromosome 7, in which the imprinting of MEST is maintained (6, 31, 32). Several transcripts were found to demonstrate allelic expression biases, suggesting the possibility of a complex imprinted gene cluster at 7q32. These transcripts are being further analyzed for their allelic expression pattern, using human tissue material to evaluate the imprinting status in vivo. Here, we have identified and characterized a new imprinted transcript located intronic to one isoform of MEST. This gene, named MESTIT1, is transcribed from the paternal allele only in the opposite direction to the MEST gene and, therefore, may be involved in regulating its expression. Li et al. (33) have also identified the same transcript as an imprinted non-coding RNA, PEG1-AS. As a first step in assessing the possible role of MESTIT1 in the imprinting of MEST, we also determined the imprinting pattern of MEST isoforms in a variety of fetal tissues and cell lines. Although MEST isoform 2 was determined previously to be biallelically expressed in the tissues and cell lines examined (20, 33), we found tissue-specific imprinting of MEST isoform 2.

**RESULTS**

**Identification of MESTIT1 as a candidate imprinted transcript**

We tested 29 genes and transcripts from a 1.5 Mb region encompassing the MEST/COPG2 locus for their allelic expression pattern by RT–PCR, using the somatic cell hybrid assay. Representative results are shown in Figure 1. Transcripts AA215684, CPA1, and CPA5 were found to demonstrate allelic differences favoring the maternal chromosome. To evaluate the imprinting status in vivo, these transcripts are being subjected to further allelic expression analysis using human tissue material. One transcript, GenBank acc. AW161444 (cDNA clone IMAGE: 2782723, part of MESTIT1, see below), reproducibly showed paternal-specific expression in different somatic cell hybrids, in which imprinting of MEST isoform 1 is maintained (Fig. 1). MEST isoform 2 unexpectedly showed maternal expression in somatic hybrids; we describe the further characterization of MEST isoform 2 later (also see Discussion).

Comparison of the complete cDNA sequence of IMAGE: 2782723 and the genomic DNA sequence containing the MEST locus (GenBank acc. AC007938) indicated that the transcript is located in an intron of MEST isoform 2 but is transcribed in the opposite direction to the MEST gene (Fig. 2A). Based on these features, it has been assigned the name MESTIT1 (MEST intronic transcript 1) by the HUGO Nomenclature committee. The name was given recognizing that the so-far established DNA sequence of MESTIT1 is antisense to the MEST locus, and not the gene itself. In order to attempt to identify the full-length transcript, RT–PCR and RACE experimentation followed by DNA sequencing was completed, yielding an assembled cDNA sequence of 3242 bp (GenBank acc. AF482998) (Fig. 2B). The DNA sequence of the 3′ end of this consensus did not contain a canonical poly(A) signal (AATAAA or AATAAA); the cDNA and 3′ RACE fragments seem to have been amplified from cDNA reverse-transcribed by oligo-dT primers hybridizing with internal poly(A) tracts. Repeated 5′ and 3′ RACE experimentation using different cDNA sources could not extend the transcript beyond 3242 nucleotides, even though a 4.2 kb message was observed upon northern blot analysis (Fig. 3; see below). Also, RT–PCR using primers A53 downR/AS2452F and primers AS445R/AS5upF failed to amplify cDNA fragments (Fig. 2B). The assembled sequence represents three-fourths of the estimated mRNA size and was used for further analyses.

We have not yet been able to establish the existence of a murine homolog of MESTIT1, since no relevant mouse cDNA or expressed sequence tag (EST) sequences (oriented in the...
same direction) are known. The human MESTIT1 gene and the equivalent syntenic region in mouse share only discontinuous stretches of DNA sequence identity (Fig. 2C). In this study, we were able to confirm the presence of isoform 2 of the murine Mest gene by characterization of the RIKEN mouse cDNA 7420402J24 (GenBank acc. AF482999). It had a related DNA sequence and genomic structure to isoform 2 of human MEST.

Therefore, MEST isoforms 1 and 2 are known in mouse, but MESTIT1 is, at present, only characterized in humans.

