DNA Hypomethylation and Germ Cell-specific Expression of Testis-specific H2B Histone Gene*

(Received for publication, July 1, 1991)

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Testis-specific H2B (TH2B) histone gene of rat is expressed during meiotic event of spermatogenic differentiation. The gene is unusual in that it has conserved the regulatory elements involved in the S phase-specific transcription of somatic H2B genes as well as the S phase-specific stabilization of histone mRNA. Genomic sequencing revealed that all analyzed CpG sites in the promoter region of TH2B gene are methylated in somatic tissues but not in testis. During spermatogenesis, these CpG sites are unmethylated as early as spermatagonia type A and up to sperm. Thus, there is a good correlation between DNA hypomethylation and germ cell-specific expression of TH2B gene. Results obtained from in vivo DNase footprinting and DNA mobility shift experiments are consistent with the hypothesis that DNA methylation inhibits gene activity by preventing the binding of transcription factors to their recognition sequences. The results show that (i) the binding of ubiquitous transcription factors to the promoter region of TH2B gene may be blocked in nuclei of liver, and (ii) DNA methylation can directly interfere with the binding of transcription factors recognizing a hexamer (ACGTCA) motif. In vitro DNA methylation and transfection experiments demonstrated that expression of TH2B gene is inhibited by DNA methylation in vivo. These findings indicate that DNA methylation may play a key role in the transcriptional repression of germ cell-specific TH2B gene.

Spermatogenesis is a complex developmental process which involves a sequential differentiation of spermatogonia to produce spermatozoa through mitosis, meiosis, and spermiogenesis. During meiotic phase of spermatogenesis, testis-specific variant H1 and core histones are synthesized, and replace their somatic histones (Meistrich et al., 1989). Although RNA analyses revealed the cell type-specific expression of somatic H2B genes as well as the S phase-specific stabilization of histone mRNA. Genomic sequencing revealed that all analyzed CpG sites in the promoter region of TH2B gene are methylated in somatic tissues but not in testis. During spermatogenesis, these CpG sites are unmethylated as early as spermatagonia type A and up to sperm. Thus, there is a good correlation between DNA hypomethylation and germ cell-specific expression of TH2B gene. Results obtained from in vivo DNase footprinting and DNA mobility shift experiments are consistent with the hypothesis that DNA methylation inhibits gene activity by preventing the binding of transcription factors to their recognition sequences. The results show that (i) the binding of ubiquitous transcription factors to the promoter region of TH2B gene may be blocked in nuclei of liver, and (ii) DNA methylation can directly interfere with the binding of transcription factors recognizing a hexamer (ACGTCA) motif. In vitro DNA methylation and transfection experiments demonstrated that expression of TH2B gene is inhibited by DNA methylation in vivo. These findings indicate that DNA methylation may play a key role in the transcriptional repression of germ cell-specific TH2B gene.

Previously, we have isolated TH2B1 gene from the testis of rat (Kim et al., 1987). Our previous studies showed that TH2B gene is expressed only in testis, in contrast to its counterpart, somatic H2B (sH2B) histone gene, which is expressed in S phase during the cell cycle of mitotically replicating cells (Kim et al., 1987; Schumperli, 1988). While transcriptional regulation of the TH2B gene appears to be different from that of the sH2B gene, the sequence organization of TH2B regulatory region shows a remarkable similarity to that of sH2B gene; octamer (ATTTTGCAT) and CCAAT sequence elements which have been shown to be important for the maximal S phase-specific transcription of sH2B genes are contained in the promoter of TH2B gene (Hwang and Chae, 1989; Fletcher et al., 1987; LaBella et al., 1988). In addition, the gene contains the elements involved in S phase-specific processing and stabilization of histone mRNA (Heintz et al., 1983; Sittman et al., 1983; Stauber et al., 1986). Indeed, gene transfer experiments showed that a cloned copy of TH2B gene is efficiently expressed in parallel with DNA replication and the mRNA declined concomitantly with decline of DNA synthesis toward the end of S phase as sH2B gene in somatic cells where the expression of endogenous TH2B gene is repressed (Hwang and Chae, 1989). These previous findings suggested that TH2B gene is potentially expressible in various tissues, but the S phase-specific regulatory elements in the promoter of TH2B gene is somehow repressed in non-expressing somatic tissues.

Tissue-specific expression of eukaryotic genes may be controlled by the tissue-specific interaction of trans-acting factors with their cis-acting elements. Numerous studies have suggested that DNA methylation and/or chromatin structure is an important regulatory element in this process (reviewed by Cedar, 1988). It appears that the inhibitory effect of DNA methylation and/or chromatin structure is mediated by preventing the binding of transcription factors to their target sequences (Becker et al., 1987; Watt and Molly, 1988; Iguchi-Arigo and Schaffner, 1989). Therefore, one possible explanation for the repression of TH2B gene is that the binding of ubiquitous transcription factors to the promoter of TH2B gene is blocked by DNA methylation and/or chromatin structure in non-expressing somatic tissues.

