CTCF binding is not the epigenetic mark that establishes post-fertilization methylation imprinting in the transgenic \textit{H19} ICR

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Imprinted expression of the mouse \textit{Igf2}/\textit{H19} locus is controlled by parent-of-origin-specific methylation of the imprinting control region (ICR). We previously demonstrated that when placed in a heterologous genomic context, the \textit{H19} ICR fragment contains an intrinsic activity that allows it to acquire differential methylation in somatic cells but not in germ cells. In the present study, we investigated the requirements for the CTCF-binding sites of the ICR in the acquisition of post-fertilization methylation. To this end, two mutant ICR fragments were introduced into the human \(\beta\)-globin locus in a yeast artificial chromosome transgenic mouse (TgM) model: 4xMut had mutations in all four ICR CTCF-binding sites that prevented CTCF binding but retained the methylation target CpG motifs, and \(-9CG\) harbored mutations in the CpG motifs within the CTCF-binding sites but each site retained constitutive CTCF-binding activity. In TgM germ cells and pre-implantation blastocysts, the absence of CTCF-binding sites (4xMut) did not lead to hypermethylation of the transgenic \textit{H19} ICR. However, after implantation, the mutations of CTCF sites (4xMut and \(-9CG\)) affected the maintenance of methylation. These results demonstrated that although the CTCF-binding sites are indispensable for maintenance of the unmethylated state of the maternal ICR in post-implantation embryos, they are not required to establish paternal-allele-specific methylation of the transgenic \textit{H19} ICR in pre-implantation embryos.

INTRODUCTION

A subset of mammalian genes is transcribed in a mono-allelic, parent-of-origin-specific manner (genomic imprinting), a process regulated by unique DNA sequences called imprinting control regions (ICRs). The ICRs are marked by DNA methylation on one of the two parental alleles and therefore, are also referred to as differentially methylated domains (DMDs). The mouse \textit{Igf2}/\textit{H19} imprinted locus is located on distal mouse chromosome 7, and the \textit{Igf2} and \textit{H19} genes are exclusively expressed from the paternally and maternally inherited alleles, respectively (1,2). The \textit{H19} ICR, located \(-2\) to \(-4\) kb relative to the transcription initiation site of the \textit{H19} gene (Fig. 1A, top), is preferentially methylated on the paternal allele (3–5) and controls imprinted gene transcription through two distinct activities: the insulator and silencer (6–10). Allele-specific insulator activity of the \textit{H19} ICR is governed by the enhancer-blocking protein, CTCF, whose DNA-binding ability is sensitive to CpG DNA methylation of its recognition motif. The CTCF-bound, hypomethylated maternal \textit{H19} ICR prohibits activation of the distal \textit{Igf2} gene from the shared enhancer located \(3'\) to \textit{H19}, resulting in exclusive \textit{H19} gene expression. In contrast, a hypermethylated paternal ICR silences \textit{H19} gene transcription by inducing epigenetic changes at the \textit{H19} gene promoter, while it prevents CTCF from binding to the ICR, thereby allowing \textit{Igf2} gene expression (6–10). Consistently, knock-in mice with disrupted CTCF-binding sites in the endogenous \textit{H19} ICR ectopically expressed the maternal \textit{Igf2} gene on the mutant allele (11–15). On the other hand, \textit{Igf2} gene expression on the paternal allele was attenuated when nine CpG motifs in the CTCF-binding sites were mutated (\(-9CG\)) to allow constitutive CTCF binding (16).

While the role of differential methylation of the \textit{H19} ICR in controlling imprinted gene transcription is established, the mechanism that discriminates the parental origin and marks
the ICR is not fully understood. The H19 ICR is methylated in sperm but not oocytes, and this distinctive, allele-specific methylation pattern is maintained throughout the development, even after fertilization (3–5,17). DNA methylation patterns depend on the shared 3′ enhancer (a gray box). The entire β-globin locus (open boxes) are shown relative to the LCR (gray box). The wild-type (WT) and mutant (4xMut or −9CG) ICR fragments (inverted orientation) were floxed (filled triangles) and introduced to the right arm of the YAC. The entire β-globin locus is contained within two SfiI fragments (8 and 100 kb). Transgene-specific probes used for long-range structural analysis in (C) are shown as solid rectangles. (B) Sequences of the CTCF-binding sites in the 4xMut, −9CG, as well as the WT ICRs. CTCF-binding motifs and CpG dinucleotides are bold-faced and underlined, respectively. Mutated nucleotides in each mutant ICR are italicized. (C) Long-range structural analysis of transgenes. DNA from thymus cells was digested with SfiI in agarose plugs, separated by pulsed-field gel electrophoresis and hybridized separately to probes in (A).

