

## Parental methylation patterns of a transgenic locus in adult somatic tissues are imprinted during gametogenesis

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### Summary

The methylation status of a mouse metallothionein-I/human transthyretin fusion gene was studied during gametogenesis in transgenic mice. In the adult tissues of this mouse line, the promoter region of the transgene on chromosome 11 is methylated when it is maternally inherited and undermethylated when it is paternally inherited. Germ cells from various developmental stages of gametogenesis were isolated, and their DNAs were assayed using methylation-sensitive restriction endonucleases and the polymerase chain reaction. Only low to nonexistent levels of transgene methylation were detected in germ cells from 14.5-day-old male and female fetuses irrespective of the parental origin of the transgene. This undermethylated state persisted in

oocytes from newborn females as well as in testicular spermatogenic cells and sperm. By contrast, the transgene promoter was completely methylated in fully grown oocytes arrested at the first meiotic prophase. The endogenous metallothionein-I gene promoter, located on a different chromosome, remained undermethylated at all stages examined, consistent with previous findings reported for a typical CpG island. Taken together, the results suggest that parental-specific adult patterns of transgene methylation are established during gametogenesis.

Key words: metallothionein-I gene, methylation imprinting, mouse gametogenesis.

### Introduction

Formerly, it was generally accepted that alleles of a given autosomal gene were functionally equal irrespective of their maternal or paternal inheritance. This fundamental notion has been the basis upon which genetic analyses have been performed in most species. Exceptions are now known to occur and, in these instances, some genes are expressed only when they are paternally inherited and others when they are maternally inherited. This phenomenon is known as genomic imprinting.

The concept of genomic imprinting stemmed from three different lines of work. First, nuclear transplantation experiments in mice demonstrated the absolute requirement of both paternal and maternal nuclei for normal development (reviewed by Surani, 1986; Surani et al., 1990; Solter, 1988). Embryos with exclusively paternal or maternal nuclei showed incomplete development and, from the observed phenotypes, it was concluded that the paternal genome is necessary for formation of extraembryonic tissues such as placenta while the maternal genome contributes to formation of the embryo proper. Second, genetic

studies of translocated chromosomes in mice showed that uniparental disomy, where homologous chromosomes or parts are derived from either parent, frequently results in developmental abnormalities in anatomy, growth, behavior and longevity of the offspring (reviewed by Cattanach, 1986; Cattanach and Beechey, 1990). In some cases, the phenotypic effects are complementary between the paternal and maternal disomies. Along this line, three endogenously imprinted mouse genes have recently been reported (Barlow et al., 1991; Bartolomei et al., 1991; Dechiara et al., 1991; Ferguson-Smith et al., 1991). Third, in some transgenic mouse strains, it was found that the methylation status of the transgene alternates depending upon the sex of the parent from which it was transmitted (Reik et al., 1987, 1990; Sapienza et al., 1987; Swain et al., 1987; Sasaki et al., 1991).

Although the nature of the imprinting signal remains unknown, previous studies have suggested several possibilities such as epigenetic methylation of DNA bases and modification of chromatin structure. The former has been studied with transgenic mice. In most cases of methylation imprinting of transgenes, the level of methylation is high

when the transgene is from the mother and low when it is from the father. Sasaki et al. (1991) produced a line of transgenic mice, designated MPA434, that carries a single copy of the human transthyretin (TTR) gene linked to a mouse metallothionein-I (MT-I) promoter. This fusion transgene is integrated into band A5 of chromosome 11. Parental-specific methylation of DNA bases in the transgene promoter has been observed for 9 generations in the BALB/c strain used as the genetic background. Knowing how and when this conversion of methylation patterns occurs from one generation to the next may shed light on the mechanism of genomic imprinting in general.

The observed parent-of-origin-dependent alteration of methylation suggests that the methylation control mechanism is sex-specific and, accordingly, may occur in the germ cell lineage. With the exception of the work of Chaillet et al. (1991), few reports have appeared dealing with an analysis of methylation status of these cells. The major difficulty has been to collect a sufficient number of germ cells for molecular analysis. In this study, we used the polymerase chain reaction (PCR) to amplify DNA from a small number of purified germ cells and then analyzed the methylation status of the transgene promoter during gametogenesis.

The MT-I promoter sequence of the transgene was originally derived from mouse genome (Glanville et al., 1981). Consequently, the MPA434 mice possess an endogenous copy of MT-I on chromosome 8 (Cox and Palmiter, 1983) in addition to the transgenic copy on chromosome 11. The endogenous MT-I promoter resembles a typical CpG island (Bird, 1986). Except for sperm, the methylation status of CpG islands in germ cells has received little attention. Therefore, we analyzed the methylation status of the endogenous MT-I promoter and compared it to the transgene MT-I promoter. The results showed that, while adult patterns of parental-specific transgene methylation were established during gametogenesis, the endogenous MT-I promoter remained undermethylated at all stages.

## Materials and methods

### Animals

MPA434 mouse, a line of transthyretin transgenic mouse (Sasaki et al., 1991), was mated with a BALB/cCrSIC mouse (Shizuoka Laboratory Animal Center, Japan) overnight. The next morning, mice with vaginal plugs were selected and defined as 0 dpc (days post coitum).