As a first step for studies on the role of chromatin structure in the transcriptional regulation of TH2B gene, we have investigated methylation of TH2B promoter as markers for active and inactive state of TH2B gene in chromatin in different tissues. We report here that there is a strong correlation between DNA hypomethylation and the germ cell-specific expression of TH2B histone gene. Genomic sequencing revealed that all analyzed CpG sites in the promoter region of TH2B gene are methylated in both male and female somatic tissues, but not in testis. In vivo footprinting and mobility shift experiments showed that the binding of ubiquitous transcription factors to the promoter region of TH2B gene may be blocked by DNA methylation in somatic tissues. Transf-
tion of in vitro methylated TH2B gene into somatic cells demonstrated that TH2B gene is inactivated by DNA methylation in both transient and long-term expression systems. These findings may explain how apparently the promoters of Hh2B and TH2B genes can be regulated in an opposite way in the same cell types. Transcription factors which are available for the transcription of the Hh2B genes as well as episomal copies of TH2B gene appear to be inaccessible to the methylated promoter region of endogenous TH2B gene. Also, our findings may provide a possible explanation for the evolutionary conservation of S phase-specific regulatory sequence elements in the promoter of TH2B gene which is mainly expressed in the absence of DNA replication.

**EXPERIMENTAL PROCEDURES**

Source of DNAs and RNAs—Sprague-Dawley rats were used. Somatic tissues were obtained from 3-4-week-old rats. Testes were obtained from various ages (6-14, 21, 28, and >60 days) of rats. Spermatozoans were isolated from the cauda epididymis and were defrosted in mannitol (900 mM) 2-3 days according to O'Brien and Bellvé (1980). Testicular cells in different stages of spermatogenesis were separated by sedimentation at unit gravity through a 2-4% gradient of bovine serum albumin using a Celsep apparatus as described previously (Bellvé et al., 1977; Wolgemuth et al., 1985). Five fractions of spermatogenic cells were isolated and combined. Elongated and elongated spermatids were isolated from testes of mature rats (>60 days), and spermatogonia type A and Sertoli cells from testes of Day 8 prepubertal rats. Embryo, placenta, and yolk sac were prepared from the uteri of normally mated female rats at day 14 of gestation. High molecular weight genomic DNA was isolated from purified nuclei as described by Saluz and Jost (1987). DNA was isolated from sperm nuclei as described previously (Shiurba and Nandi, 1979). Nuclei from various tissues were prepared according to Barberis et al. (1987), and nuclei from fractionated spermatogenic cells were isolated as described previously (Weintraub and Groudine, 1976). Total RNA was prepared from fractionated spermatogenic cells according to the method of Chomczynski and Sacchi (1987).

**Genomic Sequencing**—The genomic sequencing was performed essentially as described by Saluz and Jost (1989). Briefly, the purified genomic DNA was digested overnight at 37 °C with MspI restriction enzyme (2 units/µg of DNA) following the recommendations of the supplier (Promega Co.). The digested DNA was treated with RNase A, sevag extracted, precipitated by ethanol, and resuspended at 5 µg/µl in H2O. Fifty µg of DNA were then subjected to a partial chemical cleavage at cytosine residues as described by Maxam and Gilbert (1977) and Saluz and Jost (1987). After complete removal of piperidine by repeated lyophilization in 100 µl of H2O, the sequencing DNA was sized using an Applied Biosystems 380B oligonucleotides synthesizer. The labeled, single-stranded sequencing primer (27-mer) was then separated from the template (33-mer; 5'-AGA-CGT-TGG-AGT-GAA-GCA-CTT-TAT-CTT-AAC-CTA-ACT-G-3') by annealing a short oligonucleotide (9-mer; 5'-AAA-ATA-AGT-3') to the underlined complementary sequences in the template (33-mer; 5'-AGA-CGT-TGG-AGT-GAA-GCA-CTT-TAT-CTT-AAC-CTA-ACT-G-3'). The radiolabeled, single-stranded sequencing primer (27-mer) was then separated from the template (33-mer) by electrophoresis through a 15% polyacrylamide sequencing gel in 7 M urea. The gel was fixed, dried, and autoradiographed for 1-3 days at -80 °C.

