Tissue-specific alternative polyadenylation at the imprinted gene Mest regulates allelic usage at Copg2

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ABSTRACT

The gene Mest (also known as Peg1) is regulated by genomic imprinting in the mouse and only the paternal allele is active for transcription. MEST is similarly imprinted in humans, where it is a candidate for the growth retardation Silver-Russell syndrome. The MEST protein belongs to an ancient family of hydrolases but its function is still unknown. It is highly conserved in vertebrates although imprinted expression is only observed in marsupials and eutherians, thus a recent evolutionary event. Here we describe the identification of new imprinted RNA products at the Mest locus, longer variants of the RNA, called MestXL, transcribed >10 kb into the downstream antisense gene Copg2. During development MestXL is produced exclusively in the developing central nervous system (CNS) by alternative polyadenylation. Copg2 is biallelically expressed in the embryo except in MestXL-expressing tissues, where we observed preferential expression from the maternal allele. To analyze the function of the MestXL transcripts in Copg2 regulation, we studied the effects of a targeted allele at Mest introducing a truncation in the mRNA. We show that both the formation of the MestXL isoforms and the allelic bias at Copg2 are lost in the CNS of mutants embryos. Our results propose a new mechanism to regulate allelic usage in the mammalian genome, via tissue-specific alternative polyadenylation and transcriptional interference in sense–antisense pairs at imprinted loci.

INTRODUCTION

The proximal end of mouse chromosome 6 contains an imprinted domain associated with a growth retardation phenotype in maternal uniparental disomy (mUPD) mice (1,2). This ~220-kb domain contains the paternally expressed gene Mest (also known as Peg1) (3) and the maternally expressed gene Klf14 (4). The promoter CpG island of Mest is differentially methylated in somatic cells (5). This DNA methylation mark is directly inherited from oocytes (6) constituting the only known differentially methylated region (DMR) and gametic imprint in the entire domain. Whereas DNA methylation at Mest is associated with silencing of the maternal allele (7), expression of the maternal Klf14 was found to require maternally inherited methylation (4). A targeted mutation at Mest, the MestK0 allele (official name Mesttm1Lef) established that Mest-deficient mice exhibit two main phenotypes: intrauterine growth restriction and an abnormal maternal behavior of mutant females (7). Mest codes for a highly conserved enzyme of the α/β-hydrolase fold superfamily, although the substrate and biological function of MEST are still unknown (3,8). This evolutionary conservation contrasts with the imprinting of the locus, which has only been observed in marsupials and placentals mammals (9,10). It is plausible that selective pressures for imprinting at the locus followed acquisition of novel therian-specific functions of the locus. In accordance with this proposition, Mest is highly expressed in extra-embryonic mesoderm where it may play important roles in the establishment or function of the placental vascular network (11).

We considered the possibility that imprinted transcription at Mest might be involved in alternative functions, independent of its coding potential. For instance, the Mest locus contains the microRNA Mir335, produced from intron 2, which is implicated in the suppression of human breast cancer metastasis (12). Another striking feature of the genomic organization of Mest is its overlap with the neighboring antisense gene Copg2 (13). The imprinted status of Copg2 itself is unclear in both mouse and human. In human, where the ortholog MEST is also paternally expressed in some tissues (14),

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the allelic usage at \textit{COPG2} is disputed in the literature with a report of paternal expression (15) and another of biallelic expression and no imprinting (16). Similarly in the mouse conflicting results have been published. \textit{Copg2} was shown to be preferentially expressed from the maternal allele only in particular intra-subspecific hybrids (13) but not in interspecific hybrids involving \textit{Mus spretus} (17). Whereas \textit{Mest} is transcribed from the (+) strand of the mouse genome, \textit{Copg2} is produced from the (–) strand, and their respective final coding exons overlap such that the two transcriptional units are convergent.

In order to resolve the imprinting status of \textit{Copg2} and its relationship with imprinting at the convergent \textit{Mest} locus, we have analyzed the relationship between the two neighboring transcripts. Here we document the formation of larger \textit{Mest} isoforms, called \textit{MestXL}, which are transcribed several kilobases into \textit{Copg2}. During development, the synthesis of \textit{MestXL} is regulated by tissue-specific alternative polyadenylation in neural tissues, where these large transcripts interfere with the production of \textit{Copg2} from the paternal allele. Consequently, allelic usage at \textit{Copg2} is regulated secondarily to imprinting at \textit{Mest} in a tissue-specific manner. Moreover, we show that this type of regulation affects only \textit{Copg2} in the domain as \textit{MestXL} does not regulate allelic usage at the downstream \textit{Klf14} gene. Our results suggest a new model for the acquisition of imprinted expression, via tissue-specific transcriptional interference (TI).

**MATERIALS AND METHODS**

**Mice and genotyping**

CD-1 mice are from the Animal Care Centre and the congenic mouse line with Chr 6 \textit{Mus musculus castaneus} SNPs on the 129S1 background was derived in our laboratory ([129S1cCAST6/Lef]). The \textit{Mest KO} mouse line (MGI: 2181803, official name \textit{Mest}\textsuperscript{m1Lef}) is maintained on a CD-1 background. All animal experimentation followed the guidelines from the Canadian Council on Animal Care (CCAC) under UBC animal care license numbers A03-0289 and A03-0292.