### Isolation of germ cells from fetal genital ridges

Genital ridges were dissected from 14.5 dpc embryos. Gonads and mesonephric tubes were separated with fine forceps in Hank's solution. To dissociate the cells, the gonads were transferred to a microfuge tube (1.5 ml), washed with CMF-Tyrodé solution and incubated in 0.25% Trypsin for 15 minutes at 37°C. A single cell suspension was produced by pipetting up and down after addition of an equal volume of medium (M199 + 10% FCS). Dissociated cells were suspended in 200 µl of fresh medium, and they were cultured for 14 hours at 37°C in a multi-well plate (SUMILON, MS80480). At the end of the culture period, the solution in the well was gently agitated, and cells floating in the supernatant were removed and collected by centrifugation (Hashimoto et al., 1990). The pelleted cells were histochemically stained for alkaline phos-

phatase (Sigma Technical Bulletin, No. 85, 1976; Brinster and Harstad, 1977). Germ cells stained blue by this reaction were individually picked up with a microcapillary tube using a dissecting microscope. In this way 100-150 germ cells were collected.

### Isolation of testicular germ cells and sperm

Testicular germ cells were obtained from sexually immature males on postnatal day 17 and from mature adult males as described previously (Fujimoto and Erickson, 1982). Briefly, decapsulated testes were treated by a two-step method using collagenase (500 U/ml) and trypsin (200 U/ml). Dissociated seminiferous tubules were gently pipetted and the cell suspension was filtered through glass wool and a nylon mesh. Sperm were collected by squeezing resected epididymides (Hogan et al., 1986).

### Isolation of oocytes

Ovaries dissected from a newborn female mouse were washed with CMF-Tyrodé solution and incubated in 0.25% Trypsin in medium for 30 minutes at 37°C. Oocytes were then picked up with a micropipette. Fully grown oocytes arrested at first meiotic prophase were obtained by dissecting ovaries from a 16-week-old female mouse, transferring them to medium, and removing associated fat and mesenchymal tissues. After transferring them to fresh medium, oocytes were released by pricking the follicle with a 25-gauge hypodermic needle. Follicle cells surrounding the oocytes were carefully removed by pipetting with a micropipette, and their absence was confirmed microscopically (Donahue, 1968). Purified oocytes were then collected using a micropipette.

### Extraction of DNA

DNA was obtained from a small number of oocytes and germ cells by the guanidine-HCl extraction method (Jeanpierre, 1987). A group of 100-150 germ cells was incubated in lysis solution (6 M guanidine-HCl, 140 µl; 7.5 M ammonium acetate, 10 µl; 20% Sarkosyl, 10 µl; 20 mg/ml Proteinase K, 2 µl) at 60°C for 3 hours. The lysate was passed through a 26-gauge hypodermic needle 10 times to shear the DNA to a minimal length. DNA was precipitated with ethanol after addition of 1 µl of glycogen (20 mg/ml; Merck) and 0.5 µl of M13 RFDNA (0.1 µg/ml). The precipitate was collected by centrifugation at 16,000 revs/minute for 30 minutes, washed twice with 70% ethanol, dried in air and dissolved in 100 µl of buffer prepared for *EcoRI* digestion. DNA from blood cells, testicular germ cells and sperm was extracted by a conventional phenol procedure (Maniatis et al., 1989) with the exception that, in the case of sperm DNA, lysis was carried out in the presence of dithiothreitol (final concentration 20 mM) and a fivefold increase in Proteinase K. The final DNA precipitates were dissolved in a buffer prepared for *EcoRI* digestion.

### Restriction enzyme digestion

DNA was first cleaved at *EcoRI* sites that flank the targeted sequence in order to reduce the DNA size and thus to facilitate the PCR reaction. The digest was extracted with phenol and DNA was precipitated with ethanol. DNA was collected by centrifugation at 16,000 revs/minute for 30 minutes at 4°C, air-dried, and dissolved in a buffer prepared for *HhaI* digestion. The pooled DNA from approximately 30 cells was mixed with 25 ng of SV40 DNA and digested with 6 units of *HhaI* (Takara Shuzo, Japan) for 3 hours at 37°C in a total volume of 8 µl. To monitor completion of *HhaI* digestion, one fifth of the sample was electrophoresed in a 1% agarose gel. After staining with ethidium bromide, the gel was examined in ultraviolet light for the appearance of a SV40 DNA digestion product.

### Polymerase chain reaction

The *HhaI* digestion mixture was added to PCR buffer (10 mM

Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 200 µM dNTP) containing sense and antisense primers (99.5 µl final volume). After heating at 95°C for 5 minutes to inactivate *Hha*I, 2.5 units of *Taq*I DNA polymerase (AmpliQ, Perkin Elmer-Cetus, 250 U/50 µl) were added. The reaction mixture was overlaid with mineral oil to prevent evaporation, and the PCR was cycled 40 times with a Thermal Cycler (Perkin Elmer Cetus). Each cycle consisted of 1 minute at 95°C, 2 minutes at 60°C, and 3 minutes at 73°C. Sequence of the primers used were as follows: P1 (ACATGATGTTCCACACGTCA), P2 (CTCCAGCCCACG-CATAGTCA), P3 (CTAATGCACCAAAGCAATGAGG) and P4 (AGGAGCAGTTGGGGTCCATTCC).

#### Agarose gel electrophoresis

The PCR mixture was extracted with chloroform and 15 µl was electrophoresed in 4% Nusieve 3:1 Agarose gel (FMC Bioproducts). The gel was stained in ethidium bromide and photographed in ultraviolet light.