**Mobility Shift Assay**—Methylated and unmethylated oligonucleotides (40 bp) containing a hexamer element (ACGTCA) were synthesized using an Applied Biosystems 380B oligonucleotides synthesizer. For the synthesis of methylated oligonucleotides, 5'-cytosine-phosphoamide replaced cytosine at four CpG sites indicated in bold type (upper strand, 5'-CAT-CTT-TGG-CGT-CAT-ACT-GGC-TAC-TCCA-GG-CCC-GGC-TCC-3'). Methylated and unmethylated DNA probes were prepared by annealing end-labeled, single-stranded oligonucleotides. Binding reactions (20 µl) were carried out on ice for 15 min in 5 µl of reaction buffer, 100 µl of buffer (10 mM Tris-Cl, pH 7.4, 0.1% SDS, and 0.1 M NaCl, 3 mM MgCl2) containing 0.25 µM sucrose and 0.1 µM phenylmethylsulfonyl fluoride. DNAase I digestion was carried out at a concentration of 2 µg/ml at 37 °C for increasing times. Digestion of nuclei was then terminated by the addition of an equal volume of stop solution (20 mM HEPES, pH 7.5, 20 mM EDTA, 1% SDS, proteinase K (600 µg/ml) followed by incubation at 37 °C overnight. As a control for sequence specificity of DNAase I, deproteinized genomic DNA (50 µg) of liver and testis was digested with 0.1 µg/ml of DNAase I at 37 °C for 3 min. DNAase I-treated genomic DNA was purified by phenol-chloroform extraction, and precipitation by ethanol, and 50 µg of purified DNA was digested with MspI restriction enzyme. PCR amplification was carried out as described above. Amplified samples were analyzed on a 6% denaturing polyacrylamide gel (5 X 3 X 0.88 cm) in 0.5 X TBE and dried gels were exposed to x-ray films in the presence of an intensifying screen for 1-3 days at -80 °C.

**In Vivo Footprinting**—In vivo DNase I footprinting was performed on isolated nuclei from liver and testis of adult rats. Nuclei were purified by the method of Barberis et al. (1987), and resuspended at a concentration of 106 nuclei/ml in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2) containing 0.25 µM sucrose and 0.1 mM phenylmethylsulfonyl fluoride. DNAase I digestion was carried out at a concentration of 2 µg/ml at 37 °C for increasing times. Digestion of nuclei was then terminated by the addition of an equal volume of stop solution (20 mM HEPES, pH 7.5, 20 mM EDTA, 1% SDS, proteinase K (600 µg/ml) followed by incubation at 37 °C overnight. As a control for sequence specificity of DNAase I, deproteinized genomic DNA (50 µg) of liver and testis was digested with 0.1 µg/ml of DNAase I at 37 °C for 3 min. DNAase I-treated genomic DNA was purified by phenol-chloroform extraction, and precipitation by ethanol, and 50 µg of purified DNA was digested with MspI restriction enzyme. PCR amplification was carried out as described above. Amplified samples were analyzed on a 6% denaturing polyacrylamide gel (5 X 3 X 0.88 cm) in 0.5 X TBE and dried gels were exposed to x-ray films in the presence of an intensifying screen for 1-3 days at -80 °C.

**Blot Experiments**—RNA samples were size-fractionated on a 1.5% agarose/formaldehyde gel and transferred to GeneScreen™ membrane (Ontario New Brunswick Nuclear). The filters were hybridized to a 32P-labeled probe for 2 h at 68 °C in 5 X SSPE (1 X SSPE = 0.18 M NaCl, 10 mM NaPO4, and 1 mM EDTA) containing 5 X Denhardt's solution, 1% SDS, 200 µg/ml denatured Salmon sperm DNA. After hybridization, the blots were washed twice at room temperature for 10 min in 2 X SSC (standard concentration of NaCl and NaPO4) and 1% SDS and twice at 65 °C for 1 h in 1 X SSC, 0.1% SDS. The probe which is a 180 bp EcoRI/MspI DNA fragment containing the 5'-
untranslated region of TH2B gene (Fig. 1a) was labeled with \([\alpha^{32}\text{P}]\) dCTP (3000 Ci/mmol) by random-primer extension to a specific activity of \(5 \times 10^6\) cpm/\(\mu\)g of DNA (Feinberg and Vogelstein, 1983). For DNA blot analysis, genomic DNA was digested with restriction enzymes (10 units/\(\mu\)g) for 16 h. The digested DNA was then electrophoresed on 1% agarose gel and transferred to GeneScreenTM nylon membrane. Pre-hybridization, hybridization, and washing were done under the same conditions as Northern blots. The probe was a 310-bp Hinfl-Mspl DNA fragment recognizing the intergenic sequences between TH2A and TH2B genes.