Heterozygous \textit{Mest+/KO} males were mated to CD-1 or 129S1cCAST6/Lef female mice. For timed mating, the day of the vaginal plug is E0.5. Females were sacrificed at E14.5 and yolk-sac samples were taken for PCR genotyping and scored for lacZ expression from the \textit{Mest}\textsuperscript{KO} allele by X-gal staining as described (7,18). Genotyping of the \textit{Mest}\textsuperscript{KO} allele was performed with primers 702A and 702C. Primer sequences are given in Supplementary Table S1. Approximately 25 µg of plasmid DNA was linearized with the appropriate restriction enzymes and purified by phenol–chloroform extraction. Approximately 1 µg was transcribed by T7 or SP6 RNA polymerase (40 units, Roche) in the presence of digoxigenin (DIG)-labeled UTP (Roche) to generate sense and antisense DIG-labeled probes. The \textit{in vitro} transcription was carried out at 37°C for 2 h, incubated with DNase for 15 min to remove the plasmid DNA, and purified by precipitation with 4 M LiCl.

**RNA purification and qRT–PCR analysis**

Embryonic total RNA was purified using Trizol (Invitrogen) according to manufacturer’s directions and DNase-treated (RQ1, Promega) for at least 1 h to remove contaminating gDNA. Anywhere from 500 ng to 1 µg of RNA was reverse-transcribed with SSII (Invitrogen), using random (N15) according to SSII protocol. Q-PCR was performed on a Step-One Plus Real time PCR system (Applied Biosystems) using Eva Green (Biotium) as the dye. An amount of 1 µl of cDNA was used in a 25 µl reaction as follows: 35–40 cycles of 95°C for 30 s, 56–62°C for 30 s, 72°C for 30 s, and 79–89°C 10 s with a plate read depending on the primer reaction, followed by a melt curve. Primer sequences are given in Supplementary Table S1. Ct values of three biological replicates, obtained by the LinReg PCR program ([22]); www.linregpcr.nl], were averaged and used to calculate relative amounts of transcripts, normalized to \textit{Ppia}.

**Northern blot analysis**

For the analysis of WT embryonic and adult tissues, northern hybridization was performed on commercial blots (Clontech) containing 2 µg of poly(A)\(^+\) RNA from mouse tissues (Cat. # 7762-1) and mouse embryos (Cat. # 7763-1) using a 32P-labeled \textit{Mest} ORF clone (exons 1–12) as a probe. The blots were hybridized at 68°C overnight using Clontech quickhyb buffer and washed at maximum recommended stringency (2 × SSC/0.1% SDS, 30 min; 55°C 0.1 × SSC, 0.1% SDS 30 min).

Dynabeads (Invitrogen) were used to obtain polyadenylated RNA from total RNA from E14.5 conceptuses between female CAST (or 129S1cCAST6/Lef) or CD-1 mice and male \textit{Mest}\textsuperscript{1/KO} heterozygotes. The DNA was separated by electrophoresis on a 1% agarose, 2.2 M formaldehyde denaturing gel. Neutral transfer (20× SSC) was performed

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onto nylon membranes, followed by UV-light crosslinking. Northern blot hybridization was carried out with DIG-UTP labeled RNA probes, with an overnight incubation at 65°C in DIG hybridization buffer (50% formamide, 5× SSC, 2% blocking reagent (Roche), 0.1% Sarkosyl, 0.5% SDS and 200 μg/ml denatured salmon sperm DNA). Membranes were washed twice with 1× SSC, 0.5% SDS, 0.1% Sarkosyl for 5–10 min each, twice with 0.1× SSC, 0.5% SDS, 0.1% Sarkosyl for 30 min each, and then twice with DIG-buffer (0.1 M Maleic acid pH 7.5, 0.15 M NaCl, 0.1% Tween-20) for 5 min each to prepare for the DIG antibody. The membranes were then incubated at room temperature for 45–60 min in blocking solution [DIG-buffer with 1% blocking reagent (Roche)], and 45–60 min with DIG antibody (Roche) (1:10000 dilution) in blocking solution, then followed by two washes with DIG-buffer for 15 min. After equilibrating in 100 mM Tris pH9.5, 100 mM NaCl, chemiluminescent detection with CDP-star reagent (Amersham Biosciences) was performed for 5 min before exposing to film. Exposure times ranged from 30 s to overnight. For one blot we used an alpha 32P-CTP-labeled DNA probe (Mest exon 12) generated through linear amplification (20 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min) from a reverse primer, P1 E12 NO R1, in Mest exon 12 to generate an antisense probe. Hybridization was carried out in Church’s buffer overnight in a Leica cryostat (model CM3050 S). To prepare the sections for hybridization, they were fixed (50% formamide, 5× SSC, 5× Denhardts, 0.25 mg/ml yeast tRNA and 0.5 mg/ml single stranded DNA) overnight at 65°C with their respective probe. The following day the slides were washed in different concentrations of SSC to remove non-specific probe, put through an RNase A step, the DIG antibody (1:2000 dilution) reaction, and finally the color reaction step (usually overnight) using NBT/BCIP (Roche) as the substrate. To mount, the slides were prepared by washes with PBS, fixed for 2 h (3.7% formaldehyde, 1× MEM buffer), counterstained with nuclear fast red, dehydrated with varying amounts of ethanol concentrations, and mounted with Entellan.