Expression analysis of MESTIT1

For northern blot analysis of MESTIT1 on somatic cell hybrids, four probes were used: three cDNA probes (AS1, AS2 and AS3) and one oligonucleotide probe complementary to the MESTIT1 mRNA sequence (P#575 used in 33). The position of probes is shown in Figure 2A. Consistent with the RT–PCR results for MESTIT1 (Fig. 1), the cDNA probes AS1, AS2 and AS3 detected signals only in the paternal hybrids (P1–P4) with various levels but not in the maternal hybrids (M1–M4) or mouse A9 cells (Fig. 3A). A major band (approximately 4.2 kb in size) and two minor bands were detected on P1 and P2 hybrids. The sizes of the two minor bands were estimated to be 6.5 kb and 9.5 kb. The same pattern of hybridization signals was detected using all three cDNA probes (AS1–3) (data not shown). The probe AS3 is located downstream of the 3′ end of the cDNA clone IMAGE: 2782723 (AS2 in Fig. 2A), confirming that MESTIT1 mRNA is longer in the 3′ direction than clone 2782723 on somatic cell hybrids. The same blot was hybridized with a probe for MEST (probe S in Fig. 2A), confirming that MESTIT1 mRNA is longer in the 3′ direction than clone 2782723 on somatic cell hybrids. The same blot was hybridized with a probe for MEST (probe S in Fig. 2A). An approximately 2.5 kb band was observed only on the paternal hybrids (P1–P4), as expected (Fig. 3A). The oligonucleotide probe P#575 complementary to MESTIT1 mRNA also detected paternal-specific bands of the same sizes at those detected by

Figure 1. Somatic cell hybrid assay to screen for differentially expressed and imprinted genes on chromosome 7. PCR primers specific for MEST isoform 1, MEST isoform 2, MESTIT1 and CPA5 (GenBank acc. AF384667) were tested by RT–PCR against template cDNA from human fibroblast (Fi), somatic cell hybrid containing a paternal (P1–P4) or a maternal (M1–M4) chromosome 7, and mouse A9 cells. In each case, the expected PCR product sizes were observed, except for MEST isoform 2 in line M1, where a smaller band was reproducibly observed. Each primer pair was tested under several different PCR cycling conditions. Representative results (35, 40, 35, 35 and 21 cycles for MEST isoform 1, MEST isoform 2, MESTIT1, CPA5 and mouse Gapd, respectively) are shown. Mouse Gapd was used as a control for the amount of cDNA among the samples. The results show MEST isoform 1 to be paternally expressed, MEST isoform 2 to be maternally expressed, MESTIT1 to be paternally expressed, and CPA5 to have a reproducible differential expression pattern favoring the maternal allele. The expected PCR product size is shown on the left of each panel.
the probe AS1 for MESTIT1 (Fig. 3B). The results confirm that the 4.2 kb signals detected in paternal hybrids by the three cDNA probes (AS1–AS3) are derived from mRNA transcribed in a 7q telomere to centromere orientation (i.e. MESTIT1). Since MESTIT1 has a constant transcriptional size, it is likely that there is yet to be identified polyadenylation site.

The MESTIT1 probe AS1 was also hybridized against human multiple tissue blots (Clontech) and a 4.2 kb band was detected in many fetal and adult tissues, consistent with the detected transcript size of MESTIT1 on somatic cell hybrids (Fig. 3C). In adult heart and testis, a smaller band (approximately 1.6 kb in size) was also observed. The oligonucleotide probe used for somatic cell hybrids (P575) was also examined on the multiple tissue blot containing testis poly(A)+ RNA, but failed to detect the expression of MESTIT1. This was most likely due to the lower expression levels of MESTIT1 in human tissues than found in paternal somatic hybrids (data not shown). RT–PCR analysis detected expression of MESTIT1 in seven of 11 tissues tested (Fig. 3D). Finally, strand-specific RT–PCR using fetal brain and P1 hybrid RNAs further confirmed the transcriptional orientation of MESTIT1 to be telomere to centromere, along the long arm of chromosome 7 (Fig. 4). Our results indicate that MESTIT1 is expressed mainly as a 4.2 kb transcript, which is different from another report describing 2.4 kb of a transcript (named PEG1-AS) that overlaps with MESTIT1 (33).

Paternal-specific expression of MESTIT1 in fetal tissues and fibroblasts
A G/A SNP at nt 1347 (in GenBank acc. AF482998) within MESTIT1 exon 2 (Fig. 5A) was used to determine the origin of allelic expression of MESTIT1 in heterozygous fetal tissues. The ‘A’ variant was found at an allelic frequency of 33% in 54 chromosomes. Nine fetal samples (first trimester, ranging in gestational age from 7 to 12 weeks) heterozygous for the SNP were studied. Three were informative, in that the maternal DNA was homozygous.

