DNA Methylation-mediated Control of Sry Gene Expression in Mouse Gonadal Development

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DNA methylation at CpG sequences is involved in tissue-specific and developmentally regulated gene expression. The Sry (sex-determining region on the Y chromosome) gene encodes a master protein for initiating testis differentiation in mammals, and its expression is restricted to gonadal somatic cells at 10.5–12.5 days postcoitum (dpc) in the mouse. We found that in vitro methylation of the 5′-flanking region of the Sry gene caused suppression of reporter activity, implying that Sry gene expression could be regulated by DNA methylation-mediated gene silencing. Bisulfite restriction mapping and sodium bisulfite sequencing revealed that the 5′-flanking region of the Sry gene was hypermethylated in the 8.5-dpc embryos in which the Sry gene was not expressed. Importantly, this region was specifically hypomethylated in the gonad at 11.5 dpc, while the hypermethylated status was maintained in tissues that do not express the Sry gene. We concluded that expression of the Sry gene is under the control of an epigenetic mechanism mediated by DNA methylation.

Sry is the gene encoding the master protein essential for initiating the cascade leading to testicular differentiation in mammals (1–4). Sry is required for differentiation of Sertoli cells to induce masculinization of the embryonic gonad (5, 6). There are two types of Sry transcripts, linear and circular, with different transcription start sites (7, 8). The linear transcript is translated into protein and is expressed only from 10.5 to 12.5 dpc1 in pre-Sertoli cells in the developing male gonad (7, 9, 10). The circular form is also expressed in the genital ridge, as well as in round spermatids in adult testis and in the adult brain, but is thought to be untranscribed (9, 11–13). Typical CCAAT and TATA boxes, located in close vicinity to the transcription start site of each form, are the putative promoters (7, 8). The presence of several other putative regulatory cis-elements was indicated when sequences of the 5′-flanking region of the Sry gene from 10 different mammalian species were compared (14).

DNA methylation generally occurs at the cytosine of CpG dinucleotides in higher vertebrates; 60–70% of CpGs are methylated in the mammalian genome (15–17). Methylation of DNA is involved in various developmental processes by silencing, switching, and stabilizing genes, as well as remodeling chromatin (18–24). Regardless of the frequency of CpGs in the regulatory region, DNA methylation-mediated gene regulation occurs in various genes (22, 23). We found previously that there are numerous tissue-dependent differentially methylated regions (T-DMRs), which are widespread in the mammalian genome (25). Formation of the DNA methylation pattern is one of the epigenetic events that underlie development of various tissues in mammals (26). Analysis of several stem cells, somatic cells, germ cells, and tissues revealed that DNA methylation pattern is specific to cell type (25). To differentiate, cells must change the DNA methylation pattern. In fact, differentiation of cells is always associated with DNA methylation and demethylation, thereby forming cell type-specific DNA methylation patterns (25, 27).

To date, however, regulatory mechanisms for spatiotemporal expression of the Sry gene remain elusive.

In the present study, we first established a reporter gene assay using a primary culture of gonadal cells from mice 11.5 dpc. In the assay, in vitro DNA methylation resulted in suppression of promoter activity. In addition, CpGs in the putative promoter regions were specifically demethylated coincident with the spatiotemporal pattern of Sry gene expression. We demonstrate here that DNA methylation plays a pivotal role in the mechanism of transcriptional regulation of the Sry gene in mouse gonadal development.

**EXPERIMENTAL PROCEDURES**

**Animal Treatment and Reagents**—The experiments were carried out according to the guidelines for the care and use of laboratory animals (Graduate School of Agriculture and Life Sciences, The University of Tokyo). Mice (CD-1 (ICR) strain) were purchased from a supplier (Charles River Japan, Inc., Yokohama, Japan) and were kept under regulated temperature (22–25 °C), humidity (40–60%), and illumination cycles (14 h light and 10 h dark) throughout the experiments. Noon of the day when a vaginal plug was detected was designated as 0.5 dpc.