## Results

#### Assay of methylation status by polymerase chain reaction

The genome of MPA434 transgenic mice contains a single copy of the human transthyretin (TTR) gene linked to a mouse metallothionein-I (MT-I) promoter (Sasaki et al., 1991; Fig. 1A). *Hha*I sites, clustered in the vector plasmid pUC18 sequences and the promoter sequences derived from the mouse MT-I gene within the transgene construct, showed parental-specific methylation for nine generations (Sasaki et al., 1991). In the present experiments, we analyzed the methylation status of the promoter sequences of the transgene by the polymerase chain reaction (PCR) according to the strategy described previously (Singer-Sam et al., 1989, 1990). Briefly, *Hha*I does not cleave the targeted DNA between the PCR primers when all of its corresponding restriction sites are methylated. Consequently, PCR amplifies the targeted region, and it appears as a corresponding electrophoretic band. Amplification does not occur after *Hha*I cleavage if at least one of these sites is unmethylated. In this case, the sites are designated undermethylated.

Since the MT-I promoter sequence of the transgene was originally derived from mouse, MPA434 mice possess an endogenous copy on chromosome 8 (Cox and Palmiter, 1983) in addition to a transgenic copy on chromosome 11 (Sasaki et al., 1991). The methylation status of both of these copies was assessed by using specific primers as shown in Fig. 1A. PCR amplification with primers P1 and P3 was used to analyze the methylation status of the MPA434 transgene promoter region that showed methylation imprinting (Sasaki et al., 1991). Amplification with primers P1 and P4 determined the methylation status of the endogenous MT-I gene promoter. Primers P1 and P2 were used to analyze the common promoter region simultaneously.

Results of control experiments are shown in Fig. 1B. DNA from the blood of a MPA434 mouse was first digested with *Eco*RI and then subjected to PCR with primers P1 and P3. A 641 bp band was detected (Fig. 1B, lanes 2 and 6), and it served as a positive control for the amplification step. An identical amount of *Eco*RI-cleaved DNA was then digested with *Hha*I and processed by PCR. DNA containing the

transgene inherited from a female parent gave a positive signal (Fig. 1B, lane 5), but that from a male parent did not (Fig. 1B, lane 1). This approach represents an alternative method of expressing the data of Sasaki et al. (1991).

With the same set of DNA, the endogenous MT-I promoter was examined for methylation status with primers P1 and P4, and it was found to be undermethylated in both cases of transgene transmission (Fig. 1B, lanes 3, 4, 7 and 8). This result agrees with the constitutive undermethylation of the MT-I promoter reported previously (Bird, 1986). For subsequent determinations of the methylation status of MT-I promoter of the transgene during gametogenesis, primers P1 and P2 were used to target the 163 bp region of the promoter (Fig. 1A). In the present experiments, PCR worked more efficiently with a shorter targeted sequence when minute amounts of DNA were tested (data not shown). By targeting this region, both the endogenous and the transgenic MT-I promoters were monitored. Only when PCR gave a positive signal of 163 bp was it necessary to determine which MT-I promoter was methylated.

When no amplified band appeared after *Eco*RI/*Hha*I digestion and PCR, we concluded that both MT-I promoters were undermethylated. The PCR experiment with DNA from blood cells and with primers 1 and 2 gave identical results to those shown in Fig. 1B (Fig. 1C; compare lanes 1, 2, 5 and 6 of Fig. 1B with lanes 1, 2, 3 and 4 of Fig. 1C). The endogenous MT-I gene is located on chromosome 8 and, as yet, no imprinted genes have been reported to be on this chromosome. It would be of interest, therefore, to compare the methylation status of the same sequence in a different chromosomal location during gametogenesis.