Allelic expression was initially examined by PCR amplification of random-primed cDNAs using a forward primer within exon 1 (AS290F) and a reverse primer within exon 2 (AS1648R) followed by sequence determination of the cDNA amplicons with primers flanking the G/A polymorphism. MESTIT1 was expressed at equal levels in all tissues tested, with the exception of placenta, in which its expression was consistently lower, requiring 40 cycles of amplification. As a result, the imprinting analyses could only be performed in two samples. Monoallelic expression of MESTIT1 was observed in each fetal tissue or organ tested for all nine heterozygous fetuses studied, as visualized by a single ‘G’ or ‘A’ peak at the polymorphic site of amplified cDNA (Fig. 5B). In the three informative pairs, the allele observed in the cDNA from each individual tissue sample was opposite to the maternal allele, indicating that transcription of MESTIT1 occurs exclusively from the paternal chromosome (Table 1). Paternal expression was also confirmed in the three informative fetuses using MESTIT1 strand-specific primer (AS2227R) to generate first-strand cDNAs specifically from the MESTIT1 transcript (see example in Fig. 5B). Thus we have demonstrated that MESTIT1 is paternally transcribed in all major fetal organs and other tissues during the first trimester of gestation. All four fibroblast cell lines examined also showed exclusive paternal expression of MESTIT1 (data not shown). Our result is consistent with that of Li et al. (33), but more comprehensive in terms of numbers of samples and types of tissue.

Imprinting status of MEST isoforms in cell lines and fetal tissues
We examined the allelic expression pattern of MEST isoforms in lymphoblasts, fibroblasts, and fetal tissues, with the rationale being that there could be a relationship between the expression of MESTIT1 and MEST. In lymphoblastoid cell lines of...
Figure 3. Northern blot analysis. (A) MESTIT1 and MEST hybridized to northern blots containing poly(A)+ RNA (2 μg/lane) from somatic cell hybrids carrying paternal (P1–P4) and maternal (M1–M4) chromosome 7s and an A9 control. The results using probe AS2 (for MESTIT1), probe S (for MEST) and a 200 bp cDNA fragment (for mouse Gapd) are shown. The same blot was used consecutively for all three probes. The locations of the probes used are shown in Figure 2A and described in the Results, and Materials and Methods. (B) Detection of MESTIT1 expression using an antisense oligonucleotide probe (P#575) on an RNA blot containing P1, M4 and A9 poly(A)+ RNAs (8 μg/lane). The same blot was used consecutively for P#575, cDNA probe AS1 and Gapd. The same pattern of hybridization signals was detected by probes P#575 and AS1. Only the results for P#575 and Gapd are shown. (C) Expression analysis of the MESTIT1 gene on multiple fetal and adult tissue blots using AS1 as a probe. (D) RT–PCR analysis for MESTIT1 using 10 human adult tissues and a lymphoblastoid cell line. Primers AS270F and AS445R (see Materials and Methods) were used for MESTIT1. The PCR cycle numbers used are 40 and 27 for MESTIT1 and β-actin, respectively. The expected PCR product size is shown on the right of each panel.
individuals with paternal and maternal UPD7, it has been shown that MEST isoform 1 is imprinted (paternally expressed) but MEST isoform 2 is not imprinted (bicollally expressed) (20). We could confirm the same observation in maternal UPD cell lines (data not shown) but did not have access to a paternal uniparental disomy sample. Moreover, among four fibroblasts heterozygous for the G/C SNP in exon 12 of MEST (nt 1276 in GenBank acc. D87367) (Fig. 5A), MEST isoform 1 was expressed exclusively from the paternal allele in all four lines. MEST isoform 2 was expressed exclusively from the paternal allele in one fibroblast line, whereas it was expressed mostly from the paternal allele but was also detectable from the maternal chromosome in the other three lines (data not shown).

We determined the allelic expression patterns of MEST isoforms 1 and 2 in the five fetuses heterozygous for the G/C SNP in exon 12. Although isoform 1 showed monoallelic expression in all 13 tissues examined, isoform 2 showed biallelic expression in 11 of the 13 tissues examined. In kidney and placenta, isoform 2 was exclusively or preferentially expressed from the same allele with the expressed allele of isoform 1 (Table 2). Although we could not directly determine the parental origin of the expressed alleles of MEST isoforms 1 and 2, because mothers for the five fetuses were all heterozygous for the G/C SNP, we inferred that isoform 2 is predominantly expressed from the paternal allele in fetal kidney and placenta. The electropherograms for cDNAs (MEST isoforms 1 and 2) from brain and kidney of fetus 13 are shown as examples (Fig. 5C). The imprinting patterns for MESTIT1 and MEST isoforms in fetal tissues and cell lines tested are summarized in Table 3.