Allele-specific analysis

The allele-specific Copg2 hot-stop PCR analysis was based on a polymorphic BstUI site present in the M. m. castaneus RT-PCR product (exon 3 SNP rs36827900). The 250-bp PCR products digested with BstUI yield bands of 200 and 50 bp for the CAST allele. Random-primed cDNA was generated from dissected E14.5 embryonic organ RNA and cDNA amplification was carried out with primer reaction Copg2 ex1-5 for 35 cycles ($T_m = 58^\circ$C). An amount of 5 μl of the PCR product was transferred into another master mix containing gamma 32P-CTP to go through one final PCR cycle. The hot PCR products were digested with BstU1 (New England Biolabs) and analyzed on an 8% polyacrylamide gel. The bands were detected using the Storm Phospho-imager autoradiography system and the band densities (maternal and paternal alleles) were analyzed with the Image J software (W. Rasband, National Institute of Mental Health, Bethesda, Maryland, USA.).

As well, Copg2 ex1-5 and Copg2 ex17–19 PCR products from F1 cDNA were directly sequenced. The Copg2 ex17–19 reaction contains two additional SNPs (exon 19; rs49583711 and rs47417386). The phred program was used to read the sequence trace data and assign quality values to the bases (23), normalized to F1 gDNA.

RESULTS

Longer variants of Mest: the MestXL isoforms

The current annotation of the Mest locus presents a complex production of different isoforms of the mRNA. First, the expressed sequence tag (EST) data suggest variability in promoter usage, with three minor isoforms (42–4) transcribed from upstream promoters, relative to the main isoform 1, which initiates at the differentially methylated chromatin of the MestXL locus.
CpG island defining exon 1 (Supplementary Figure S1A and B) (5). Second, by mapping SAGE tags from the Mouse Atlas of Gene Expression (21) for the Mest mRNA, we also identified variability in the size of the 3'-UTR of exon 12 leading to the production of three isoforms (A to C; Supplementary Figure S1C). The majority of Mest transcripts (74%) are processed by transcriptional termination and polyadenylation at the most downstream termination signal within exon 12, yielding a terminal exon of 1406 bp.

The production of these isoforms in adult tissues and during development was assessed by northern blotting using a random-primed dsDNA probe against the Mest coding region (Figure 1). Our results show that the main ~2.5 kb isoforms of Mest (isoforms A, band IV) are broadly expressed in the samples analyzed (see Supplementary Figure S1D for molecular weights of isoforms). Although this analysis cannot resolve the isoforms 1 to 4 generated by alternative promoter usage, most ESTs support formation of isoform 1 in adult and embryonic tissues (96.6% of all ESTs), and ESTs for isoforms 2 and 3 suggest that these promoters are egg-specific. The results also identified lower molecular weight RNA species consistent with the structures of the isoforms B (band V) and C (band VI) produced by alternative polyadenylation within exon 12 and supported by the LongSAGE data (Supplementary Figure S1C). Surprisingly, we also detected polyadenylated transcripts larger than the fully spliced Mest mRNA. In adult tissues, the mature Mest mRNA of 2.5 kb is detected at high levels in heart and brain. The larger molecular weight species (bands I to III) were mostly detected in brain, where they represent close to 30% of the transcripts (Figure 1A). These larger transcripts represent variants >10.5 kb (band I) and ~6.5 kb of transcribed sequences (bands II and III). During development, the main Mest transcript of 2.5 kb is very abundant at all stages analyzed (Figure 1B, band IV). In addition to the shorter isoforms with alternative polyadenylation within exon 12 (band VI, ~1.7 kb; band V, ~2.3 kb) embryos also show the two higher molecular forms at ~6.5 kb (bands II and III) at all stages analyzed.

The annotation of the Mest-Copg2 loci on the Ensembl browser (NCBI37) presents evidence for two clusters of ESTs, transcribed from the (+) strand of chromosome 6, like Mest, and extending from the end of exon 12 of Mest all the way into intron 20 of the neighboring antisense gene Copg2 (Supplementary Figure S2). Imprinted transcription from the paternal allele has previously been described for these two EST clusters (13). The transcripts from intron 20 of Copg2 were referred to as Mit1, encompassing EST clones covering >5 kb of intron 20 sequences. This region of the current mouse genome sequence (Build 37/mm9) still contains a gap within intron 20 of Copg2, the size of which is set arbitrarily and not supported by physical data (24). Spliced Mest transcripts with exon 12 variants extending up to this gap would make transcripts of a predicted size of ~6.8 kb. Those extending beyond the gap, assuming a negligible size for this gap and absence of splicing, and up to exon 20 of Copg2 would span ~13 kb (Supplementary Figure S2). We refer to these long transcripts as the MestXL isoforms.