Blastocysts at 3.5 dpc, 8.5-dpc embryos, 11.5-dpc gonads separated from mesonephros and liver, and 15.5-dpc testis and liver were collected. In promoter assays, the 11.5-dpc gonads separated from mesonephros and liver were pipetted to single cells in trypsin and collagenase solution to disperse them. Separated cells were then cultured at 37 °C in Dulbec-co's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum.

The 11.5-dpc gonadal somatic cells used for bisulfite sequencing were dispersed and cultured for 3 h. Then the cells were washed three times in phosphate-buffered saline to remove germ cells and blood cells and then were collected. All samples were frozen in liquid nitrogen and stored at −80 °C until use. All reagents, unless otherwise stated, were purchased from Wako Pure Chemicals (Osaka, Japan).

**Analysis of CpG Frequency in the Sry Gene**—The GC content and

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1 The abbreviations used are: dpc, days post-coitum; T-DMRs, tissue-dependent differentially methylated regions; RT, reverse transcription; -as, antisense; ppc11.5, primary cultured gonadal cells of the 11.5-dpc fetus; 4.4kb-cont, construct comprising the 4.4 kb of rat Dnmt1 cDNA fragment with supposedly no transcription regulatory activity.

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DNA Methylation-mediated Gene Silencing of Sry

CpG frequency of the DNA sequences of the Sry gene were examined with a program (CpG View, version 1.4.6) provided by the National Institute of Infectious Diseases (www.nihs.go.jp/yoken/genebank/binaryFileMac).

RNA Extraction and RT-PCR—Total RNA was isolated from tissues and cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA synthesis was performed using Superscript first-strand synthesis system for RT-PCR (Invitrogen) with hexamer random primers and 1.0 μg of total RNA in 20 μl according to the manufacturer's instructions. Amplification by PCR was performed with Taq polymerase (TOKYO, Tokyo, Japan) and 2 μl of cDNA. Primer sequences were 5'-AAGCCGCCCATGATGCTTTATGGT-3' and 5'-ACACCTTGACCTCCGATG GGCTGA-3' for Sry (9) and 5'-GACAAGCTCTCCGATGCAAGAAG-3' and 5'-TTACGCGTTGGCCTTAGGTTTCAG-3' for β-actin (28). The thermocycling program used was an initial cycle of 95 °C for 1 min, followed by 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, for 33 and 28 cycles for Sry and β-actin, respectively.

Real Time Quantitative RT-PCR—Expression of Sry mRNA was analyzed quantitatively using SYBR Green I real time PCR (29) with ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The reaction mixture contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 18 pmol each of the forward and reverse primers of Sry (9) or β-actin (28) described above, and 5 μl of 1:4 diluted cDNA in a total volume of 20 μl. The thermocycling program was 45 cycles of 95 °C for 15 s and 60 °C for 1 min with an initial cycle of 95 °C for 10 min. Because SYBR Green I is not specific for the PCR product, after the PCR, a dissociation curve (melting curve) was constructed from the range of 60 to 98 °C to ensure that primer-dimers and other nontarget products had been eliminated. To estimate mRNA levels, a standard curve was generated from a dilution series of the control. The slope of the standard curve generated for each detection approach was placed into the following equation to determine PCR efficiency. PCR efficiency = 10 (-1/slope) – 1. All quantitative data, including cycle number when a reaction reaches threshold, the cycle threshold (CT), were analyzed using ABI PRISM 7000 SDS software. Expression level was calculated using the following equation: the value = 1/(1 + PCR efficiency)1/2. The Sry mRNA levels were normalized by dividing concentrations of the PCR products from Sry mRNAs by those from the β-actin mRNA. Experiments were performed at least three times independently.