DISCUSSION
We have identified a novel imprinted transcript, MESTIT1, which is widely expressed at low levels in an opposite orientation to MEST and imprinted (paternally expressed) in all fetal tissues examined. MESTIT1 joins the MEST gene and CIT1 (also called COPG2IT1) within intron 20 of the COPG2 gene as the third apparently independent transcript, within this 60 kb region of human chromosome 7q32, demonstrating paternal chromosome-specific expression (Fig. 2A). Since MEST and COPG2 also overlap at their 3' ends and murine Copg2 appears to be maternally expressed (not yet shown in humans), all lines of evidence suggest that transcriptional regulation at this genomic locus is highly complex. Recently, Li et al. (33) also identified a smaller but overlapping transcript to MESTIT1 (called PEG1-AS) as an imprinted PEG1/MEST antisense transcript expressed predominantly in human testis and in mature spermatozoa. We also detected strong expression of MESTIT1 in testis by northern...
analysis and RT–PCR (Fig. 3). However, through exhaustive analysis, we determined the major transcript size of MESTIT1 to be 4.2 kb, which is larger than the 2.4 kb message described for PEG1-AS (33). One may postulate that the MESTIT1 locus contains two isoforms (2.4 and 4.2 kb). To reconcile the difference between the two studies, we tried four probes for northern analysis, including an oligonucleotide probe (Pr#575) complementary to MESTIT1 (and also PEG1-AS). Our three cDNA probes and Pr#575 consistently detected 4.2 kb signals on paternal somatic cell hybrids. The probe AS1 detected 4.2 kb signals in a variety of human tissues and 1.6 kb signals in heart and testis, but failed to detect 2.4 kb bands. Further characterization of the gene structure of MESTIT1 (i.e. defining the 3′ end with a polyadenylation signal) may help reconcile the two studies.

Our data suggest that MESTIT1 is a paternally expressed non-coding RNA, as is the case in seven other previously identified antisense/intronic transcripts at imprinting loci in human and mouse (24, 28, 34–43). In the cases of Igf2ras (Air) and LIT1, deletions that block the transcription of antisense transcripts resulted in the loss of imprinting of the sense transcripts (34, 44, 45). However, the function of the other antisense transcripts, including MESTIT1, remains to be elucidated.

In this study, we performed comparative sequence analysis for MEST and the MESTIT1 locus between human and mouse. Although exon 2 of MESTIT1 contains four regions showing greater than 75% sequence similarity, the overall conservation between human and mouse sequences is not significantly high (50.6%) (Fig. 2C). This value is lower than that of the 5′ or 3′ UTRs of MEST (69.3% for 5′ UTR of isoform 1, 52% for 5′ UTR of isoform 2, and 62.3% for 3′ UTR). Thus, the evolutionary constraint for the MESTIT1 locus is weaker than that of UTRs of protein-coding genes (46, 47). This observation suggests that expression of MESTIT1 itself from a specific promoter may be more critical than the content of the transcribed sequence.

Low sequence conservation in non-coding imprinted transcripts between human and mouse seems to be a common feature. The human IGF2AS mRNA (2.1 kb in size) contains three exons, whereas mouse Igf2as mRNA (4.8 kb in size) contains at least four exons. Although both human exon 1 and mouse exon 1 of IGF2AS/Igf2as overlap with IGF2/Igf2 with similar length (91 bp in human and 97 bp in mouse), the sequence similarity between IGF2AS and Igf2as mRNAs is only 50% (42). Our analysis of COPG2IT1 (GenBank acc. AF038190) and Copg2as2 (also called Mit1/Mb9) (GenBank acc. AF217545), which are paternally expressed transcripts located within intron 20 of COPG2/Copg2, indicates a sequence similarity of 59.7%. In the case of GNASAS/Nepsas, the former has five exons (36), whereas the latter is intronless (41). Although a 269 bp region forming a part of GNASAS exon 1 and a 5′ upstream region are highly conserved

<table>
<thead>
<tr>
<th>Fetus</th>
<th>Fetal age (weeks)</th>
<th>Tissue samples</th>
<th>Maternal genotype</th>
<th>Expressed allele</th>
<th>Imprinting status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Brain, heart, lung, skin</td>
<td>G</td>
<td>A</td>
<td>Paternally expressed</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>Heart, intestine, skin</td>
<td>G</td>
<td>A</td>
<td>Paternally expressed</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>Eye, skin, stomach, umbilical cord</td>
<td>A</td>
<td>G</td>
<td>Paternally expressed</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>Brain, heart, intestine, liver, placenta</td>
<td>G/A</td>
<td>G</td>
<td>Monoallelic (UI)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Brain, heart, intestine, skin, tongue, placenta</td>
<td>G/A</td>
<td>A</td>
<td>Monoallelic (UI)</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>Lung, pancreas</td>
<td>No DNA</td>
<td>G</td>
<td>Monoallelic</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>Brain, heart, kidney</td>
<td>G/A</td>
<td>G</td>
<td>Monoallelic (UI)</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>Brain, eye, heart, intestine, lung, umbilical cord</td>
<td>G/A</td>
<td>G</td>
<td>Monoallelic (UI)</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>Brain, heart, tongue</td>
<td>G/A</td>
<td>G</td>
<td>Monoallelic (UI)</td>
</tr>
</tbody>
</table>