**RESULTS**

**Tissue-specific Methylation Patterns of CpG Sites in the Promoter Region**—There are 10 CpG dinucleotides in the 5'-untranslated region shared by TH2A and TH2B genes (Fig. 1a). Since no MspI and HpaII site (CCGG) for methylation analysis is present in this region, genomic sequencing was used to determine the methylation patterns of five CpG sites in the promoter region (within \(-140\) bp) of TH2B gene in DNA from various tissues. We have previously shown that the minimal promoter of TH2B gene is contained within 140 bp from the transcription initiation site (Hwang and Chae, 1989). The sequence elements and the CpG sites present in this region is shown in Fig. 1b. Fig. 2 shows the genomic sequencing analysis of the top (sense) strand of the TH2B promoter region. Since 5-methylcytosine does not react with hydrazine in the chemical sequencing reaction (Ohmori et al., 1978; Miller et al., 1978), the absence of the corresponding band in the sequencing ladder is indicative of the methylation cytosine. Fig. 2 shows that all five CpG sites are fully methylated in both male and female somatic tissues, but unmethylated in the enriched fraction of pachytene spermatocytes and the whole testis. Also, three CpG sites on the top strand in the promoter of TH2A and five CpG sites on the bottom strand in the 5' body of TH2B gene (Fig. 1) showed the same tissue-specific methylation patterns (data not shown). Thus, these results reveal that there is a good correlation between DNA hypomethylation and germ-cell specific expression of TH2B gene.

**Methylation Patterns of CpG Sites in the Promoter Region in Testicular, Embryonic, and Extraembryonic Cells**—In order to determine when the unmethylated pattern of the TH2B promoter has been established during spermatogenesis, DNA was isolated from germ cells at various stages of spermatogenesis using a Celsep apparatus. The analyzed cell types included spermatogonia type A, early spermatids, and spermatozoa. Since previous studies have suggested that there is substantial less DNA methylation in extraembryonic tissues than in embryonic and adult somatic tissues (Razin et al., 1984; Young and Tilghman, 1984), we have also examined the methylation patterns of the TH2B promoter in DNA from embryonic and extraembryonic tissues (yolk sac and placenta) at day 14 of gestation. However, the TH2B gene was only methylated in all analyzed germ cells, while in Sertoli cells which are somatic cells in testis the same sites were fully methylated. Relatively weak bands in the lane of spermatogonia type A were due to the lower purity of the isolated cell fraction. These results indicate that the promoter region of TH2B gene was unmethylated as early as spermatogonia type A and up to spermatozoa during spermatogenesis. Since previous studies have suggested that there is substantially less DNA methylation in extraembryonic tissues than in embryonic and adult somatic tissues (Razin et al., 1984; Young and Tilghman, 1984), we have also examined the methylation patterns of the TH2B promoter in DNA from embryonic and extraembryonic (yolk sac and placenta) tissues at day 14 of gestation. However, the TH2B gene was fully methylated in both embryonic and extraembryonic tissues. Thus, TH2B gene is methylated in somatic tissues as early as day 14 of gestation.

**Changes in the TH2B mRNA during the Prepubertal Development of Rat Testis**—Since the promoter region of TH2B gene is unmethylated in all analyzed spermatogenic cells and our previous in situ cytohybridization results showed that TH2B gene is expressed only in pachytene spermatocytes (Kim et al., 1987), there seemed to be a temporary uncoupling of the relationship between DNA hypomethylation and TH2B
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**FIG. 3.** Methylation patterns of five CpG sites in the promoter region of TH2B gene in testicular, embryonic, and extraembryonic cells. Testicular cells in different stages of spermatogenesis were fractionated as described under “Experimental Procedures.” Embryo, placenta, and yolk sac were prepared from the uteri of normally mated female rats at Day 14 of gestation. From these cells, high molecular weight genomic DNA was isolated and genomically sequenced as described under “Experimental Procedures.” Arrowheads indicate the positions of cytosine residues in CpG dinucleotides (Fig. 1).

**FIG. 4.** Changes in the TH2B mRNA during the prepubertal development of rat testis. Panel A, total RNA (15 μg) isolated from thymus (lane T) and the testes of rats at 6, 8, 10, 12, and 14 days after birth (age matches numbers above each lane) was analyzed by RNA blot hybridization as described under “Experimental Procedures.” Panel B, total RNA (10 μg) was purified from the enriched fraction of spermatogenic cells and subject to Northern blot analysis. S, Sertoli cells (~95%); SA, spermatogonia type A (~60%); P, pachytene spermatocytes (~90%); ES, elongated spermatids (~95%); TE, total RNA from 30-day-old rats.