Sry Promoter Assay in Vitro—The 5'-flanking fragment of the Sry gene was isolated from genomic DNA of CD-1 (ICR) strain mice by genomic PCR. The position of the translation start site was designated as +1. The 4.4 kb of the 5'-flanking region (from −4420 to −58: LC), the 2.0-kb upstream region of the linear transcription start site (from −2090 to −58: Lin), and 2.4 kb of the upstream region of the circular transcription start site (from −4420 to −2063: Cir) of the Sry gene were subcloned into pGL3-Basic vector (Promega, Madison, WI). Constructs into which each genome fragment was inserted in the opposite direction were used as controls (LC-as, Lin-as, and Cir-as) (Fig. 3A). The construct into which the 4.4-kb cDNA fragment of rat Dmrt1 was inserted was also used as a control. Cloning of all constructs was carried out using the bacterial strain SCS110 (Stratagene, La Jolla, CA), which is deficient of two methylases found in most strains of Escherichia coli, Dam and Dem methylases. Reporter constructs were methylated in vitro with 3 units of SssI methylase (New England BioLabs, Inc., Beverly, MA) per μg of DNA in the presence of 160 μM S-adenosylmethionine at 37 °C for 3 h. Bisulfite restriction mapping and methylcytosine-sensitive restriction enzymes were used to confirm that methylation at CpG sites was complete. The 11.5-kbp gonadal somatic cells, liver cells, and NIH 3T3 cells were collected and cultured on a 24-well plate (1.6 × 104 cells/well). Subsequently, the cells were transfected with 0.165 pmol of reporter constructs with Effectene transfection reagent (Qiagen GmbH, Hilden, Germany). To normalize firefly luciferase activity of the reporter constructs, an internal control plasmid (0.015 pmol) expressing Renilla luciferase (pRL-TK vector, Promega) was co-transfected into the cells. Promoter activity was measured at 12 h after the transfection because a peak of activity was observed at 12 h after transfection when activity was measured between 6 and 48 h after transfection (data not shown). The activities of both luciferases were determined by means of a Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Experiments were performed at least three times independently.

Genomic DNA Preparation and Genomic PCR—Tissues (≤20 mg) and cells were incubated in 250 μl of lysis solution (10 mM Tris-HCl (pH 8.0), 150 mM EDTA, 1% SDS, 100 μg/ml proteinase K) for 20 min at 55 °C. Then 100 μl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TE) and 30 units of RNase were added, and the mixture was incubated for 60 min at 37 °C. After two rounds of phenol-chloroform extraction, 150 μl of 1 M Na-acetate (pH 7.0) were added, and the DNA was ethanol-precipitated. The purified DNA (≤3.0 μg) was digested with HindIII. Then DNA fragments of 3.5–4.5 kb in size were gel-extracted using QIAquick gel extraction kit (Qiagen GmbH) to remove the downstream inverted repeat sequence of the Sry gene, and fragments were eluted in 30 μl of TE. In genomic PCR, the reaction contained 1 unit of AmpliTaq Gold (PerkinElmer Life Sciences), 1X AmpliTaq Gold buffer, 2.5 mM MgCl2, 0.2 mM each dNTPs, 2 μM each forward and reverse primer, and 2 μl of adult ear genomic DNA solution in a total volume of 20 μl. The primers used were as follows: for Region I, 5'-GTTTGTTGT- CATTAAAGTTTGGTTTAAACC-3' and 5'-CTCCCTTCTCATTTGTGCAGCAG-3'; and for Region II, 5'-TAAAGGAAATGACAGCCAGCCCATA-3' and 5'-GTCAGACAAACCCCCCTCAAGTCTTGTG-3'. The thermocycling program was 45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with an initial cycle of 95 °C for 10 min. The PCR product was cloned and sequenced.

Bisulfite Restriction Mapping and Sequencing—The bisulfite reaction, in which cytosine is converted to uracil and 5'-methylcytosine remains non-reactive (30), was carried out as described previously (22, 23). In brief, the HindIII-digested genomic DNAs were initially denatured with 0.3 M NaOH. Then sodium metabisulfite solution (pH 5.0) and hydroquinone were added at a final concentration of 2.0 M and 0.5 mM, respectively. The reaction mixtures were incubated under mineral oil in the dark at 55 °C for 16 h. The denatured DNA was purified with Wizard DNA purification resin (Promega), and modification was then terminated by treatment with 0.3 M NaOH at 37 °C for 15 min, followed by ethanol precipitation. The DNA was suspended in 30 μl of TE. Two μl of each sample were amplified with AmpliTaq Gold (PerkinElmer Life Sciences) using the primer sets IBF/IBR or IIBF/IIBR1 under the same conditions as genomic PCR described above. Half of the PCR products were digested with 5 units of TaqI (Roche Diagnostics) at 65 °C or with 5 units of HpyCH4 IV (New England Biolabs) at 37 °C for