Table 1. Imprinting of MESTIT1 during first-trimester fetal development

The gestational age and source of tissues from fetuses heterozygous for a G/A polymorphism in MESTIT1 are listed. The maternal genotype, allele detected in the MESTIT1 cDNA and inferred parental expression profile are shown. UI, uninformative. Monoallelic expression of MESTIT1 was identified in the following tissues: brain (6/6), eye (2/2), heart (7/7), intestine (4/4), kidney (1/1), liver (1/1), skin (4/4), stomach (1/1), tongue (2/2) lung (3/3), pancreas (1/1), placenta (2/2) and umbilical cord (2/2).
between human and mouse (87% identity over 285 bp) (36), identity between exons 2 to 4 was not maintained. An imprinted non-coding RNA H19 showed 60.23% similarity between human and mouse (mRNA sequences from GenBank acc. M32053 and X07201 were used for comparison). The XIST/Xist (X inactive specific transcript) shows low sequence similarity (48–49%) between human and mouse, although there is significantly higher conservation for limited regions (48).

Based on the analysis of non-coding RNAs mentioned above, the following features can be described: (1) these human and mouse genes are less conserved (approximately 50–60%) than their protein coding counterparts; (2) common exon–intron structure is not always maintained between two species; and (3) only small regions of sequence similarity were maintained.

Our analysis of the human and mouse MEST locus revealed the structural similarity between the two species, with both having two isoforms of MEST and highly conserved regions between portions of MESTIT1. The latter observation may imply the existence of MESTIT1 in mouse, and/or the importance of the intervals as cis-regulatory elements. At the moment, it is not clear if mouse Mestit1 exists (analysis in progress). The expression of mouse Mest isoform 2 seems to be more limited than that of human MEST isoform 2. Whereas 26 of 412 ESTs correspond to isoform 2 in Unigene Hs.79284 (human MEST), BB580007, derived from in vitro fertilized eggs, is the only EST corresponding to isoform 2 in 655 ESTs in Unigene Mm.1089 (mouse Mest).

We performed imprinting analysis of MEST isoforms using a variety of fetal tissues and two types of cell line for the following reasons. First, although paternal expression of MEST has been shown by three groups (12,17,18), the imprinting status of MEST isoform 2 had been examined only in lymphoblastoid cell lines (20) when we initiated the analysis. Furthermore, because MEST isoform 2 showed maternal-specific expression in somatic cell hybrids (Fig. 1), we considered the possibility of ‘reciprocal imprinting’ of MEST isoform 2 and MESTIT1. Therefore, second, we aimed to examine whether the expression pattern observed in A9 somatic cell hybrids reflected the true imprinting status of MEST isoform 2 in vivo (or whether it might be an artificial phenomenon in somatic cell hybrids).

Our results showed that MEST isoform 1 is paternally expressed exclusively in all tissues and cell lines examined, whereas MEST isoform 2 is preferentially paternally expressed in fetal placenta, kidney and fibroblast lines but is biallelically expressed in other fetal tissues and lymphoblastoid cell lines. Our results indicate that the minor level of maternal expression previously observed for the human MEST gene (14) is most likely derived from the biallelic expression of MEST isoform 2, and that the imprinting of the human MEST isoform 1 is as strictly regulated as that of the equivalent mouse gene (19). MESTIT1 also showed exclusive paternal expression in all tissues and cell lines examined (Table 3). Interestingly, it has been shown that the 1.5 kb region between MESTIT1 (PEG1AS) and exon 1 of MEST isoform 1 has promoter activity in both the sense and antisense directions (33).

| Table 2. Allelic expression analysis of MEST isoforms in human fetal tissues |
|-----------------|----------------|----------------|----------------|----------------|----------------|
|                  | 8              | 10             | 11             | 12             | 13             |
| MEST isoform     | 1              | 2              | 1              | 2              | 1              | 2              |
| Fetal tissues    |                |                |                |                |                |
| Brain            | G              | Bi             | G              | Bi             | G              | Bi             |
| Eye              |                |                |                |                |                |
| Heart            | G              | Bi             | G              | Bi             | G              | Bi             |
| Intestine        | G              | Bi             | C              | Bi             | G              | Bi             |
| Kidney           |                |                |                |                |                |
| Liver            |                |                |                |                |                |
| Lung             | G              | Bi             | G              | Bi             | C              | Bi             |
| Placenta         | C              | C              | G              | G > C          | C              | C > G          |
| Skin             |                |                |                |                |                |
| Spinal cord      | G              | Bi             | G              | Bi             | G              | Bi             |
| Stomach          |                |                |                |                |                |
| Tongue           | G              | Bi             | G              | Bi             | G              | Bi             |
| Umbilical cord   |                |                |                |                |                |

All five fetuses are heterozygous for the G/C SNP in MEST exon 12. The expressed allele is shown. Bi, biallelic expression; G > C and C > G, preferential expression.