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Figure 1. Generation and structural analysis of H19 ICR TgM. (A) (Top) Genomic structure of the mouse Igf2/H19 locus. The Igf2 and H19 genes (open boxes) are approximately 90 kb apart, and the expression of both genes depends on the shared 3′ enhancer (a gray box). The H19 ICR is located within a SacI (Sa)–BamHI (B) fragment lying approximately 1.8 to −4.7 kb relative to the transcription initiation site of the H19 gene. The solid rectangles in the enlarged map indicate the position of the CTCF-binding sites. (Bottom) Schematic representation of the YAC transgenes. The positions of the β-like globin genes (open boxes) are shown relative to the LCR (gray box). The β-like globin genes are expressed in both the LCR and the e-globin gene in the human β-globin YAC. SfiI restriction enzyme sites are located 5′ to the LCR, within the LCR and in the right arm of the YAC. The entire β-globin locus is contained within two SfiI fragments (8 and 100 kb). Transgene-specific probes used for long-range structural analysis in (C) are shown as solid rectangles. (B) Sequences of the CTCF-binding sites in the 4xMut, −9CG, as well as the WT ICRs. CTCF-binding motifs and CpG dinucleotides are bold-faced and underlined, respectively. Mutated nucleotides in each mutant ICR are italicized. (C) Long-range structural analysis of transgenes. DNA from thymus cells was digested with SfiI in agarose plugs, separated by pulsed-field gel electrophoresis and hybridized separately to probes in (A).
that the paternally, but not maternally, inherited wild-type transgenic ICR was highly methylated at the blastocyst stage, indicating that methylation imprinting in the transgenic H19 ICR was established before implantation. Importantly, even in the absence of CTCF binding to the H19 ICR, the transgenic ICR in blastocysts was differentially methylated. These results clearly demonstrated that the CTCF-binding sites were essential to maintain but not to establish differential methylation of the transgenic H19 ICR.

RESULTS

Generation of YAC TgM

To elucidate a possible role of CTCF in the post-fertilization acquisition of parent-of-origin-dependent methylation of the transgenic H19 ICR, we introduced two types of mutations into the ICR fragment. In 4xMut, all four CTCF-binding sites in the ICR were mutated and loss of CTCF binding to the sites was expected irrespective of their methylation status (Fig. 1B; 11). For −9CG, nine of 10 CpG motifs within four CTCF-binding sites were mutated. It was therefore predicted that CTCF would constitutively bind to the −9CG ICR regardless of the methylation status of the remaining CpG motifs outside of the CTCF sites (Fig. 1B; 16). These mutant ICRs were independently inserted into a human β-globin YAC at a position 3' to the locus control region (LCR) (Fig. 1A, bottom). We microinjected purified YAC DNA into fertilized eggs from CD1 (ICR) mice and generated two TgM lines for each construct. The copy number and long-range structural analyses of the TgM showed that both 4xMut lines (No. 74 and 228) had a single and intact copy of the integrated transgene (Fig. 1C, left and data not shown). Among the −9CG TgM lines, No. 493 had one intact and one truncated (the 5' portion of the YAC, including the LCR and −9CG ICR, was lost) YAC copy, whereas No. 828 had two copies of the intact and one 3'-truncated (whole β-globin locus was retained) copy of YAC (Fig. 1C, right and data not shown).

Genomic imprinting in adult somatic cells

To examine the methylation status of the transgenic ICRs in nucleated erythroid cells of the adult spleen, we first analyzed genomic DNA by Southern blotting with methylation-sensitive restriction enzymes (Fig. 2A). The wild-type transgenic ICR fragment in the β-globin locus (ICR, Fig. 2B, left) was more heavily methylated when paternally inherited, which was consistent with our previous observations (22). In contrast, the 4xMut ICR was substantially methylated regardless of its parental origin (4xMut, Fig. 2B, middle). In the −9CG TgM (−9CG, Fig. 2B, right), the difference in the methylation level between the paternally and maternally inherited mutant ICR fragments became less obvious when compared with the wild-type ICR fragment (ICR), which was because the methylation of the paternally inherited mutant ICR was partially lost. We confirmed these results in the other 4xMut or −9CG TgM lines (data not shown).