Fig. 1. Genomic organization of mouse Sry. A, genomic structure of mouse Sry and positions of primers. Open and filled circles indicate CpG dinucleotide sequences in 129 mouse strain. Two moderate clusters of CpG sites (arrows). Filled circles represent polymorphic sequences, which are not CpG dinucleotides in the CD-1 (ICR) strain. TaqI and HpyCH4 IV restriction enzyme sites are marked by filled and open triangles, respectively. The position of the translation start site is designated as +1. ORF, open reading frame. B, schematic diagram of the predicted bands after bisulfite restriction mapping. PCR products amplified using the primer sets, IBF/IBR or IIBF/IIBR1, are digested when template genome DNA is methylated.
3 h for Regions I and II, respectively. The other half was used for undigested control. TaqI and HpyCH4 IV recognize 5'-TCGA-3' and 5'-ACGT-3' sequences, respectively. Therefore, after the bisulfite reaction, the unmethylated DNA remains intact following TaqI or HpyCH4 IV digestion (see Fig. 5A, U), whereas the methylated DNA is cleaved (see Fig. 5A, Me). The intensity of each band was determined using an image-analyzing program (NIH Image, version 1.6.1) provided by the National Institutes of Health (ftp://rsbweb.nih.gov/pub/nih-image/nih-image161_fat.hqx). The degree of methylation in each sample was calculated by the formula $I_{Me}/(I_{U}/H11003 \times 541/573 + I_{Me}) \times 100$ for Region I and $I_{Me}/(I_{U} + I_{Me}) \times 100$ for Region II, where $I_{Me}$ and $I_{U}$ represent the intensity of methylated and unmethylated bands, respectively, in each sample. The results from at least three independent experiments were quantified and averaged. In the case of sodium bisulfite sequencing, each PCR product amplified using primer sets IBF/IBR or IIBF/IIBR was cloned into pGEM T-Easy vector (Promega), and 5 or 10 clones were sequenced, then methylation status of individual CpGs was determined. As a control, plasmid DNA, which is unmethylated, was amplified and then subjected to the same analysis in parallel to confirm the completion of the bisulfite reaction. The primers used were as follows: for Region I, IBF, 5'-GTTTGTGTTATTAAATGTTTTGAAATT-3' and IBR, 5'-TATACCTTTAACATTCTCCAAAATA-3'; and for Region II,
Fig. 3. Promoter assay using primary cultured gonadal cells of 11.5-dpc fetus. A, schematic diagram of reporter constructs. Top, schematic diagram of Sry gene locus. I, constructs containing the 4.4-kb fragment of the 5′-flanking region of Sry gene including putative linear and circular promoter regions, in proper (Lin) or opposite (Lin-as) directions. II, constructs containing the putative linear promoter region in proper (Lin) or opposite (Lin-as) directions. III, constructs containing the putative circular promoter region in proper (Cir) or opposite (Cir-as) directions. Bottom, the 4.4kb-cont containing the 4.4-kb fragment of rat Dnmt1 cDNA. Open circles indicate CpG dinucleotide sequences. ORF, open reading frame; Luc, luciferase. B, promoter activities of LC and LC-as in 11.5-dpc gonadal cells, 11.5-dpc liver cells, and NIH 3T3 cells are shown. Filled, open, and hatched bars represent 4.4kb-cont, LC-as, and LC, respectively. C–E, promoter activities of all constructs in 11.5-dpc gonadal cells (C), in 11.5-dpc liver cells (D), and in NIH 3T3 cells (E) are indicated. Average activity of 4.4kb-cont in 11.5-dpc gonadal cells was arbitrarily set as 100%. All results are shown as the mean ± S.E. *, difference between constructs is statistically significant (p < 0.001, Student’s t test). Experiments were replicated at least three times in each case.
RESULTS

Gene Structure and CpG Dinucleotides of the Sry Gene—According to the registered nucleotide sequence in the GenBank™/EBI data base (accession number X67204), there were 16 CpGs in the 4.5-kb 5′-flanking region of the mouse Sry gene (Fig. 1A). These CpGs were not evenly distributed in this region but formed two approximate clusters: four CpGs in the region immediately upstream of the transcription start site of the linear form (7 CpGs in the region immediately upstream of the transcription start site of the circular form (7 CpGs in the region immediately upstream of the transcription start site of the linear form (8 CpGs in the region immediately upstream of the transcription start site of the circular form (Fig. 1A)).