The gestational ages (week) for the fetuses are 8, 5.5, 10, 11 and 6.
sense and antisense imprinted transcripts, Nesp55 and Gnas-as (Nesp-as). Although the two transcripts show reciprocal imprinting (maternal expression of Nesp55 and paternal expression of Gnas-as) in many tissues, the imprinting of Nesp55 sense transcripts was strictly maintained even in the absence of the Gnas-as transcripts (37). This suggests that the imprinting of the sense transcript (Nesp55) is not controlled directly by the antisense transcript (Gnas-as).

Our new data have revealed further complexity of imprinting mechanisms at the human MEST locus, including tissue- and isoform-specific imprinting. Our results contribute to the elucidation of molecular mechanisms controlling MESTIT1 and MEST expression and their possible roles in development and disease. For example, considering the growth retardation phenotype in Mest-deficient mice, loss-of-function mutations in the paternal allele of the MEST gene could cause a similar phenotype in humans. Although two independent groups screened for mutations in MEST in RSS patients, the studies did not identify any mutations (13,14). However, it is still possible that loss of the paternal copy of MEST in RSS patients may contribute to the pre- and postnatal growth retardation phenotype in the patients. Therefore, if MESTIT1 is involved in the regulation of MEST expression (and dysfunction of MESTIT1 results in loss of MEST expression), MESTIT1 could be involved in the etiology of RSS. Mutational analysis for MESTIT1 in RSS patients is in progress to assess the potential involvement of the transcript in the etiology of RSS.

MATERIALS AND METHODS

Identification of genes/transcripts from a 1.5 Mb interval between D7S530 and D7S649 flanking MEST/COPG2

Unigenes localized between D7S530 and D7S649 (25) were subjected to sequence clustering analysis using software in GCG Wisconsin Package. Also, the genomic DNA sequence GenBank acc. AC007938 containing MEST was repeat-masked using Repeat Masker, and used for a BLASTN search against dbEST to identify transcripts. The identified ESTs were analyzed further. Twenty-nine EST clusters that (1) had exon–intron structure or (2) were composed of two or more ESTs were analyzed further. PCR primers for radiation hybrid (RH) mapping (GeneMap '99 at NCBI) were used for the following 13 transcripts: WI-6171 (UBE2H), WI-11310, stSG21174, SGC35496, stSG3642, A006N09 (KIAA0265), sGC32605, WI-18390 [an alternative isoform of MEST at its 3' end (23)], IB3560, WI-17971, SGC33996, WI-16323 and WI-14634. Primer sequences can be found at http://www.ncbi.nlm.nih.gov/genemap99/. Primers for 10 EST clusters and six known genes were designed by Primer 3 (http://www.ebi.ac.uk/Tools/pmc3/cgi-bin/primer3www.cgi) using the sequences of following GenBank accession numbers (primer sequences available upon request): L22454 (NRFI), AA215684, AI016231, AI271622, AI829971, W23173, AA644574, AA300014, AA405520, AW161444 (MESTIT1), T95999, X67318 (CPA1), U19977 (CPA2), AF095719 (CPA4), AF384667 (CPA5) and D87367 (MEST).

RT–PCR analysis using RNA from somatic cell hybrids and human tissues

Two micrograms of total RNA from somatic cell hybrids and human adult tissues (Clontech, Palo Alto, CA, USA) was used for reverse transcription (RT) with random primers using SuperScript II (Invitrogen, Burlington, Canada) (1% of the RT reaction was used for PCR). The following conditions were used for PCR: initial denaturation at 94°C for 3 min, followed by 18–40 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s. Primers PEG33/34 and PEG36/34 were used for MEST isoforms 1 and 2, respectively (20). Primers AS270F (5'-CCACGGACACTGAGGAAAAT-3') and AS445R (5'-CCACTTGATCCATGTTGGA-3') were used for MESTIT1. Primers 5'-CAGACCTTTGGGGAGAAGGAT-3' and 5'-ACACTGGGAGATATGCTTC-3' were used for CPA5. The primer sequences for mouse Gapd were described previously (6). The primer sequences for β-actin are 5'-CTCTGGCCATCACCAGAACTA-3' and 5'-AAGCCATGCAATCTCATTAC-3'. The PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

RT–PCR and RACE to determine cDNA sequence of MESTIT1

Primers for RT–PCR were 5'-GGGCGAGGTCTCCCTTTACAGC-3' for AS5'upF, 5'-TAAAGGATCCCTTTACGC-3' for AS1F, 5'-CCACCTTGATCCATGTTGGA-3' for AS445R, 5'-TGTTGAAACCCCGTCTC-3' for AS2452R, 5'-TCTTGGCTGGAGCGAATC-3' for AS3242R, and 5'-CCCCCGC-

Table 3.