To obtain further experimental evidence for these longer variants, we analyzed data from SAGE tags libraries from the Mouse Atlas of Gene Expression Project, which provide the location and abundance of ESTs located close to terminal or internal poly(A) stretches (21). We identified six main SAGE tags expressed from the (+) strand of the genome in the region of overlap between Mest and Copg2 (Figure 2A). The first four tags (tags 1 to 4) map within the exon 12 of Mest and represent expression of the short isoforms of Mest, with tag 4 marking the main site of polyadenylation. They are expressed at low levels in adult tissues (notably in brain, pituitary and adrenal glands) but show high levels of expression during development (Figure 2B). Their expression patterns in

**Figure 1.** Northern blot analysis of Mest expression. A dsDNA probe of the entire Mest open reading frame (spanning exons 1–12) was hybridized on mouse northern blots containing 2 μg of poly(A)+ mRNA per lane. (A) Adult tissues. (B) RNA samples from whole mouse embryos at the indicated developmental stages. The different Mest isoforms detected were labeled I to VI. Band IV corresponds to the main isoform A of Mest (Supplementary Figure S1).
Figure 2. Analysis of SAGE tags for the Mest and MestXL transcripts. (A) Schematic representation of the Mest-Copg2 overlap region, drawn to scale. The position of the gap in the genome sequence is indicated (below) but omitted in the estimation of the size of intron 20 of Copg2. Above the (+) strand, on which terminal Mest exons are represented by black rectangles, six SAGE tags are positioned (arrows). Tags 1 to 4 map to exon 12 and represent the short isoforms of Mest. Tags 5 and 6, located between the gap and exon 20 of Copg2 represent the larger MestXL isoforms. Probes used in northern and ISH analyses are labeled d to k (gray lines). (B) Gene expression profiles for SAGE tags 1 to 6 in adult and embryonic LongSAGE (un-amplified) libraries from the given tissues, presenting the tag counts according to the shading scale defined below. Statistics for each tag is from 136 different LongSAGE libraries. (C) Expression levels of tags 1-6 in adult and embryonic SAGELite (amplified) libraries from the given adult tissues. For the embryos, whole head RNA was analyzed from E8.5 to E11.5, and whole brain from E12.5 to 16.5. Statistics for each tag is from 77 different libraries. (D) Pearson’s correlation coefficient for the six SAGE tags in the 136 LongSAGE libraries.
136 different adult and embryonic LongSAGE libraries (19) show high levels of correlation (Figure 2D), suggesting that the ratios of isoforms A to C are rather constant across tissues and do not represent tissue-specific isoforms.

The SAGE tags 5 and 6 map within intron 20 of Copg2, beyond the gap in the mouse genome sequence, relative to exon 12 of Mest (Figure 2A), and within the Mit1 cluster of ESTs (Supplementary Figure S2). We refer to these tags as representative of longer isoforms of Mest, the MestXL variants. Expression of tags 5 and 6 show the highest degree of correlation amongst the tags analyzed in the 136 LongSAGE libraries (Figure 2D). They show moderate to low levels of expression in adult and embryonic tissues expressing the short Mest isoforms, but they are also sometimes expressed at higher levels than tags 1 to 4 (for instance in adult visual cortex and lung, Figure 2B). The analysis of amplified libraries (SAGELite (20)) showed that transcripts terminating at tags 5 and 6 are often more abundant than the short isoforms in several regions of the adult brain (Figure 2C). During embryogenesis we see a gradual shift from Mest to MestXL polyadenylation in the developing brain, the latter of which is first detected at developmental stage ~E13 (Figure 2C).

We also analyzed all the ESTs from the (+) strand at Mest (from UniGene entry Mm.335639) and within the two EST clusters on either side of the gap (Supplementary Tables S2 and S3). Whereas only 35% of Mest ESTs are from libraries generated from central nervous system (CNS) tissues, most ESTs from the 5'- and 3'-clusters are from the CNS (77 and 84%, respectively; Supplementary Figure S3). Together with the northern blot analysis, these results suggest the existence of longer isoforms of Mest, and that the production of MestXL transcripts is under developmental and tissue-specific regulation.

Loss of MestXL transcripts from the mutant MestKO allele

The targeted allele Mestm[1/2ef] (or MestKO) is based on a promoter-trap construct in which exons 3 to 8 and part of exon 9 of Mest were replaced by a splice–acceptor–IRES–βgeo–pA cassette (Figure 3A) (7). Transcription from this mutant allele is imprinted and expression of the βgeo reporter faithfully recapitulates Mest expression in paternal heterozygotes (7). Based on the structure of the mutant allele, transcriptional termination is predicted to occur upstream of the remaining exon 9 sequences and to produce truncated Mest transcripts. To provide support for this prediction we performed northern blots on poly(A)+ RNA from WT and MestKO E14.5 embryos and adult brain tissue (Figure 3B and Supplementary Figure S4). Since only the paternal allele of Mest is active for transcription, all Mest transcripts are from the mutant KO allele in MestKO heterozygotes (7). We used strand-specific RNA probes hybridizing to different regions of Mest, to the mutant allele (neo probe), and to three different positions along the MestXL isoforms (BB692936 EST, BY247745 EST, and Mit1). Probe locations are presented in Figures 2A and 3A.