Sry Expression in Vivo and in Vitro—Using RT-PCR, we analyzed the stage-specific expression of Sry during development using a primer set that detects both forms of the Sry transcript. At 11.5 dpc, expression of Sry was detected in the gonad but not in liver (Fig. 2A). Expression was not detected in 8.5-dpc embryos and in all 15.5-dpc tissues examined (data not shown except for testis and liver). Thus we confirmed previous reports that expression of Sry is restricted to limited tissues and to a limited period of time. We then examined Sry expression in primary cultured gonadal cells of the 11.5-dpc fetus (pgc11.5). As expected, Sry expression was observed throughout the culture period. After 24 h of culture, however, expression became very weak and was barely detectable at 48 h of culture (Fig. 2B). We also confirmed the presence of transcript by RT real time quantitative PCR (Fig. 2C). No transcript was detectable in fetal liver cells or NIH 3T3 cells. The kinetics of Sry expression in vitro recapitulated the transient expression of Sry in vivo. Based on these results, it appears that pgc11.5 maintained normal transcriptional activity during the early culture period; therefore, it may be possible to evaluate promoter activity using pgc11.5.

The Promoter Activity of the Sry Gene—In this study, we established a reporter gene assay based on the finding that pgc11.5 expressed endogenous Sry in vitro. For the assay, six reporter constructs were made. These were (i) construct LC, containing the 4.4-kb genomic fragment (−4424 to −58) spanning promoter regions of both linear and circular forms, (ii) construct Lin, containing the 2.0-kb fragment (−2090 to −58) including the promoter region of the linear form, and (iii) construct Cir, containing the 2.4-kb fragment (−4424 to −2063) including the promoter of the circular form (Fig. 3A). The three fragments, oriented inversely, were used as controls for each construct (LC-as, Lin-as, and Cir-as). The construct 4.4kb-cont, containing the 4.4 kb of rat Dmmt1 cDNA fragment with supposedly no transcription regulatory activity, was also used as a control.

Although activity of the pGL3-Basic vector was not significantly different from that of 4.4kb-cont both in fetal liver cells and in NIH 3T3 cells, it was exceptionally higher in pgc11.5 (data not shown). Therefore, 4.4kb-cont was considered to be a more adequate control than the pGL3-Basic vector. LC showed an equivalent expression level (104%) to 4.4kb-cont, whereas activity of LC-as was less than 45% of that of 4.4kb-cont (Fig. 3B) in pgc11.5. The activities of both LC and LC-as in fetal liver and NIH 3T3 cells were also less than 50% of 4.4kb-cont. These results suggest that the 4.4-kb upstream region of the Sry gene contains suppressive element(s) and that the gonadal cells of the 11.5-dpc fetus are capable of inducing Sry expression despite suppressive activity. Then activities of the linear and circular promoter regions were examined separately (Fig. 3, C and D). In pgc11.5, Lin showed −83% expression level of LC, and activity of Cir was strongly repressed. Conversely, activity of Lin-as was barely detectable, and that of Cir-as was suppressed to a level comparable with that of LC-as. In fetal liver cells and NIH 3T3 cells, expression of all constructs was strongly suppressed. Therefore, both linear and circular promoter regions likely contain suppressive element(s). The 2.0-kb upstream region of the linear form transcription start site contains expression-inducing element(s). However, the 2.4-kb upstream region of the circular form transcription start site demonstrated cryptic promoter activity in the inverse direction in pgc11.5.