<table>
<thead>
<tr>
<th>Tissues/Cells</th>
<th>MEST Isoform 1</th>
<th>MEST Isoform 2</th>
<th>MESTIT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal tissues</td>
<td>P</td>
<td>P, P &gt; M or Bi*</td>
<td>P</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>P</td>
<td>P or P &gt; M</td>
<td>P</td>
</tr>
<tr>
<td>Lymphoblastoid cells from UPD7 patients</td>
<td>P</td>
<td>Bi*</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Somatic cell hybrids containing either paternal or maternal chromosome 7</td>
<td>P</td>
<td>M</td>
<td>P</td>
</tr>
</tbody>
</table>

P, paternal; M, maternal; Bi, biallelic; P > M, preferential paternal expression.

*The detailed results are described in Results and Table 2.

* Determined using UPD7 cell lines by Kosaki et al. (20), and result for maternal UPD7 cells confirmed in the present study.
GACAGAGTTAGT-3’ for AS3’downR (Fig. 2B). The following conditions were used for RT–PCR: initial denaturation at 94°C for 3 min, followed by 30–40 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 30 s, and extension at 72°C for 40–90 s. cDNA derived from the P1 hybrid cell line (containing a paternal chromosome 7) was used for template DNA. Marathon ready cDNA from testis (Clontech) was used for 5’ and 3’ RACE, following the manufacturer’s protocol. Gene-specific primers were used as 5’GSP-1 (5’-TGGG-TAGACATGTTCCATGGCCCTCA-3’), and 5’GSP-N (nested) (5’-TTGCTCTTACATGGATCTGGTGFT-3’) for 5’ RACE, 3’GSP-N-1A (5’-TCAGCCAGAAACCTTGGTACAACA-3’) and 3’GSP-NA (5’-AATCACATCGAGCCCGCTCTCAAC-3’) for 3’ RACE-1, and 3’GSP-1B (5’-TGGTTGTTCCTTCATCTTGGATGCTGGTGT-3’) and 3’GSP-NB (5’-CAGACGAGGGAAGCAGCTGG-AACTGA-3’) for 3’ RACE-2. The amplified DNA fragments were purified and sequenced directly.

Northern blot analysis

Total RNA from somatic cell hybrids was extracted with guanidine thiocyanate followed by cesium chloride centrifugation. Poly(A)+ RNA was purified using FASTTRACK 2.0 kit (Invitrogen). For the RNA blot for cDNA probes (Fig. 2A and 3A), two microgram per lane of poly(A)+ RNA samples were subjected to electrophoresis through 1% formamide–agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham, Piscataway, NJ, USA). cDNA probes were labeled with [γ-32P]ATP and used for strand-specific hybridization (Fig. 3). Probe AS1 for MESTIT1 is a 211 bp cDNA fragment (nt 411–621 in GenBank acc. AF482998) free from repetitive sequences (Fig. 2A, B). Probe AS2 for MESTIT1 and probe S for MEST were prepared from cDNA clones IMAGE: 2782723 and IMAGE: 251818, respectively (Fig. 2A). Probe AS3 for MESTIT1 is a cDNA fragment containing 117 bp of MESTIT1 sequence (nt 2755–2871 of GenBank acc. AF482998) amplified in 3’ RACE-2 (Fig. 2A, B). For probe AS2, human placenta DNA was used to suppress the hybridization of repetitive sequences in the probe. A probe for mouse Gapd (200 bp in size) was prepared by RT–PCR followed by gel purification. The hybridization and washing conditions were described previously (6).

A 36 bp oligonucleotide P#575 (5’-TGTACTCAGGGTT-C TTCCAAACAGAATATGCTTTTCC-3’) (33) is complementary to MESTIT1 mRNA and corresponds to nt 688–653 of GenBank acc. AF482998. P#575 was end-labeled with [γ-32P]ATP and used for a strand-specific probe for MESTIT1. An RNA blot containing 8 µg each of poly(A)+ RNA from P1 hybrid, M4 hybrid and mouse A9 cells was hybridized with the end-labeled P#575 at 55°C using Expresshyb solution (Clontech) for 3 h. The blot was washed successively with 2 × SSC/0.1% sodium dodecyl sulfate (SDS), 1 × SSC/0.1% SDS, and 0.2 × SSC/0.1% SDS at 55°C for 15 min each.