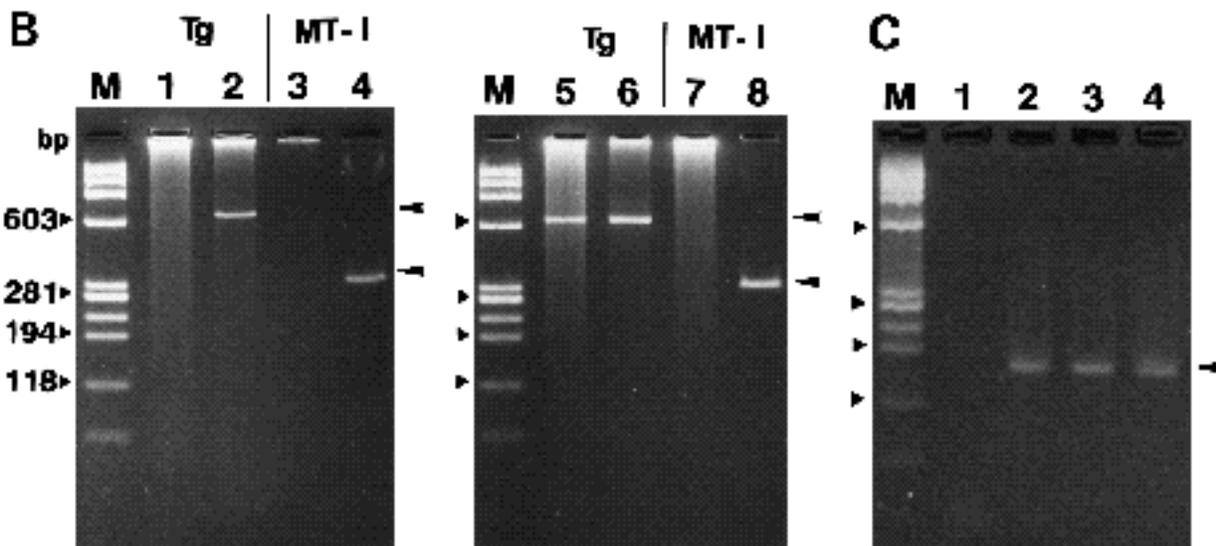
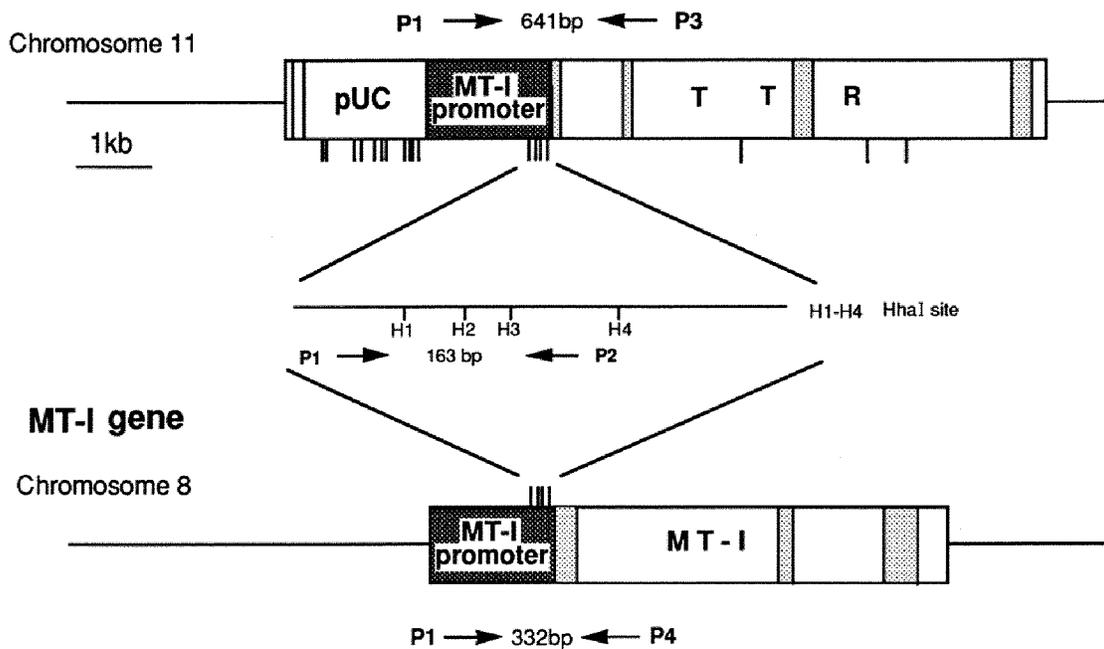
#### Analysis of transgene promoter in fetal germ cells

Germ cell DNA from 14.5 dpc male and female fetuses was similarly tested with primers P1 and P2. The paternally inherited transgene promoter showed no amplified 163 bp DNA fragment from either male or female fetuses (Fig. 2A, lanes 1 and 5), indicating that some of these *Hha*I sites were not methylated. The transgene promoter in somatic cells from gonads was also undermethylated (Fig. 2A, lanes 3 and 7). By contrast, fetuses with a maternally inherited transgene had fully methylated transgene promoters in somatic cells from gonads (Fig. 2B, lanes 3 and 7), while the corresponding sites in germ cells were undermethylated (Fig. 2B, lanes 1 and 5). With the same sample of DNA from gonadal somatic cells, P1 and P4 primers showed no amplified band (data not shown). This result indicates that the endogenous MT-I promoter is undermethylated in these cells. An identical state was found in germ cells from 12.5 dpc fetuses with a maternally inherited transgene (data not shown).

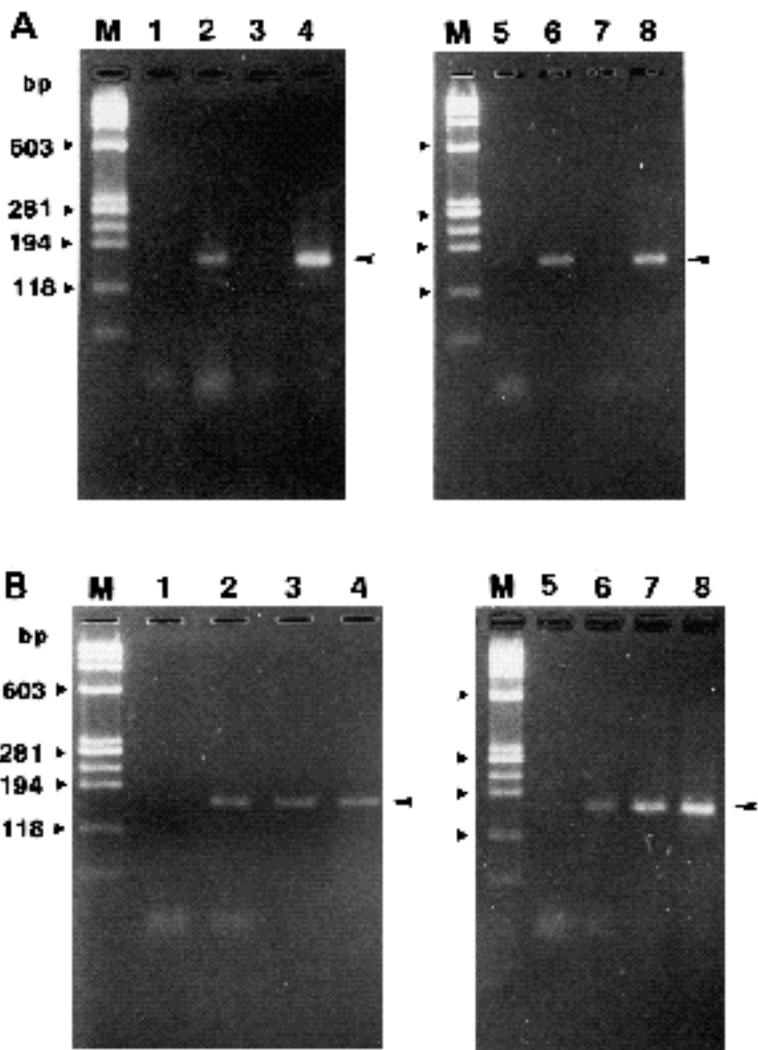
#### Analysis of testicular germ cells and sperm