DNA Methylation Suppressed Sry Activity—Next, we examined whether DNA methylation affects promoter activity. In vitro methylation of LC-as strongly inhibited activity in pgc11.5 (Fig. 4A, columns 1 and 2). Activity of LC also was suppressed by DNA methylation to the level of LC-as (Fig. 4A, columns 3 and 4). Likewise, in vitro methylation repressed reporter activity of both Lin and Lin-as in pgc11.5 (Fig. 4B, columns 1-4). In the circular promoter region, promoter activity was difficult to detect, except for Cir-as in pgc11.5, and DNA methylation also suppressed this activity (Fig. 4C). In any case, DNA methylation clearly suppressed activities of all constructs. Given these results, hypomethylation likely is required to sustain Sry expression in gonadal cells.

Tissue- and Stage-specific Changes in DNA Methylation Status of the Putative Promoter Regions of the Sry Gene—The DNA
methylation status of Regions I and II during development was analyzed by sodium bisulfite restriction mapping (Fig. 5A). Bands representing the methylated forms predominated in 8.5-dpc embryos and 11.5-dpc fetal liver for both Region I and Region II. Region II was also predominantly methylated in the fetal testis and liver at 15.5 dpc. It should be noted that both regions in 11.5-dpc gonad were predominantly unmethylated. Semiquantitative analysis of the band image by NIH Image software (version 1.6.1) revealed that the DNA methylation level of Region I was less than 3% at the blastocyst stage (Fig. 5B). Methylation level dramatically increased to more than 75% in the 8.5-dpc embryo. The hypermethylated status was maintained in liver of the 11.5-dpc fetus (62.0%), while the level was severely decreased to 3.0% in the gonad. Region II was also hypomethylated in the gonad at 11.5 dpc (2.3%). These results indicate that the Sry gene has T-DMRs in the putative promoter regions. Given that gonadal expression of Sry is restricted to a defined developmental period peaking at 11.5 dpc, expression is likely associated with hypomethylation.

FIG. 5. DNA methylation status of Regions I and II at different developmental stages. A, bisulfite restriction mapping. Region I and Region II of blastocyst and fetal tissues were amplified by genomic PCR following sodium bisulfite reaction, and then the PCR products were digested with TaqI or HpyCH4 IV restriction enzyme. When the genomic DNA contains methylated CpG in the recognition sites, the PCR products are fragmented (Me), whereas the PCR products from the unmethylated DNA are intact (U) (shown in Fig. 1B). U, uncut; D, digested with restriction enzyme; M, DNA 100-bp ladder marker; Con, adult testis genomic DNA used as a control. B, methylation ratio was estimated based on the intensity of each band after restriction mapping. All results are shown as the means ± S.E. Experiments were performed at least three times in each case.

We further analyzed the methylation status of each CpG in the T-DMRs of the Sry gene by sodium bisulfite sequencing. As expected, methylation status of almost all CpGs in Regions I and II corresponded with the results of restriction mapping (Fig. 6). These CpGs were unmethylated at the blastocyst stage, while they were heavily methylated in 8.5-dpc embryos. The hypermethylated status was maintained in 11.5-dpc fetal liver. In contrast, with all but one exception, these CpGs in gonadal somatic cells as well as in the gonads were unmethylated at 11.5 dpc. Thus we confirmed that CpGs in the 5'-upstream region of Sry are methylated/demethylated in a tissue- and temporal-specific manner.

DISCUSSION

A reporter assay for Sry gene transcription has not been developed previously due to unavailability of adequate cell lines and low activity of the intrinsic promoter. In this experiment, we established the Sry promoter assay system and showed that the 4.4-kb upstream region of the Sry gene, even though its activity was very weak, has promoter activity and contains suppressive element(s). Results presented here suggest that there is a positive regulatory element of Sry expression within the 2.0-kb upstream region of the linear form transcript. Also, there are at least two negative regulators, one in the same region as the positive regulatory element in Lin and the other in the upstream region of the circular form transcript. The relatively high activity of Cir-as may be due to cryptic promoter activity residing in the inverted repeat sequence. The positive and negative elements remain to be identified.