To further evaluate the methylation status of the mutant ICRs, two distinct regions of the transgenic ICR (regions I and II, Fig. 2A) were amplified by transgenic allele-specific nested PCR and analyzed by bisulfite sequencing. In 4xMut TgM (Fig. 2C, middle), both regions were heavily methylated irrespective of whether they were paternally or maternally inherited. In −9CG TgM (Fig. 2C, right), the paternally inherited transgenic ICR had a moderate decrease in methylation compared with the wild-type control (ICR, Fig. 2C, left), especially at region I, which was compatible with the Southern blot result (Fig. 2B, right). Therefore, these results suggested that CTCF binding to the sites antagonistically interfered with the acquisition of methylation within the ICR in adult somatic cells, which was also the case for the endogenous locus.

We next examined the effects of mutating the CTCF-binding sites in the H19 ICR on its enhancer-blocking insulator activity in regulating β-globin gene expression. Total RNA was prepared from anemic adult spleens of the TgM, and β-globin gene expression was analyzed by semiquantitative reverse transcription PCR (RT–PCR) (Fig. 2D). When the wild-type ICR was inserted between the LCR enhancer and the β-globin gene (ICR and Fig. 1A), paternally inherited β-globin gene expression was much higher, which was governed by the allele-specific insulator activity of the maternal, hypomethylated ICR (22). In the 4xMut TgM, the β-globin gene was highly and equally expressed regardless of its parental origin, suggesting that mutating the CTCF sites disrupted the insulator activity of the H19 ICR. In both −9CG TgM lines, transgenic β-globin gene expression was significantly higher when it was paternally inherited. However, the difference between paternal and maternal transgenic β-globin gene expression was not as pronounced as that seen in the TgM with the wild-type ICR, suggesting that CTCF became capable of binding to the paternally inherited −9CG ICR to exhibit insulator activity.

DNA methylation of the mutant ICRs in the testis

Unlike the endogenous H19 ICR, the transgenic H19 ICR in the β-globin gene locus was unable to acquire methylation in the testis (22). We therefore examined if the CTCF-binding motifs in the H19 ICR contributed to the lack of methylation in the testis. Southern blot analyses of testis DNA from the 4xMut or −9CG TgM revealed that these mutant ICR fragments were hypomethylated (Fig. 3A, middle and right), as was seen in the wild-type ICR fragment (ICR, Fig. 3A, left), suggesting that the CTCF motifs in the transgenic H19 ICR played neither a positive nor a negative role in establishing the methylation status in male germ cells.

A more detailed bisulfite sequencing analysis of the 4xMut or −9CG ICRs confirmed the Southern blot results (Fig. 3B). In addition, combined bisulfite restriction analysis (COBRA) of the transgenic H19 ICR in oocytes revealed that neither mutation in the CTCF sites in the ICR affected its unmethylated status (data not shown), demonstrating that the unmethylated state of the transgenic H19 ICR in both male and female germ cells was not determined by CTCF binding to the ICR. Therefore, the effects of mutating the CTCF sites on methylation in the transgenic H19 ICR in adult somatic cells must have occurred after fertilization.
DNA methylation of the transgenic H19 ICR in embryos

Parent-of-origin-dependent methylation of the transgenic H19 ICR in the β-globin locus was established after fertilization (22). In addition, the above results suggested that the presence or absence of CTCF binding to the H19 ICR could modulate its methylation status in somatic cells. It is therefore conceivable that CTCF is involved in establishing allele-specific, post-fertilization methylation of the transgenic H19 ICR. As a first step to test this possibility, we examined the timing of the acquisition of paternal-allele-specific methylation of the wild-type transgenic ICR in embryos (ICR, Fig. 4A). Bisulfite sequencing of the paternally inherited transgenic ICR revealed substantial methylation at region I (Fig. 4A, top). In contrast, the maternally inherited transgenic ICR was almost devoid of methylation (Fig. 4A, bottom). These results suggested that paternal-allele-specific methylation of the transgenic ICR in...
the β-globin locus was established prior to implantation. In 9.5 dpc embryos (Fig. 4C), although differential methylation was still observed, the spread of the DMD (i.e. extent of the region that is methylated only in the paternally inherited ICR) apparently narrowed compared with that in 3.5 dpc embryos because the most 5' CpGs (located further upstream of CTCF site 1) were additionally methylated in the maternal ICR (Fig. 4C). These 5' CpGs on the maternal transgenic ICR were already substantially methylated in 7.5 dpc embryos (Fig. 4B). Since the position of this border between DMD and non-DMD is the same as that in adult erythroid cells (Fig. 2C, left; 22), these results suggested that the spread of the DMD was established in post-implantation embryos.