Paternally inherited transgene promoters were not fully methylated in somatic cells of the progeny (Figs 1B, 7). Previous data suggested that transgene methylation sites were unsaturated in sperm and their germ cell precursors (Sasaki et al., 1991). To confirm these findings, testicular germ cells and sperm were examined. Normally, spermatocytes are predominant in the postnatal day-17 male, but

## A Transgene



**Fig. 1.** PCR assay of methylation status of the MT-I promoter region of MPA434 transgene and the endogenous MT-I gene. (A) Schematic diagram of the transgene in MPA434 transgenic mice and the mouse endogenous MT-I gene. The MPA434 transgene is located on chromosome 11 (Sasaki et al., 1991) and the MT-I gene is located on chromosome 8 (Cox and Palmiter, 1983). These two genes have in common the same promoter (MT-I promoter), part of which is expanded. The 641 bp region containing 4 *HhaI* sites (H1-H4) of the MT-I promoter as well as the first exon of the TTR gene was targeted by PCR with primers P1 and P3 to analyse the methylation status of the transgene promoter region which showed methylation imprinting (Sasaki et al., 1991). The 332 bp region containing 4 *HhaI* sites (H1-H4) of MT-I promoter and the first exon of the MT-I gene was targeted by primers P1 and P4 to analyse the methylation status of the endogenous MT-I gene promoter region. The 163 bp region containing three *HhaI* sites (H1-H3) was targeted by PCR with primers P1 and P2 to analyse the common MT-I promoter regions simultaneously. pUC, pUC18 plasmid sequence; MT-I promoter, mouse metallothionein-I promoter; MT-I, mouse metallothionein-I gene; TTR, human transthyretin gene. (B) PCR analysis of the methylation status of the transgene promoter and the endogenous MT-I promoter regions. The left panel shows the results for a transgene inherited from a male parent, the right panel from a female parent. Tg, PCR amplification of 641 bp MPA434 transgene promoter sequence with primers P1 and P3; MT-I, PCR amplification of 332 bp endogenous MT-I promoter sequence with primers P1 and P4. Lanes 1, 3, 5 and 7 *EcoRI/HhaI* digest; lanes 2, 4, 6 and 8 *EcoRI* digest; M, DNA size marker. Arrowheads on the right side of each panel show the position of amplified products; upper, 641 bp and lower, 332 bp. (C) PCR analysis of the methylation of the common MT-I promoter regions. Blood DNA of MPA434 mouse bearing the transgene transmitted from a male (lanes 1 and 2) or a female (lanes 3 and 4) parent was analyzed by PCR. Lanes 1 and 3, *EcoRI/HhaI* digest; lanes 2 and 4, *EcoRI* digest as a control for amplification. M, DNA size marker. Arrowhead on the right side of the panel shows the position of an amplified product (163 bp).

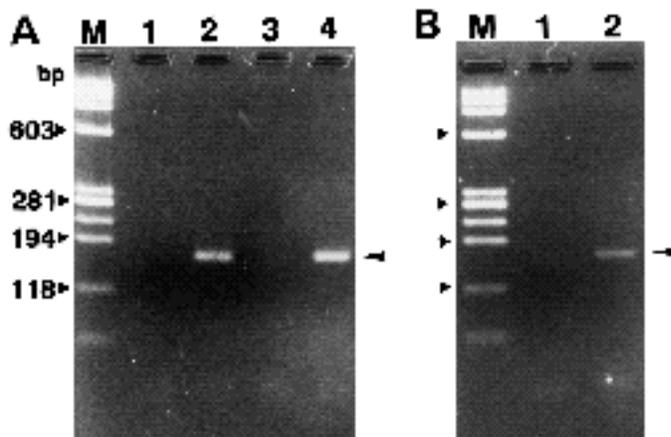


**Fig. 2.** Methylation status of the common MT-I promoter regions in fetal germ cells. PCR analysis was done on DNA extracted from fetal germ cells or from somatic cells in gonads at 14.5 days of gestation. Transgenes inherited from either a male parent (A) or a female parent (B) are shown. In both cases, the left panel refers to a male fetus, and the right panel refers to a female fetus. Lanes 1, 3, 5 and 7, *EcoRI/HhaI* digest; lanes 2, 4, 6 and 8, *EcoRI* digest. M, DNA size marker. Arrowhead on the right side of each panel shows the position of an amplified product (163 bp).