Within the Mest locus, probes against exons 3–8 (probe b) and exon 12 (probe d) detected the Mest and MestXL isoforms in WT E14.5 head RNA (Figure 3B). These blots are over-exposed so that we could observe the weaker higher molecular weight signals. Since these exons are deleted in the MestKO allele, the absence of signal with the exons 3–8 probe on RNA from MestKO embryos confirms that only the paternal allele is active for all the isoforms detected in the WT embryos. Premature transcriptional termination at the inserted poly(A) cassette of the MestKO allele is confirmed by the absence of signal with the exon 12 probe on RNA from MestKO embryos as well as the detection of the expected 5-kb mutant RNA using a probe against the neo marker. We did not obtain a good signal for the mutant transcript using a small exons 1–2 probe on embryonic RNA although the 5-kb mutant mRNA was detected with this probe on adult brain RNA (Supplementary Figure S4), as well as with the neo probe. Furthermore, qRT–PCR analysis of the same region showed that the Mest KO transcripts are expressed at ~50% of WT levels (Supplementary Figure S5) and rapid amplification of cDNA end (5′-RACE) experiments confirmed that the mutant transcript indeed initiates at exon 1 of Mest (Supplementary Figure S6). It is therefore possible that the formation of stable mutant transcripts including exons 1 and 2 is affected by abnormal processing of the intron 2 Mir335 during development.

We also considered the possibility that the higher molecular weight isoforms might represent the pre-mRNA (10.5 kb) or partially spliced forms of Mest. To address this, we used a genomic probe against intron 11 (probe c). The overexposed signal presents a smear that ranges from 2.8 to 7 kb with no prominent bands detected (Figure 3B). The absence of the higher molecular weight bands detected with Mest cDNA probes suggests that they represent processed Mest isoforms and not unspliced primary transcripts. The absence of signal on the mutant RNA sample provides further evidence for the absence of read-through from the inserted polyA cassette, a result which is also supported with qRT–PCR analysis (Supplementary Figure S5).

To analyze the formation of the MestXL isoforms, we used three probes (h-j) from intron 20 of Copg2 (Figure 2A). These probes, which span >7.5 kb of genomic sequences, detect RNA bands of ~4.8 kb, ~6.0 kb, as well as higher molecular weight forms (Figure 3B). Notably, the probe against the EST BB692936, located at the end of Copg2 intron 20 and 5′ of the gap (probe h), detects the higher molecular weight RNA isoforms corresponding to bands I-III seen with the Mest CDNA probes. More importantly, all the isoforms detected with these intron 20 probes are absent in MestKO embryos, in which transcription terminates at the inserted polyA signal (Figure 3B).

Our northern blot analysis shows that Mest and MestXL transcripts are imprinted and expressed only from the paternal allele during development. In MestKO embryos, Mest transcription terminates at the inserted polyA cassette of the mutant allele and production of the MestXL isoforms is concomitantly eliminated.
MestXL transcripts are produced in the developing nervous system

To look at the developmental expression pattern of MestXL transcripts in more detail, we performed in situ hybridization experiments on sagittal sections of E14.5 embryos (Figure 4), at a stage when both Mest and MestXL SAGE tags are detected in the developing brain (Figure 2C). First, we used antisense RNA probes to look at the expression of Mest in WT and Mest+/KO embryos. We also analyzed expression of the neo marker present in the /C12-geo cassette of the MestKO allele. Our results confirm the widespread mesodermal expression of Mest during development (3,25) as well as the previously described expression in neuronal tissues (7,8). In the E14.5 spinal cord, immunohistochemistry experiments show that both Mest and Mest KO are broadly expressed in NCAM1-positive neurons (Supplementary Figure S7). The ISH signal is seen for all the antisense Mest cDNA probes assayed, but not for the intron 11 probe (Figure 4A). For the exon 1–2 probe (a), we detected weaker expression for the mutant allele than for the WT transcript, which is consistent with our qRT–PCR data (Supplementary Figure S5). Together these results show that mRNA levels from the promoter-trap MestKO allele are lower than those detected in WT embryos as a consequence of the insertion of the /C12-geo cassette. Our results also show absence of transcription from the WT maternal allele in Mest+/KO heterozygotes, using the exon 3 to 8 probe, which hybridizes to coding sequences deleted in the MestKO allele. We evaluated the amount of read-through beyond the inserted polyadenylation signal of the MestKO allele by using an exon 12 probe. In agreement with our northern blot and qRT–PCR results, very little transcription was detected downstream of the MestKO poly(A) site.