DNA methylation of the mutant H19 ICR in embryos
To determine whether CTCF binding is required to establish post-fertilization, parent-of-origin-dependent methylation of the transgenic H19 ICR, we examined the methylation status of the 4xMut ICR in blastocysts. Similar to the wild-type transgenic ICR, the paternally inherited 4xMut ICR was highly methylated (Fig. 4D, top), whereas the maternally inherited 4xMut ICR was marginally methylated (Fig. 4D, bottom). These findings indicated that the 4xMut ICR is differentially methylated at the blastocyst stage. However, in 7.5 dpc embryos, the maternally inherited 4xMut ICR became substantially methylated (Fig. 4E). In 9.5 dpc embryos, both the paternal and maternal 4xMut ICRs were equally and almost completely methylated (Fig. 4F). These results implied that the CTCF-binding sites were not essential to establish post-fertilization, differential methylation at the H19 ICR in the transgenic β-globin gene locus, whereas they were required to maintain its status after implantation.

DISCUSSION
In order to understand the process of genomic imprinting, it is important to determine how the ICR acquires and maintains differential methylation. It has been reported that introduction of mutations into the CTCF-binding sites of the H19 ICR led to aberrant methylation of the endogenous ICR in somatic cells (11–16), whereas these mutations did not change the methylation pattern in germ cells (11,14–16). In addition, deleting all or most of the H19 ICR from the endogenous locus had little impact on the methylation of the remaining H19 upstream region in male germ cells or in pre-implantation embryos, although differential methylation was lost afterwards (24–26). Accordingly, while the ICR is indispensable for paternal-allele-specific methylation in somatic cells, it may not be required to control methylation acquisition at the H19 locus in male germ cells, and the activity introducing methylation within the H19 ICR and its surrounding sequences during spermatogenesis may reside outside the H19 ICR. Consistently, we previously demonstrated that a transgenic H19 ICR (2.9 kb) in the heterologous β-globin YAC transgenic locus was paternally methylated in adult somatic cells despite the absence of methylation in male germ cells (22), demonstrating that this differential methylation was acquired after fertilization. Park et al. (27) also reported that when a 2.4 kb H19 ICR fragment was knocked into the alpha-fetoprotein (Afp) locus, it was methylated in somatic cells when paternally inherited, but not during spermatogenesis. Based on these results, we infer that (i) methylation acquisition
in germ cells and somatic cells, at least in the Igf2/H19 locus, may be separable events, and that (ii) the H19 ICR may be marked by an epigenetic modification other than DNA methylation in germ cells, and allele-specific methylation can be acquired after fertilization by referring to this ‘primary mark’. Our recent finding that paternally inherited H19 ICR fragments, randomly integrated into the mouse genome, acquired methylation imprinting in somatic cells, independent of their variable methylation status in male germ cells (28) is consistent with this notion.

Evidence for the existence of such a primary mark would be impossible to detect in the endogenous Igf2/H19 locus, since the H19 ICR is methylated during spermatogenesis by a presumptive extrinsic activity. It is therefore essential to dissect the H19 ICR sequences and place them in a heterologous gene locus to identify this putative epigenetic signal (primary mark). Our YAC TgM strategy is therefore unique and provides a powerful tool for deciphering the fundamentals by which we can dissect the underlying imprinting mechanism(s) in transgenic, as well as at the endogenous, loci.

In the current study, we further examined the methylation status of the H19 ICR in the β-globin locus and found that the paternally but not maternally inherited ICR was substantially methylated in blastocysts, at least at the 5′ region (region I) of the ICR (Fig. 4A). These findings suggest that methylation acquisition of the transgenic H19 ICR begins at or before this developmental stage. Unlike imprinted methylation, the whole genome undergoes global demethylation after fertilization with the lowest methylation levels at the morula stage. In blastocysts, the genome begins to reacquire methylation (29,30). We assume that an underlying de novo methylation mechanism of this phenomenon may be used in the endogenous H19 ICR to ensure allelic methylation when it failed to fully acquire methylation in male germ cells or partially lost methylation owing to incomplete maintenance after fertilization. If this is the case, the transgenic H19 ICR may exploit this same activity to acquire allele-specific, post-fertilization methylation.