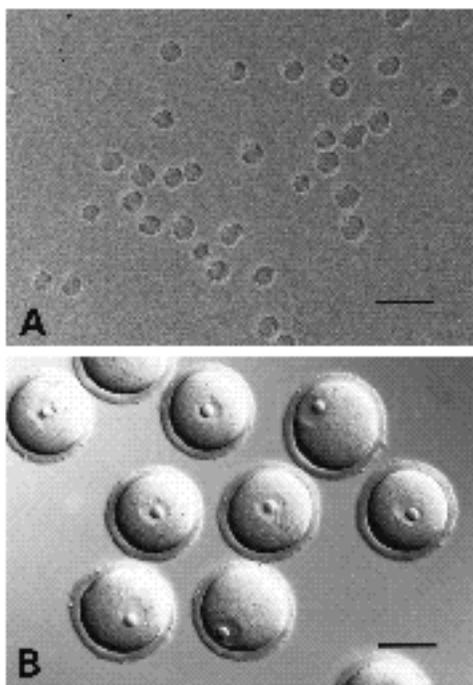
in adults more than 70% of the cells are spermatids (Thomas et al., 1989). Testicular germ cell DNA was isolated from these two stages and analyzed. *HhaI* sites in the targeted 163 bp region were undermethylated at both stages, even when the transgene derived from a female parent (Fig. 3A). Transgene promoters were also undermethylated in sperm (Fig. 3B), corresponding to the paternal pattern found in adult somatic cells. In contrast, transgene promoters from a female parent were completely methylated in the somatic cells of offspring (Fig. 1B; Sasaki et al., 1991).

*Methylation of transgene promoter during oocyte growth*

Since undermethylated transgene promoter sites were found in fetal germ cells, testicular germ cells and sperm, the time of appearance of the adult patterns was investigated in female gametes. Oocytes were isolated from a newborn mouse and from a 16-week-old female mouse (Fig. 4A,B). Oocytes from the former were at the beginning of diplotene stage of first meiotic prophase. Those from the latter were fully grown oocytes arrested at diplotene stage of first meiotic prophase. As shown in Fig. 4, oocytes grow extensively during this period. In oocytes from a newborn mouse, the transgene promoter was undermethylated (Fig. 5A). Nev-



**Fig. 3.** Methylation status of the common MT-I promoter regions in testicular germ cells. PCR analysis was carried out on spermatogenic cells and sperm that carried the transgene from a female parent. (A) Spermatogenic cells from postnatal day 17 and adult males. Lanes 1 and 2, postnatal day 17; lanes 3 and 4, adult. Lanes 1 and 3, *EcoRI/HhaI* digest; lanes 2 and 4, *EcoRI* digest. (B) Sperm from epididymides. Lane 1, *EcoRI/HhaI* digest; lane 2, *EcoRI* digest. M, DNA size marker. Arrowhead on the right side of each panel shows the position of an amplified product (163 bp).



**Fig. 4.** Photomicrographs of isolated mouse oocytes. (A) Newborn oocytes. (B) Fully grown oocytes. Photography was by a Nomarski optics. Scale bar, 50  $\mu$ m.

ertheless, all three *Hha*I sites of transgene promoter were methylated in fully grown germinal-vesicle-stage oocytes (Fig. 5B, lane 1). The amplified 163 bp band did not result

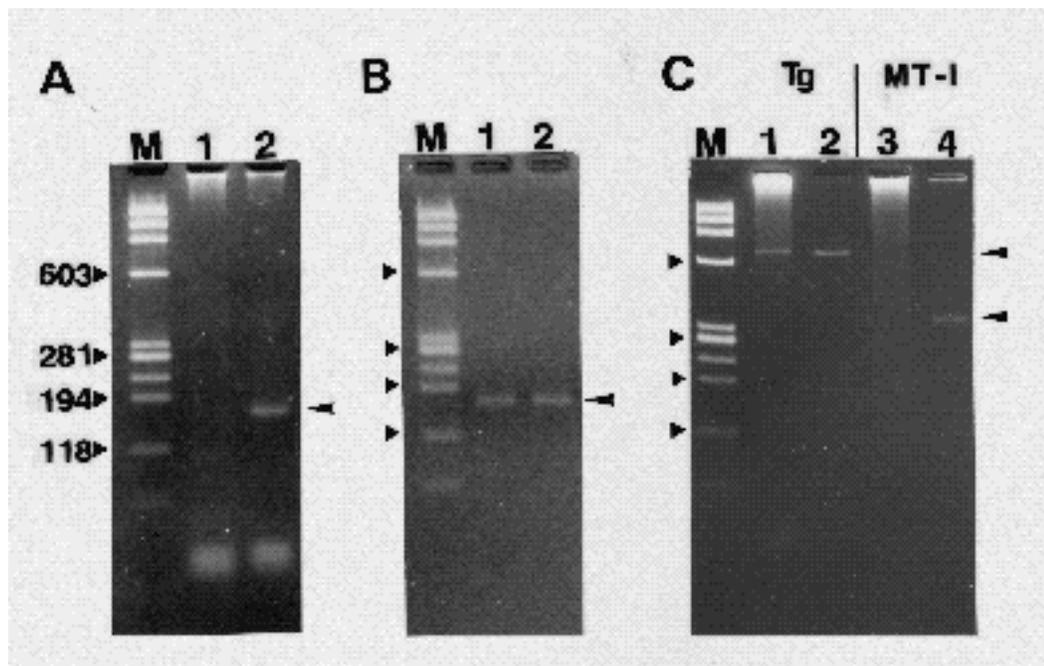
from the methylated state of the corresponding sites of the endogenous MT-I promoter, because the PCR assays with primers P1 and P4 gave no amplified signal (Fig. 5C, lane 3) and transgene promoter-specific primers P1 and P3 gave a positive signal (Fig. 5C, lane 1). This indicated that de novo methylation of the transgene promoter occurred during the growth phase of the oocytes prior to meiotic metaphase I (Fig. 6).