**FIG. 5.** In vivo footprinting experiments reveal testis-specific protein-DNA interactions in the promoter region of TH2B gene. Nuclei from liver and testis were digested with DNase I for increasing times (lanes 1, 1 min; 2, 2 min; 3, 4 min; 4, 8 min; 5, 16 min). As a control for the background due to cleavages by endonucleases, nuclei were incubated at 37 °C for 1 min in the absence of DNase I (lane 0). The probe used to visualize the genomic sequences is depicted in Fig. 1. Control reactions with protein-free genomic DNA are also shown (lane F). A size marker, the pT7 plasmid containing TH2B gene was sequenced at cytosine residues (lane C). Cap site and the location of regulatory sequences in the TH2B promoter are indicated on vertical lines; TATAA (~26 to ~30), hexamer (~46 to ~51), CCAAT (~71 to ~75), and octamer (~93 to ~100). DNase I-sensitive sites and the protected sequences are indicated by arrows and open arrowheads, respectively. Numbers on the left side indicate the distance from the transcription initiation site of TH2B gene in base pairs.
Methylation at a CpG Site within the Hexamer Sequence Inhibits the Binding of Transcription Factors—To determine whether DNA methylation can directly interfere with the binding of transcription factors to the TH2B promoter region, we have performed mobility shift assay with methylated synthetic oligonucleotides. Since there is a CpG dinucleotide within the hexamer sequence element (ACGTCA) and in vivo footprinting analysis showed that the binding of transcription factor(s) to this element may be blocked in liver, the hexamer sequence element was chosen for this analysis. The methylated oligonucleotides (40-mer) containing four CpG dinucleotides of which a CpG site is located within the hexamer motif, 100-fold molar excess of DNA fragment containing base substitutions in the hexamer motif, 100-fold molar excess of competitor. The methylated oligonucleotide (40-mer) corresponded to the site containing the hexamer motif, 100-fold molar excess of DNA fragment containing base substitutions in the hexamer motif (ACGTCA to ACTCGA) was included in all reaction mixtures. In fact, the addition of the DNA fragment resulted in the subtraction of several protein-DNA complexes which may recognize the sequences other than hexamer motif in the oligonucleotide probe (data not shown). The reaction mixture assayed in lane F did not contain nuclear extracts. Arrowhead, protein-DNA complex; P, free oligonucleotide probe.

In Vitro DNA Methylation Inhibits Gene Expression in Vivo—To obtain more direct evidence for the role of DNA methylation in the repression of TH2B gene, we have performed in vitro DNA methylation and transfection experiments. The pTHAB plasmid containing TH2B gene was methylated in vitro at all cytosine residues in CpG dinucleotides using SsSI methylase, and the effect of DNA methylation on TH2B transcription was assayed by both transient and stable transfection experiments. In a transient assay, the methylated or nonmethylated TH2B constructs was introduced into mouse C3H 10T1/2 cells with RSVneo as a cotransfection control. Transfected cells were synchronized and S phase cells were harvested 4 h after release from the aphidicolin block at which time TH2B mRNA reaches its maximum level (Hwang and Chae, 1989). RNA was extracted from both S and non-S phase cells, and the expression of TH2B gene was analyzed by Northern blot experiments. Fig. 7 shows that TH2B gene was totally inactivated by DNA methylation, whereas nonmethylated TH2B gene is expressed efficiently in an S phase-dependent manner in somatic cells. To further confirm that DNA methylation causes inhibition of TH2B transcription, the same constructs were stably cotransfected into mouse C3H 10T1/2 cells using the neo gene as a selective marker. DNA and RNA samples were prepared from pools of 200–400 stable clones and subjected to Southern and Northern analysis. Methylation patterns of TH2B gene were determined by restriction analysis with methylation-sensitive enzyme HpaII and subsequent blot analysis. Fig. 8A shows that DNA from stable clones containing the methylated TH2B construct is poorly cleaved by HpaII enzyme, indicating that the methylation patterns were faithfully maintained in those cells. On the other hand, a 408-bp band was detected following HpaII digestion of DNA from cells containing the nonmethylated TH2B construct, indicating that these restriction sites remain hypomethylated during cell division. Somatic tissue-specific methylation of TH2B gene is also shown in Fig. 8A and further confirmed by additional blots (data not shown). In our initial experiments, we observed that the growth of stable clones containing TH2B construct was not appreciable for about 4–5 days after pools of stable clones were replated, and further maintenance in a selective medium resulted in the selective enrichment of cells expressing TH2B gene at a low level, suggesting that the production of TH2B gene is testis-specific. Since our in vitro footprinting analyses have previously shown that the transcription factors recognizing CCAAT, hexamer, and octamer sequence elements in the TH2B promoter are ubiquitously present in various tissues (Hwang et al., 1990), it is likely that the binding of transcription factors to the TH2B promoter, notably to CCAAT and hexamer sequence elements, is somehow blocked in liver, but not in testis. We also observe protected regions in DNase I digestion patterns of samples from liver. It is possible that the methylated promoter region of TH2B gene in somatic cells is not free as in naked DNA, but occupied by certain proteins, probably structural proteins, thereby excluding the binding of transcription factors to the TH2B promoter. For the analysis of footprints from germ cells, the whole testis containing both somatic (mainly Sertoli) and germ cells was used. However, it is unlikely that the observed footprint patterns have been obtained from Sertoli cells since (i) footprints from the testis of 21-day-old rats in which more than 70% of cells are germ cells are essentially the same as those from testis of adult rat, while footprints from the testis of 7-day-old rats which contains mainly Sertoli cells (~85%) exhibit DNase I digestion patterns similar to those of liver (data not shown), and (ii) the promoter of TH2B gene in Sertoli cells which are somatic cells in testis is heavily methylated as in liver (Fig. 3).