As described earlier, the H19 ICR fragment knocked-in at the Afp locus also acquired paternal-allele-specific methylation in somatic cells (27). In this case, however, the paternal H19 ICR was methylated after implantation at the earliest stage, but not in blastocysts. It is possible that the size of the H19 ICR fragments in each experiment or the sequences surrounding the ICR fragment (β-globin YAC versus endogenous Afp loci) affect the timing of methylation onset at the ectopic H19 ICR in early embryos.

To elucidate whether CTCF binding to the H19 ICR controlled the post-fertilization acquisition of differential methylation in the β-globin transgene, we introduced CTCF site mutations into the H19 ICR (4xMut and −9CG) and examined their effects on methylation. In both the TgM testis and oocytes, these mutations did not affect the methylation status of the transgenic ICRs (Fig. 3 and data not shown), suggesting that CTCF binding is neither necessary nor inhibitory to establish the unmethylated state of the transgenic H19 ICR in the germline. In blastocysts, both wild-type and 4xMut transgenic ICRs equally acquired paternal-allele-specific methylation (Fig. 4A and D). We therefore concluded that the CTCF
sites were not essential for initially establishing allele-specific methylation of the transgenic H19 ICR after fertilization (Fig. 5, left). In both 9.5 dpc embryos (Fig. 4F) and adult erythroid cells (Fig. 2B and C), the maternally inherited 4xMut ICR was aberrantly methylated and the state of this ICR was indistinguishable from the paternally inherited allele. In contrast, methylation in the paternally inherited −9CG ICR in adult erythroid cells was partially lost (Fig. 2B and C), presumably because of its ability to constitutively bind to the CTCF protein and hence protect the ICR from methylation (16). Thus, as was shown with these two mutations in the endogenous H19 ICR (11–16), the CTCF sites were important to protect the maternally inherited transgenic H19 ICR from de novo methylation after implantation (Fig. 5, right). We therefore assume that the functional roles of the CTCF protein and its binding sites are essentially the same for the endogenous and transgenic H19 ICRs.

In the endogenous H19 gene locus, the spread of the DMD was shown to change during embryogenesis; it becomes smaller in somatic cells than in germ cells, and the position of the border between DMD and non-DMD, at least at the 5′ end of the H19 ICR, appears to be established by 13.5 dpc of the embryonic stage (4,5). Based on our bisulfite sequencing results, the 5′ region (region I, Fig. 2A) of the maternally inherited transgenic ICR was almost devoid of methylation in blastocystcs (Fig. 4A), while the 5′-most CpGs in region I of the maternal transgenic ICR were substantially methylated in 7.5 dpc embryos (Fig. 4B), demonstrating that the spread of the DMD changed also in the transgenic β-globin locus. In addition, the positions of the methylation borders (region I) in both transgenic (7.5 and 9.5 dpc, Fig. 4B and C, and adult, Fig. 2C; 22) and endogenous H19 ICRs (4,5) were the same, implying that an activity that defines the methylation border is intrinsic to the H19 ICR. Collectively, judging from the fact that the transgenic 4xMut ICR acquired de novo DNA methylation after implantation, it is presumable that the CTCF sites within the H19 ICR may help determine the positions of the final methylatin borders and protect the maternal ICR from de novo DNA methylation (Fig. 5, right), both in the endogenous and transgenic loci.

It is intriguing to speculate how differential methylation of the transgenic H19 ICR is acquired after fertilization, in the absence of germline methylation. Two distinct, but not mutually exclusive, working hypotheses may explain the observed phenotypes. First, in pre-implantation embryos, the maternally inherited H19 ICR is either bound by an unknown factor other than CTCF or subject to an epigenetic modification, which inhibits de novo DNA methylation of the ICR. This mark is set theoretically within the ICR in oocytes but not sperm, and this state is heritable through fertilization and early embryogenesis. In post-implantation embryos, CTCF may take over the function of the putative epigenetic mark and becomes responsible for maintaining the unmethylated state of the maternal ICR. Alternatively, an unidentified epigenetic modification, other than DNA methylation, is set in the H19 ICR in male germ cells and is retained until the post-fertilization period. DNA methyltransferase or its cofactor then recognizes this putative mark to selectively and actively introduce DNA methylation on the paternal H19 ICR. In this scenario, CTCF binding may be required to prevent the maternal ICR from acquiring allele-non-specific methylation in post-implantation embryos. In either case, the allele discrimination mark is likely set during gametogenesis.