## Discussion

### *Adult patterns of transgene methylation are imprinted during mouse gametogenesis*

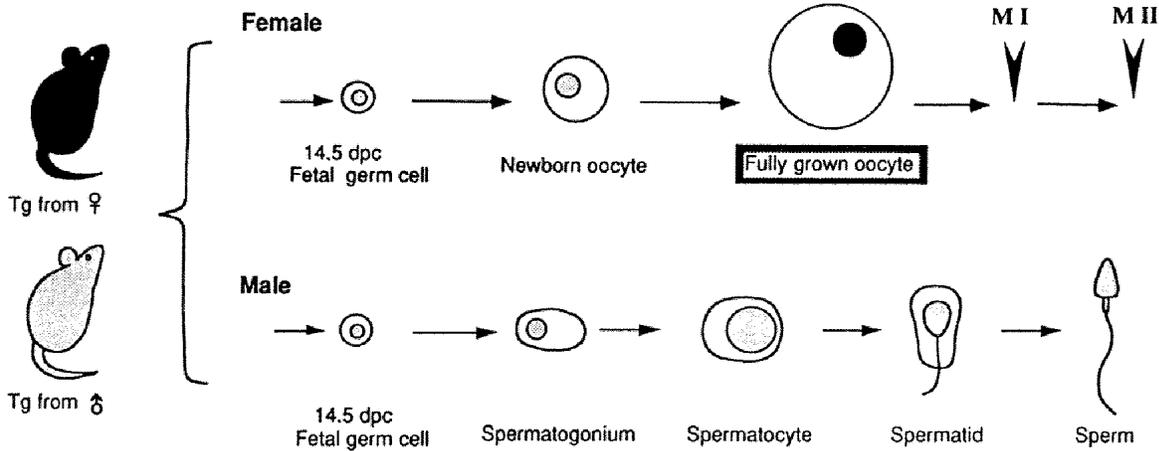
Based on the observation that the methylation status of the transgene promoter in MPA434 mice changes in a germline-dependent manner, we attempted to determine period of gametogenesis during which erasure and re-establishment of methylation patterns occurs. Difficulties in obtaining sufficient number of germ cells were overcome by applying the PCR method to a very small number of cells. The fact that the MPA434 mouse genome bears a single copy of the TTR gene made the interpretation of the results unambiguous. Thus, within the region of the transgene promoter examined, parental patterns of methylation were erased in fetal germ cells and adult patterns were re-established during gametogenesis.

Chaillot et al. (1991) studied a RSV-Ig-myc transgene construct that exhibited methylation imprinting and analyzed its methylation status during gametogenesis as well as early embryogenesis. With regard to the transgene transmitted as a maternal allele, their results showed that



**Fig. 5.** Methylation status of the MT-I promoter regions of the transgene and the endogenous MT-I gene in oocytes. PCR analysis was done on newborn oocytes, and fully grown oocytes that carried the transgene from a female parent. (A) Oocytes from newborn mouse. Lane 1, *Eco*RI/*Hha*I digest; lane 2, *Eco*RI digest. (B) Fully grown germinal-vesicle-stage oocytes. Lane 1, *Eco*RI/*Hha*I digest; lane 2, *Eco*RI digest. In A and B, PCR was done with primers P1 and P2. (C) Fully grown germinal-vesicle-stage oocytes.

Tg, specific PCR amplification of 641 bp transgene promoter sequences with primers P1 and P3; MT-I, specific PCR amplification of 332 bp endogenous MT-I gene sequence with primers P1 and P4. Lanes 1 and 3, *Eco*RI/*Hha*I digest; lanes 2 and 4, *Eco*RI digest. For A-C, M indicates DNA size marker, and the arrowheads on the right side of the panel indicate the position of amplified products.



**Fig. 6.** Summary of the changes in methylation status of the imprinted transgene during gametogenesis. Only in the female gonad does the transgene become highly methylated between the newborn oocyte stage and the fully grown germinal vesicle oocyte stage. Stippled area represents the undermethylated state; black area shows the highly methylated state.

imprinting was established during gametogenesis, a finding in agreement with the present study. The MPA434 transgene promoter was methylated in oocytes arrested at the diplotene stage of the first meiotic prophase in contrast to the RSV-Ig-myc transgene, which showed a high level of methylation in unfertilized eggs (metaphase II oocyte). This apparent difference in results may simply reflect the fact that earlier stages in oogenesis were not examined by Chaillot et al. (1991).

In the case of the paternally transmitted transgene, the results of the current work and those of Chaillot et al. (1991) were clearly different, however. Chaillot et al. (1991) found that the RSV-Ig-myc transgene was undermethylated at the prospermatogonial stage. It was methylated during spermatogenesis, becoming highly methylated in sperm. At the blastocyst stage of zygotic development, the transgene was undermethylated, finally acquiring the adult methylation pattern through a postimplantation *de novo* methylation process. By contrast, the MPA434 transgene remained undermethylated throughout the process of development of prospermatogonium to sperm. The pattern of undermethylation persisted after fertilization up to the blastocyst stage (data not shown).

One reason for the disparity may be that the methylation status of the paternal alleles of these transgenes were different in adult somatic cells. The MPA434 transgene promoter was completely undermethylated in the paternal allele (Sasaki et al., 1991), but the RSV-Ig-myc transgene was partially methylated. Another possibility may be that the region analyzed in the MPA434 transgene construct was a CpG island in the MT-I promoter. Chaillot et al. (1991) did not specifically focus their analysis on CpG islands in the RSV-Ig-myc transgene. Some genes in sperm have an unmethylated CpG island surrounded by methylated sequences (Ghazi et al., 1990; Shemer et al., 1990). Taken together, the present results suggest that parental specific methylation patterns are first erased in fetal germ cells. Thereafter, the corresponding adult patterns are re-established in developing gametes.