The expression pattern of MestXL was studied using RNA ISH probes complementary to the (+) or (–) strand that hybridize downstream of the Mest isoform 1A to ESTs annotated in the genome browsers. In addition, we generated one probe for which there is no

![Figure 3. Loss of MestXL expression from the mutant MestKO allele. (A) Schematic diagrams of the Mest and MestKO alleles. The exons are represented by black rectangles. The MestKO allele consists of a splice acceptor-IRES-lacZ.neo fusion-poly(A) cassette (white rectangle) replacing Mest exons 3–8, and part of exon 9. The positions of the RNA probes a to e are show above the alleles (gray lines). The intronic Mir335 is shown below each allele (black line). (B) Strand-specific northern blot analyses of Mest, MestKO, and MestXL expression. Various DIG-labeled RNA probes were hybridized to mouse northern blots containing 1.5 μg of poly(A)+ RNA purified from E14.5 WT and Mest+/KO embryo heads. See Figures 2A and 3A for probe positions, identified by the letters given below the bots. BB and BY are (+) strand ESTs (BB692936 and BY247745, probes h and i, Figure 2A) mapping 5' and 3' of the gap, respectively. The position of molecular weights marker bands in kb (MW) are indicated on the left sides of each blot.](https://academic.oup.com/nar/article-abstract/40/4/1523/2411544)
supporting EST data, the Copg2 intron 23 probe (probe g, Figure 2A). The BB692936 EST and Mit1/AF217545 are located in intron 20 of Copg2 and transcribed from the (+) strand. The four probes designed to detect MestXL show a localized expression signal in the developing nervous system (Figure 4C, probes f to j). More specifically, MestXL appears to be expressed in most parts of the developing mid/hind brain and in the spinal cord, but not in the developing forebrain. This CNS-specific transcription is absent in Mest+/−/KO embryos, confirming imprinting of MestXL and suggesting that it represent longer Mest transcripts, produced by tissue-specific alternative polyadenylation (Figure 4D). A probe designed to detect transcription downstream of Mit1 (Copg2 in18-ex20, probe k) shows that transcription of MestXL terminates in intron 20, which is supported by SAGE tag 6 (Figures 2A and 4D).

The reciprocal probe partners detecting transcription from the (−) strand, either the Copg2 mRNA or primary mRNA, were analyzed as well. For all probes assayed,
minimal expression was observed for Copg2 mRNA or primary mRNA transcripts in both WT and Mest+/KO heterozygous embryos with the exception of the Copg2 exons 14–20 and 3’-UTR cDNA probes (Supplementary Figure S8). As observed for MestXL, Copg2 is mainly expressed in the developing neural tissues.

Together with our northern data, the expression analysis by ISH suggests that MestXL is imprinted, produced exclusively in the developing and adult CNS, and lost in Mest+/KO mutants. These conclusions are also supported by extensive RT–PCR experiments detecting brain-specific transcripts in the Mest-Copg2 region only in WT samples (Supplementary Figure S9). Our analysis of expression profiling by high-throughput sequencing (RNA-seq) also revealed transcription extending from exon 12 of Mest to exon 20 of Copg2, specifically in developing and adult neuronal tissues (Supplementary Figure S10).

Allelic usage at Copg2, but not Klf14, is regulated by MestXL

The relationship between Mest/MestXL and Copg2 is of particular interest due to their 3’-end overlap, which is conserved in humans (13,16). Copg2 was reported to be predominantly maternally expressed in a tissue-specific manner, specifically in tissues where we document the formation of MestXL transcripts (13). To investigate whether the production of MestXL via alternative polyadenylation at Mest has an effect on allelic-usage at Copg2 we examined allele-specific expression of Copg2 in Mest+/KO heterozygous animals, in which production of MestXL is abolished. Having established that the MestXL transcripts are mainly detected in the CNS in the developing mouse embryo, we investigated the parental allele contributions and expression of Copg2 in a tissue specific manner.

We first investigated the imprinting pattern of Copg2 in four E14.5 WT and Mest+/KO samples: two where MestXL is expressed, the MHB and spinal cord (SC), and two where MestXL is not expressed; the heart and liver. To determine the maternal and paternal allele contributions from Copg2 we used hot-stop PCR/RFLP analysis (Figure 5A). Labeling the PCR products only in the final cycle is advantageous since this circumvents the formation of un-digestible heteroduplexes (26). Our analysis relied on a SNP in exon 3 of Copg2 (rs36827909) which creates a BstUI site unique to the M. m. castaneus allele. By measuring the band densities of the maternal (CAST) and paternal (CD-1) alleles we obtained quantitative measurements and calculated maternal to paternal (M/P) allele ratios for WT and Mest+/KO samples purified from F1 hybrid embryos. In WT samples, the MH shows the highest Copg2 M/P ratio as the maternal allele is expressed 2.3 times more than the paternal allele (Figure 5B). When MestXL expression is lost in the Mest+/KO MHB, this allelic bias is lost and the M/P ratio plummets to 0.86. A similar situation occurs in the SC but it is not as striking; the WT and Mest+/KO M/P ratios are 1.40 and 0.92, respectively. In tissues where MestXL is not expressed, the heart and liver, the M/P ratios basically stay the same between WT and Mest+/KO samples. Here the WT and Mest+/KO ratios are 1.38 and 1.49 for the heart and 0.78 and 0.71 for the liver.

We also directly sequenced the exon 3 SNP and two additional SNPs in exon 19 (rs49583711 and rs47471386). The quantification for this method involves using the phred software (23) to calculate the area under the called base (SNP) tracing. After normalizing the read-outs to the F1 genomic DNA tracing, the M/P ratios were calculated by averaging the reads for each SNP in 4 WT and 6 Mest+/KO samples from each E14.5 tissue. The results showed similar patterns to the hot-stop PCR analysis and suggest that allelic usage at Copg2 is regulated by MestXL (Figure 5C).