**FIG. 6.** Effect of CpG methylation on the binding of transcription factors recognizing hexamer (ACGTCA) motif. Nuclear extracts from liver and testis were incubated with methylated or unmethylated 32P-labeled, double-stranded oligonucleotides (unmethylated, UM; methylated, M) with (+) or without (−) 100-fold excess of competitor. The methylated oligonucleotide (40-mer) corresponding to the region spanning the nucleotides from −30 to −69 contains four methylated cytosines at positions of −34, −50, −60, and −62 on the top strand (Fig. 1). To increase the specificity of the binding toward the site containing the hexamer motif, 100-fold molar excess of DNA fragment containing base substitutions in the hexamer motif (ACGTCA to ACTCGA) was included in all reaction mixtures. In fact, the addition of the DNA fragment resulted in the subtraction of several protein-DNA complexes which may recognize the sequences other than hexamer motif in the oligonucleotide probe (data not shown). The reaction mixture assayed in lane F did not contain nuclear extracts. Arrowhead, protein-DNA complex; P, free oligonucleotide probe.

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histone protein could be toxic to somatic cells. Therefore, pools of stable clones were allowed to grow for only 3 days before cells were synchronized, released, and harvested. Nevertheless, the average number of integrated TH2B copies (~2 copies) was much lower in stable clones transfected with nonmethylated TH2B construct compared to that in clones containing methylated construct (~30 copies). Although more copies are present in stable clones carrying methylated TH2B construct, it is clear that most of them were severely inactivated by DNA methylation (Fig. 8B).

**DISCUSSION**

Our results presented here indicate that DNA methylation may play a causal role in the repression of TH2B gene in somatic cells. In *vitro* methylation of TH2B gene led to the complete repression of TH2B transcription in *vitro* in gene transfer experiments. In *vitro* footprinting experiments revealed that the binding of ubiquitous transcription factors to the promoter region of TH2B gene is somehow blocked by DNA methylation in non-expressing somatic tissues. Moreover, DNA mobility shift experiments showed that DNA methylation can directly interfere with the binding of transcription factor(s) to the hexamer motif (ACGTCAT). Such direct effects of DNA methylation on the binding of transcription factor(s) have been reported from studies on Hela cell transcription factors which bind to the adenovirus major late promoter (Kovesdi et al., 1987; Watt and Molloy, 1988), the factor binding to the late E2A promoter (Hermann et al., 1989), a specific factor binding to the CAMP responsive sequence element (Iguchi-Ariga and Schaffner, 1989), and a transcription factor of the rat tyrosine aminotransferase gene (Becker et al., 1987). In addition to the direct inhibitory effect of DNA methylation, transfection and microinjection experiments have suggested that the inhibitory effect of DNA methylation may be indirectly mediated by inducing or stabilizing an inactive chromatin structure (Keshet et al., 1986; Buschhausen et al., 1985, 1987). More recently, Bird and co-workers (Meehan et al., 1989; Antequera et al., 1989) described a methyl-CpG-binding protein (MeCP) which binds to methylated DNA in *vitro*, and provided strong evidence that inhibitory effect of DNA methylation may be indirectly mediated by MeCP (Boyes and Bird, 1991). Therefore, it is likely that cytosine methylation may alter the affinity of DNA sequences for transcription factors or structural proteins, thereby inhibiting the binding of transcription factors to methylated DNA sequences directly, or indirectly by affecting the local chromatin structure surrounding the methylated DNA sequences. Although the direct inhibitory effect of DNA methylation has been demonstrated for the hexamer-binding proteins in this study, it is possible that the TH2B gene in somatic tissues is locked into an inactive chromatin structure since (i) DNA methylation may be unable to prevent the binding of other transcription factors to their recognition sequences such as CCAAT which do not contain CpG dinucleotides, and (ii) in *vitro* footprint patterns of liver nuclei showed several protected regions, notably around TATAA sequence and the cap site, which indicate that certain proteins, probably structural proteins, are bound to these regions.