#### *De novo methylation during oocyte growth*

The undermethylated MPA434 transgene promoter in newborn oocytes was converted to a highly methylated state in fully grown oocytes. This suggests that the introduction of methyl groups occurred in oocytes during oogenesis. Newborn oocytes and fully grown oocytes are both at the diplotene stage of meiosis (McLaren, 1988). *De novo* methylation may, therefore, have taken place in the absence of DNA synthesis.

Howlett and Reik (1991) recently reported that low-copy number genes such as the Intracisternal A Particle (IAP) gene and the Murine Urinary Protein (MUP) gene are highly methylated in unfertilized eggs, and they suggested the possibility of *de novo* methylation at the oocyte growth stage. It is also conceivable that the RSV-Ig-myc transgene of Chaillot et al. (1991) became highly methylated during oocyte growth. It is interesting to note that a 400-fold increase in the concentration of DNA methylase is found in fully grown oocytes and MII-stage oocytes as compared to somatic cells (Howlett and Reik, 1991). High DNA methylase activity is also associated with unfertilized eggs (Monk et al., 1991). These observations, together with our current results, are inconsistent with previous findings that showed a high level of methylation in sperm and a low level in eggs in L1, IAP and MUP sequences, and in bulk DNA (Sanford et al., 1987; Monk et al., 1987; Monk, 1990).

The undermethylated state of DNA as a whole in primordial germ cells of either sex, together with the findings of undermethylated egg DNA and methylated sperm DNA, led to the notion that *de novo* methylation occurs only during spermatogenesis (Cedar and Razin, 1990). However, satellite DNA sequences in sperm are in an undermethylated state (Zimmerman and Wolgemuth, 1984; Sanford et al., 1984), and there may be different control mechanisms for DNA methylation of transgenes as compared to endogenous differentiation-related genes, house keeping genes, and heterochromatin. The realization that *de novo* methylation happens during the oocyte growth phase may help

reopen investigation of DNA methylation during gametogenesis.

#### *Methylation status of CpG islands in germ cells*

It was once thought that CpG island sequences have no methylated bases except for those on the inactive X chromosome (Antequera et al., 1989; Lewis and Bird, 1991). Recently, Migeon et al. (1991) showed that a specific site in the 5' CpG island of the P3 and the hypoxanthine phosphoribosyl transferase (HPRT) genes on the active X chromosome is indeed methylated at 6-9 weeks of human gestation. They also found that subsequent demethylation during development establishes the adult pattern of undermethylation. This suggests that methylation occurs in CpG islands in a given tissue at a specific developmental stage and makes it important to reconsider the possibility that CpG island methylation takes place in germ cells.

Aside from the work of Driscoll and Migeon (1990), systematic analyses of CpG islands methylation in germ cells has been confined to sperm and testis (Bird, 1986, 1987; Kolsto et al., 1986; Schemer et al., 1990). From analyses of male and female germ cells in this study, it is apparent that the endogenous MT-I promoter region (CpG island) located on chromosome 8 is constitutively undermethylated during spermatogenesis and oogenesis. Driscoll and Migeon (1990) also found that CpG islands in X-linked genes remained undermethylated in both testis and ovary at 8-21 weeks of human gestation, although the testicular and ovarian tissues were analyzed without isolating the germ cells. Shemer et al. (1991) reported that the CpG island in the apolipoprotein AI gene is undermethylated in sperm and oocytes and, in the present study, CpG islands were undermethylated in sperm, immature oocytes, and 12-14.5 dpc fetal germ cells as well. This suggests that the CpG island never becomes methylated de novo but rather maintains its undermethylated state. Shemer et al. (1990) hypothesized that a CpG island has a signal sequence within its structure for protection against de novo methylation (Tazi and Bird, 1990).

Why does the CpG island sequence in the transgene MT-I promoter become methylated while the same sequence in the endogenous MT-I gene does not? Evidence from PCR experiments shows that four *HhaI* sites in the transgene MT-I promoter region behave in a similar fashion with respect to their methylation status (data not shown). Accordingly, *HhaI* sites in the pUC18 sequence may be expected to behave like H1-H4 sites in the MT-I promoter. Chromatin encompassing this region may behave as a block during the methylation process similar to what occurs when CpG islands are packaged into heterochromatin in the course of X-chromosome inactivation. It may be of interest to analyze the chromatin structure of this region with regard to its methylation status.

#### *Comparison with endogenously imprinted genes*

It is not clear at present whether the methylation conversion of transgenes during gametogenesis has some direct connection with endogenous genomic imprinting. Three endogenously imprinted mouse genes have been reported thus far. They are the insulin-like growth factor type 2 receptor gene (Igf2r; Barlow et al., 1991), the insulin-like

growth factor 2 gene (Igf2; Dechiara et al., 1991), and the H19 gene (Bartolomei et al., 1991). In each case, expression of the gene depends on the sex of the parent from which it was transmitted. In at least two of these genes, a detailed analysis of genomic clones revealed sites whose methylation was determined by its parental origin (Igf2, Chaillot et al., 1991; Igf2r, Barlow et al., unpublished data), a phenomenon very similar to transgene methylation imprinting. Analysis of methylation status of these sites during gametogenesis may help to determine whether methylation control mechanisms similar to that for transgene methylation also operate on endogenously imprinted genes. Genomes of cells destined to differentiate into germ cells, which in turn constitute the next generation, are thought to replicate in an undermethylated state. It is reasonable to suppose that methylation occurs in specific regions of their genes during gametogenesis and that this methylation variation constitutes the basis for the phenomenon of genomic imprinting.

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