Strand-specific RT–PCR

One microgram each of total RNA from the P1 hybrid cell line (containing a paternal chromosome 7) or human fetal brain (Clontech) was used in a reaction for RT using the ThermoScript RT–PCR System (Gibco-BRL). The RT reaction was performed at 58°C with a strand-specific primer AS270F or AS621R. As a positive control, RT by random priming was performed for the same RNA material using SuperScript II (Gibco-BRL). All RT reactions were performed with or without reverse transcriptase (RT+/−). One-twentieth of the RT solution was subjected to PCR amplification with primers AS307F and AS445R, yielding a cDNA fragment from MESTIT1 which is 139 bp long. The cycling conditions were initial denaturation at 94°C for 3 min, followed by 30–40 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s.

Allelic expression analysis for MESTIT1 using fetal tissues and fibroblast cell lines

Multiple fetal tissues and maternal DNA samples were acquired as described previously (10). Local ethics approval for collection of paired samples for the study of genes involved in growth-related disorders was granted by the Research Ethics Committee of the Royal Postgraduate Medical School (96/4955).

Genomic DNAs from samples were genotyped for a novel transcribed G/A polymorphism within exon 2 of MESTIT1 (nt 1347 of GenBank acc. AF482998), to identify informative samples for imprinting analyses of MESTIT1 (Fig. 5A). Primers AS1648R (5’-TCAGGGATATGGTTGGTGAA-3’) and AS1129F (5’-GATTCACACGATGGATG-3’) flanking the polymorphism were used to PCR amplify a 520 bp product from genomic DNA. The cycling conditions were: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. The PCR products were purified and sequenced directly with the original primers. Total RNA was extracted from individual tissues of fetuses or fibroblast cell lines using the guanidine isothiocyanate extraction technique. Two micrograms of total RNA was treated with DNase I, and used for RT with random hexamers, to synthesize first-strand cDNA. In the case of informative sample pairs, where the fetus was heterozygous and the maternal DNA was homozygous, cDNA synthesis was repeated using a MESTIT1 strand-specific primer, AS2777R (5’-GGAA-GACTGAGCTGTAATT-3’) in place of random hexamers. Duplicate sets of samples, with RT omitted, were prepared for the detection of any contamination of the cDNAs with genomic DNA. One-tenth of the RT reaction was used as template for PCR amplification of a 1359 bp MESTIT1 cDNA product across intron 1 and the polymorphic site, using primer AS1648R and AS290F (5’-GGAGGAAACTACCCTTATAA-3’) (Fig. 5A). PCR amplification was performed within the log-linear phase for 38–40 cycles, using the same conditions as described for genomic DNA. The amplified cDNAs were purified and sequenced using primers AS1129F and AS2227R flank ing the polymorphism to determine the allelic origin of expression.

Allelic expression analysis for MEST isoforms using fetal tissues and fibroblast cell lines

Genomic DNAs from samples were genotyped for a transcribed G/C polymorphism within exon 12 of MEST (at nt 1276 of...
GenBank acc. D87367), to identify informative samples for imprinting analyses of MEST isoforms (Fig. 5A). Primers S1134F (5'-AGTGTCATCTCGGATGCC-3') and S1414R (5'-TCAACCTTATCAGAGTCC-3') were used for the amplification of a 281 bp fragment from genomic DNA. The cycling conditions were: initial denaturation at 94°C for 3 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 40 s. First-strand cDNA was synthesized with random hexamers as described in the previous section. Primers S206F (5'-CATGGGAATACGCCGCCCCAGT-3') and S1414R were used for the amplification of a 1209 bp fragment for isoform 1 from fetal cDNA. The cycling conditions for MEST isoform 1 were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. Primers exon A-F (5'-GGTCTACCTGAGCTAGT-3') and S1414R were used for the amplification of an 1183 bp fragment for isoform 2 from fetal cDNA. The cycling conditions for MEST isoform 2 were: initial denaturation at 94°C for 3 min, followed by 43 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90 s. All PCR reactions were performed in 50 µl volumes containing 1.5 mM betaine. The PCR products were purified and sequenced. For fibroblast cDNAs, primers PEG33 and S1398R (5'-GTCGATTCTGGATGACC-3') (for MEST isoform 1) and primers PEG36 and S1398R (for isoform 2) were used for PCR. The cycling conditions for isoform 1 were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s. The same conditions except for the cycle number (40 cycles) were used for MEST isoform 2.

ACKNOWLEDGEMENTS

We acknowledge technical support from Robert Edge and others in The Centre for Applied Genomics (http://tcag.bioinfo.sickkids.on.ca/) at The Hospital for Sick Children. We also thank Professor Y. Hayashizaki of RIKEN, for providing clones for our work. K.N. is a Research Fellow of the CIHR. S.W.S. is a Scientist of the Centre for Applied Genomics (http://tcag.bioinfo.sickkids.on.ca/).

REFERENCES