Also, it is important to note that TH2B gene contains a CpG island which is characterized by a high G + C content and a high density of CpG dinucleotides (Fig. 1a). CpG islands

**Fig. 7. Effect of DNA methylation on TH2B transcription in transient assays.** Seven micrograms of methylated (+) and nonmethylated (−) TH2B constructs were transiently introduced into mouse embryo fibroblast C3H 10T1/2 cells with 5 µg of pRSVneo plasmid as a cotransfection control. The cells were synchronized, released, and harvested at 4 h after release from the aphidicolin block. The non-S phase cells were incubated for 24 h with aphidicolin. Total cellular RNA (15 µg) from S and non-S phase cells were fractionated on a 1.5% formaldehyde gel, blotted onto nylon membrane, and hybridized to 3²P-labeled probes specific for TH2B and neo transcripts. Transfection efficiencies among different dishes were normalized by the amount of neo transcript. The first lane on the left is a control RNA sample from the testes of 14-day-old rats.

**Fig. 8. Inhibition of TH2B transcription by DNA methylation in stably transfected clones.** Genomic DNA and total cellular RNA were isolated from stably transfected cells containing the methylated (+) or mock-methylated (−) pTHAB construct, and subjected to blot analyses. Panel A, methylation patterns of TH2B gene in tissues and stable clones. Methylation status of TH2B gene was determined by using methylation-sensitive enzyme HpaII and its isoschizomer MspI (both HpaII and MspI enzymes recognize the sequence CCGG, but HpaII cannot cleave CCGG). Total genomic DNA (15 µg) from liver and testis of adult rats or stably transfected clones was digested with HpaII and its isoschizomer MspI enzymes, fractionated on a 1.5% formaldehyde/agarose gel, blotted, and hybridized to the probe specific for TH2B transcript. RNA from the testis of 10-day-old rats was included as a positive control.
which are frequently found at the 5′ ends of most housekeeping genes and some tissue-specific genes are known to be methylation-free in many cell types (reviewed by Bird, 1986). When methylated in vitro and transfected into animal cells (Keshet et al., 1985; Boyes and Bird, 1991), or de novo methylated in many cell lines (Antequera et al., 1990), leading to the suggestion that methylation of CpG islands is incompatible with gene activity. Recently, it was shown that McCP-1 is involved in the methylation-mediated repression of four genes harboring CpG islands, favoring the indirect mechanism accounting for the inhibitory effect of DNA methylation (Boyes and Bird, 1991). Unlike many other genes with CpG islands including several testis-specific genes (Ariel et al., 1991), the TH2B gene is normally methylated in non-expressing somatic tissues. Therefore, it is possible that TH2B gene may be inactivated by DNA methylation indirectly via McCP-1 or similar proteins. If so, a high density of CpG dinucleotides in the body of TH2B gene may be important for the tight repression of TH2B gene in somatic cells.

Our analysis of the TH2B gene expression during spermatogenesis indicated that the TH2B gene is expressed at a relatively low level in pre-meiotic spermatogenic cells and the messenger RNA level is drastically elevated in pachytene spermatocytes. While it has been shown that the cooperative interaction between the CCAAT sequence element at −71 bp and the hexamer element at −46 bp may be involved in the transcription of TH2B gene (Antequera et al., 1990), it is not yet clear which transcription factors are involved in the expression of TH2B gene in spermatogenic cells. On the other hand, expression of TH2B gene appears to be independent of DNA replication in pachytene spermatocytes, in which very little DNA synthesis occurs (Chiu and Irvin, 1985; Meistrich, 1987). Also, synthesis of testis-specific histone variants is not affected by inhibitors of DNA synthesis (Chiu and Irvin, 1985). These suggest that different sets or forms of transcription factors might be involved in the expression of the TH2B gene in spermatogenic cells. While it has been shown that the cooperative interaction between the CCAAT sequence element at −127 bp and the octamer at −95 bp is important for the efficient transcription of cloned TH2B gene in mitotically replicating cells, it is possible that TH2B gene is also expressed in an S phase-dependent manner in spermatogonial cells. On the other hand, expression of TH2B gene appears to be independent of DNA replication in pachytene spermatocytes, in which very little DNA synthesis occurs (Chiu and Irvin, 1985). Also, synthesis of testis-specific histone variants is not affected by inhibitors of DNA synthesis (Chiu and Irvin, 1985). These suggest that different sets or forms of transcription factors might be involved in the expression of the TH2B gene in spermatocytes. While it has been shown that the cooperative interaction between the CCAAT sequence element at −71 bp and the hexamer at −46 bp may be involved in the transcription of TH2B gene in spermatogenic cells. Also, it is important to note that the level of TH2B mRNA is markedly increased in pachytene spermatocytes, indicating the presence of pachytene-specific enhancer and/or the stabilization of TH2B mRNA in pachytene spermatocytes. Another possibility is repression of TH2B gene expression in spermatogenic cells. We found that there is a protein factor which binds to a site between the TATA box and the transcription initiation site of TH2B gene in cells enriched with spermatogonia. Such a protein can interfere with the binding of RNA polymerase to the TH2B promoter. These possibilities are being tested in our laboratory by introducing the TH2BCAT reporter gene containing β-actin substitutions at different cis-acting sequences into primary culture of spermatocytes.