Considering that the paternal allele of Copg2 is aberrantly expressed in Mest+/KO embryonic neural tissues, our next question was whether this leads to an overall increase in Copg2 expression levels, as suggested by the allele-specific data. qRT–PCR analysis of Copg2 levels was performed on cDNA from four Mest+/+ (WT) and six Mest+/KO E14.5 embryos from two F1 (CAST6/CD-1) litters. The results showed striking differences between WT and mutant samples only in tissues where MestXL is expressed (Figure 5D). In the mutant MHB, Copg2 was expressed 1.4× higher than in the WT (P = 0.012), a significant difference between the two genotypes. In the Mest+/KO SC, Copg2 is expressed 1.2× higher than in the WT, a difference that approaches significance (P = 0.09). On the other hand, in the heart and liver, Copg2 expression levels remain similar between the Mest+/+ and Mest+/KO samples (P = 0.81 and 0.62, respectively). Our results show that Copg2 expression exhibits tissue-specific allele-specific bias which are regulated by the formation of MestXL.

We also analyzed whether MestXL is involved in regulating Klf14, located ∼60-kb downstream from the Copg2 promoter and transcribed from the (–) strand (4). A previous report demonstrated that Klf14 itself does not harbor any of the known imprinting hallmarks such as a DMR or differential histone modifications that would mediate its imprinting, but is regulated by maternal methylation (4). A mechanism whereby the maternally methylated Mest DMR might regulate this imprinting is through a possible long-range effect by paternally expressed MestXL in cis. To investigate this possibility we again took advantage of our Mest+/KO line that contains a truncated MestXL. Allele-specific analysis and qRT–PCR on Mest+/KO E14.5 tissues show that MestXL is not implicated in the regulation of imprinting at Klf14 (Supplementary Figure S7). This result is in agreement with our observation that MestXL transcription terminates before exon 20 of Copg2, therefore supporting a local cis effect. In support of this model, we additionally found that MestXL is not localized to the nucleus, a characteristic feature of regulatory non-coding RNAs such as Air, Kcnj10l and Xist that are thought to mediate long-range regulation of epigenetic silencing (Figure 5E).

DISCUSSION

We show here that transcription at the imprinted Mest locus is regulated by tissue-specific alternative
polyadenylation, resulting in the production of longer variants, the MestXL transcripts. These CNS-specific transcripts extend far into the 3′-end of the convergent Copg2 locus and regulate the allelic usage at Copg2 in these specific tissues. The mechanism of this tissue-specific alternative polyadenylation at Mest remains to be elucidated but is certainly not unique to Mest. Over half of mammalian genes contain multiple polyadenylation sites that lead to variants with different 3′-UTRs or coding regions (27). Using SAGE data, several mRNAs expressed in neuronal tissues were shown to have progressively longer 3′-UTRs throughout embryonic and postnatal developmental stages (28), a characteristic shared by MestXL. Examples of neuronal-specific larger variants of imprinted genes have also been documented. Dlk1 was found to produce a transcript in brain that is 2.7 kb larger than the main transcript (29). Longer 3′-UTRs for genes expressed in the brain have been involved in translational control/repression and differential RNA localization (30–32). Whether MestXL is involved in the
translational repression of Mest, besides its role in regulating allelic usage at Copg2 remains to be determined.

The Mest locus consists of a variety of alternatively spliced transcripts coupled with alternative promoter usage. Together with our findings that it is also under the regulation of tissue-specific alternative polyadenylation, the Mest locus appears to be more complex than previously thought. We showed that the two higher molecular weight isoforms detected on our northern blots include the Mest ORF or part of it. This suggests that MestXL consists of the mature Mest mRNA with an extended terminal exon, a conclusion supported by our results using downstream probes proximal to the genomic gap. Using additional probes detecting transcripts from the (+) strand distal to the gap, we observed not only higher molecular bands that appear to be >10 kb, but also prominent bands at sizes such as 4.8 and 6.5 kb suggesting these transcripts are either differentially processed isoforms or cleavage products. This type of regulation has been observed for many genes (33). Interestingly, the Mest and MestXL probes seem to share the 6.5-kb band perhaps suggesting they could all be part of the same transcript. Given that a 4.8-kb band is not detected from the Mest probes, it is likely that this transcript does not include the Mest mRNA transcript and is comprised of sequences mostly on the distal side of the gap. The highest molecular weight transcripts themselves that we detect could also be processed transcripts if the gap is very large. The gap might complicate and impede our understanding of how the MestXL transcripts are regulated and processed throughout this region, but our results provide evidence that their expression relies on transcription from the Mest locus and are under the regulation of alternative polyadenylation.