The relationship between the developmental kinetics of Sry expression and DNA methylation status is illustrated in Fig. 7. Regions I and II are hypomethylated in the blastocyst, and then they become hypermethylated by 8.5 dpc. By 11.5 dpc, Regions I and II become hypomethylated again in the gonad, while the hypermethylated level is maintained in other tissues. Importantly, expression of Sry occurs in association with temporal hypomethylation in the sex-determining period. After this period, Region II becomes hypermethylated again, although Region I remains hypomethylated. The hypomethylation status of Region I may not have functional
DNA Methylation-mediated Gene Silencing of Sry

It has been proposed that vertebrate DNA methylation mainly has a protective role in limiting the expression of foreign DNA elements and endogenous transposons (36). Induction of human SRY expression in a human prostate cancer cell line treated with a DNA methylation inhibitor, 5-aza-dC, has also been reported (37). Sry has no introns in human and mouse, implying that the gene might have been integrated into the chromosome by a retro-transposition event during evolution. If this were the case, such genes might be targets for DNA methylation to repress ancestral retro-transposition of the genes. In the HMG-box domain of Sry, there is high homology between several species, but curiously, most of the nucleotide sequences are not conserved in the 5′-flanking region (14). However, importantly, the CpG-rich region in the 5′-flanking region is exceptionally conserved in animals including human and bovine (data not shown). The unique feature of DNA methylation of the Sry gene provides a clue for evolutionary initiation and consequences in acquiring and establishing the epigenetic system for gene regulation and sex determination in mammals.

Sry is the gene encoding the master protein initiating the cascade of testicular differentiation. We found previously that there are numerous T-DMRs, which are widespread in the mammalian genome (25). Analysis of several stem cells, somatic cells, germ cells, and tissues of the mouse revealed that the DNA methylation pattern is specific to cell type. Differentiated cells must accurately replicate the DNA methylation pattern on the newly synthesized DNA strand to maintain the cellular phenotype during cell proliferation. We have proposed that genome-wide methylation may be maintained through the formation of methyl-CpG-binding protein 2 (MeCP2)-DNA methyltransferase 1 (Dnmt1) complexes at the replication foci (38). For differentiation, in contrast, cells must change the DNA methylation pattern through demethylation (25–27). Sry is required for the differentiation of Sertoli cells to induce masculinization of the embryonic gonad. Thus, DNA methylation is an important system for Sry regulation and cellular differentiation.

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The Sry T-DMRs are demethylated in developing gonadal cells during a limited period, while they are heavily methylated in other somatic cells. Release from CpG methylation-mediated gene silencing appears to allow gonadal somatic cells to express Sry during the sex-determining period. It is clear that stage-specific demethylation of the 5′-flanking region, particularly Region II, appears to be required for Sry expression in utero. Thus, there is a time window of DNA demethylation of the promoter region of linear Sry during the sex-determining period. In addition, the observation that DNA methylation suppressed the promoter activity of the Sry gene supports a role for DNA methylation in gene regulation. Based on these results, we propose that Sry gene expression is regulated epigenetically by DNA methylation-mediated gene silencing.

The Sry T-DMRs are demethylated in developing gonadal cells during a limited period, while they are heavily methylated in other somatic cells. Release from CpG methylation-mediated gene silencing appears to allow gonadal somatic cells to express Sry during the sex-determining period. It is clear that stage-specific demethylation of the 5′-flanking region of the Sry gene is a critical mechanism for Sry expression. Interestingly, DNA methylation markers on imprinted genes such as Igf2, Igf2r, H19, and Snrpn are erased at 11.5–12.5 dpc (31–34). The demethylation kinetics of the Sry gene were similar to that of imprinted genes; therefore, the temporal demethylation mechanism seems to apply to several genes associated with development of germ cells and the gonad.

Most studies on DNA methylation-mediated gene repression have dealt with TATA-less and GC-rich promoters (17, 23, 35). However, we have revealed previously that exclusive expression of the rat placental lactogen 1 (rPL-I) gene, which has a TATA box and poor CpG sites in the upstream region, is under the control of DNA methylation (22). Thus, tissue-specific and developmentally regulated genes with few CpGs are also targets of DNA methylation-mediated control.
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