In summary, paternal-allele-specific methylation of the transgenic H19 ICR is initiated in pre-implantation embryos, and the CTCF-binding sites appear to be dispensable to establish this methylation status. On the other hand, the CTCF sites are indispensable for maintaining the unmethylated state of the maternal H19 ICR after implantation.

**MATERIALS AND METHODS**

**Yeast targeting vector and TgM**

The mutant ICR fragments were generous gifts from Christopher J. Schoenherr (4xMut, University of Illinois) (11) and Marisa S. Bartolomei (−9CG, University of Pennsylvania) (16). The 4xMut or −9CG fragments were excised with BglII and subcloned into BglII-digested pHS1/loxPw+/ICR (22) to generate pHs1/loxPw+/4xMut or pHs1/loxPw+/−9CG, respectively. These plasmids were linearized with SpeI and used to mutagenize the human β-globin YAC (A201F4.3) (31). All vector sequences were verified by DNA sequencing, and successful homologous recombination in *Saccharomyces cerevisiae* was confirmed by Southern blot analyses with several combinations of restriction enzymes and probes.

To generate TgM, purified YAC DNA was microinjected into fertilized mouse eggs from CD1 mice (ICR; Charles River Laboratories). Tail DNA from founder offspring was screened first by PCR and then by Southern blotting. Several independent TgM lines for 4xMut (lines 74 and 228) and −9CG (lines 493 and 828) were generated and maintained by mating with CD1 mice. The structural analysis of the YAC transgene was performed as described elsewhere (31). For analyses of DNA methylation or gene expression levels of the transgene, male or female TgM were crossed with...
female or male wild-type CD1 mice, respectively, to obtain Tg offspring that inherited the transgene paternally or maternally. Although these TgM in the CD1 (outbred) genetic background were supposed to exhibit some degree of heterogeneity in their genetic background, we obtained constant results from multiple transgenic animals from multiple lines. Therefore, the observed phenotype was considered to be attributable to a genetic manipulation, rather than to an individual or specific genetic background.

Animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Methylation analysis by Southern blotting

Genomic DNA was prepared from nucleated erythroid cells of anemic spleens or whole testes from 1- to 2-month-old TgM using standard procedures. DNA was digested with BamHI followed by methylation-sensitive HpaII, HhaI or BstUI or methylation-insensitive MspI. DNA was separated on an agarose gel and transferred to a nylon membrane. The membrane was hybridized with the α-32P-labeled probe (Fig. 2A) and subjected to X-ray film autoradiography.

Bisulfite sequencing

Genomic DNA was extracted from nucleated erythroid cells of anemic spleens (pool of two to four) or whole testes (pool of two) from 1- to 2-month-old TgM and digested with XhoI. Genomic DNA was also extracted from either 9.5 dpc (four embryos from a litter were pooled, two litters analyzed) or 7.5 dpc embryos (excluding the ectoplacental cone; several embryos from a litter were pooled, two litters analyzed). The DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit following the manufacturer’s instructions (Zymo Research). Blastocysts were flushed from the uteri with M2 medium at 3.5 dpc and washed with M2 medium followed by phosphate-buffered saline. Three or four blastocysts were embedded in agarose beads. The agarose beads were treated with sodium bisulfite as described previously (28) and were separately and directly used for nested PCR amplification of the transgenic ICR. Transgenic ICR-specific nested PCR, PCR product cloning and sequence analysis were performed as described previously (22,28). All embryos were obtained by natural mating between TgM and wild-type animals.

Semiquantitative RT–PCR

Total RNA was extracted from phenylhydrazine-induced anemic adult (1 to 2 months old) spleens using ISOGEN (Nippon Gene) and converted to cDNA with ReverTra Ace (Toyobo). The PCR conditions were described previously (32). An aliquot of each PCR product was electrophoresed on 8% polyacrylamide gels, dried and subjected to X-ray autoradiography and phosphor-imaging for quantitative analysis.

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Conflict of Interest statement. None declared.

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