The allele-specific expression of Copg2 shows great variability in different genetic backgrounds of F1 hybrid mouse strains. Three different kinds of hybrids have been used to study the imprinting at Copg2 and all have given different results. In an intraspecific hybrid between C57BL/6 and M. m. molossinus, Copg2 is more exclusively maternally expressed in the developing embryo and adult brain tissue (13). However, in an interspecific F1 hybrid between C57BL/6 and M. spretus, Copg2 was biallelically expressed in all adult tissues analyzed including in the brain (17). For our studies, we used a different intraspecific F1 hybrid cross between CD-1 and M. m. castaneus and found that Copg2 is preferentially maternally expressed in a tissue specific manner. Our results suggest that Copg2 transcription itself is not primarily imprinted. Although not studied in great detail, and despite the claims of the authors, Copg2 does not appear to have a promoter-proximal DMR that would mediate its imprinting (17). Instead we propose that Copg2 transcription is secondarily imprinted due to the production of larger Mest variants, MestXL, that are under the regulation of alternative polyadenylation (Figure 6). As a consequence, these variants would interfere and regulate the allelic usage at Copg2. The variability observed in different hybrid strains suggests that whatever mechanism is controlling the imprinting at Copg2 is possibly rapidly evolving and, considering our results, perhaps the variation is at the level of alternative polyadenylation from the Mest locus.

Our results support a model in which allelic usage at Copg2 is regulated by the production of larger variants at the Mest locus. In tissues that express MestXL, Copg2 is preferentially maternally expressed due to the TI on the paternal chromosome. This imprinting is lost in MestXL/ko tissues where MestXL is truncated due to the introduced poly(A) signal in the MestXY allele (Figure 6). The effect is an overall increase in Copg2 expression levels which may in fact contribute to the phenotype described for MestXY/ko mice. In tissues where Mest is basal expression such as in the developing liver, Copg2 is biallelically expressed because there is no competing TI. Previous microarray expression data from Dmnt3f E8.5 embryos, which do not inherit proper methylation marks from the maternal germline, provides further evidence for this model (34). Since the maternal Mest promoter is not methylated in these embryos, Mest is upregulated 1.5 times presumably due to biallelic expression. A probe located downstream of Mest that maps to BB692936, a MestXL EST studied in our experiments, shows a 1.8-fold upregulation which is most likely because the maternal allele is transcribed as well. Most importantly, Copg2 is downregulated 2-fold in the mutants. We propose that it is the biallelic expression of MestXL which interferes with the transcription of Copg2 in these mutant embryos.

The mechanism of this TI is unknown but current models propose two possibilities. The ‘collision’ model suggests that collisions between converging sense and
antisense elongation complexes in cis can lead to premature termination of the transcriptional progress of one or both complexes in yeast (35). Elongation of the regulatory MestXL Pol II complex could dislodge the target Copg2 Pol II complex terminating its transcription. Alternatively, the overlapping double-stranded 3'-UTRs could lead to adenosine–inosine editing of the paternal Copg2 3'-UTR which might target it for degradation (36,37). Perhaps the termination complex of MestXL interferes with changes in chromatin structure needed for transcription of Copg2, an idea previously proposed (38,39).

We demonstrated that MestXL regulates allelic usage at Copg2 but not at Klf14. Since Klf14 is exclusively expressed from the maternal allele, does not carry a germline imprint, and is silenced in absence of maternal methylation imprints, it was previous proposed that Klf14 might be under long-range regulation via the Mest promoter DMR (4). Since loss of MestXL does not abrogate imprinting at Klf14, our results indicate that the regulation of imprinting within this domain involves a combination of different mechanisms.

The Mest imprinting domain shares homology with human chromosome 7q32 and there is supporting data that the human MEST locus produces MESTXL variants. Yamasaki et al. identified an alternative 3'-UTR for MEST that is at least 2.7 kb larger than the main 3'-UTR in the four fetal tissues analyzed (not neural specific) and they additionally demonstrated that COPG2 intronic transcript 1 (CIT1), an antisense transcript located in intron 20 of COPG2, was paternally expressed (16). The mouse and human sequences for intron 20 of Copg2 are 80% identical, although both genomic sequences are incomplete and contain a gap. This MEST/ COPG2 region has been associated with growth retardation and Silver-Russell syndrome (SRS) in maternal uniparental disomies (mUPD) for this chromosome 7 segment, where both copies are inherited from the mother. Nearly 10% of SRS cases are associated with mUPD for this region presumably due to the loss of paternally expressed genes (40,41). As a consequence, MEST was designated as a key candidate locus for the etiology of SRS in these cases yet extensive sequencing and methylation studies have failed to provide any evidence and the SRS etiology associated with UPD at 7q32 remains ambiguous and unknown (42,43). Since MestXL is paternally expressed and its loss affects Copg2 expression, this raises the possibility that increased COPG2 levels might be implicated in the etiology of SRS.

To conclude, this is the first report to demonstrate that the Mest locus produces larger transcripts, MestXL, that include the previously reported transcript Mit1. Furthermore, we document for the first time that they have a role in regulating the allelic usage at Copg2 and propose a model for allelic interactions between neighboring transcripts as a new mechanism for the regulation of imprinted expression in mammals. The analysis presented in this study further shows that growth retarded Mest+/KO mutant mice (7) are not only deficient for the MEST protein, but also lack the MestXL transcripts, a conclusion which might have important implications for the role of this region in SRS in humans.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary methods, Supplementary Tables 1–3, Supplementary Figures 1–11, Supplementary references [7,22,44–48].

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