Our genomic sequencing analyses showed that TH2B gene is unmethylated as early as spermatogonia type A and up to sperm. In somatic tissues, the TH2B gene is methylated as early as 14 day of gestation. Thus, the methylation patterns of TH2B gene in somatic and germ cells appear to be determined during early embryogenesis. So far, only limited information is available for the DNA methylation events which occur during embryo development due to the limiting amount of embryo cells for the methylation analysis. One possibility is that TH2B gene in somatic cells, but not in germ cells, is selectively methylated during early embryogenesis, as suggested by Monk et al. (1987) from studies on overall DNA methylation in genomic DNA from early embryonic lineages.

TH2B gene is a variant H2B histone gene which is expressed only in testis. While the germ cell-specific expression of TH2B gene requires a quite different regulatory mechanism, the promoter and 3′-processing element of TH2B gene show a remarkable similarity to those of sH2B gene (Hwang and Chae, 1989). Also, TH2A and TH2B genes are closely associated like sH2A-H2B pair. These suggest that a TH2A-TH2B cluster might have been evolved from a somatic H2A-H2B pair by gene duplication event (reviewed by Maxon et al., 1985; D’Andrea et al., 1985). To accomplish germ cell-specific expression of both TH2A and TH2B genes, they might have exploited DNA methylation around their transcriptional sites rather than created new cis regulatory elements in the promoter region, as suggested by our results presented here. Also, the regulatory sequences of the testis-specific H1 (H1t) gene show a similarity to those found in somatic H1 genes (Cole et al., 1986). These suggest that testis-specific histone genes might share a common evolutionary pathway from their somatic counterparts and a common regulatory mechanism underlying the tissue-specific expression of these genes. Further experiments toward the characterization of chromatin structure surrounding TH2B gene and the identification of sequence elements necessary for the testis-specific expression of TH2B gene will provide important insights into the coordinate and germ cell-specific expression of testis-specific histone genes as well as the evolutionary relationship between testis-specific and somatic histone genes.

Acknowledgments—We thank Inhwan Hwang and Kyu Lim for many helpful discussions. Aphidicolin was provided by the Natural Products Branch, National Cancer Institute.}

REFERENCES

Antequera, F., Macleod, D., and Bird, A. P. (1989) Cell 58, 509-517
Antequera, F., Boyes, J., and Bird, A. (1990) Cell 62, 503-514
Ariel, M., McCarrey, J., and Cedar, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2317-2321
Barberis, A., Superti-Furga, G., and Bussinger, M. (1987) Cell 50, 347-359
Becker, P. B., Ruppert, S., and Schütz, G. (1987) Cell 51, 435-443
Bellve, A. R., Cavicchia, J. C., Millette, C. F., O’Brien, D. A., Bhatnagar, Y. M., and Dym, M. (1977) J. Cell. Biol. 74, 68-85
Bird, A. P. (1986) Nature 321, 209-213
Boyes, J., and Bird, A. (1991) Cell 64, 1123-1134
Buschhausen, G., Graessmann, M., and Graessmann, A. (1985) Nucleic Acids Res. 13, 5503-5513
Buschhausen, G., Wittig, B., Graessmann, M., and Graessmann, A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1177-1181
Cedar, H. (1988) Cell 53, 3-4
Chirgwin, J. M., Przybylsa, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
Chiu, M., and Irvin, J. L. (1985) Arch. Biochem. Biophys. 236, 260-265
Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-167
Cole, K. D., Kandala, J. C., and Kistler, W. S. (1986) J. Biol. Chem. 261, 7178-7183
D’Andrea, R. J., Coles, L. S., Lesnikowski, C., Tabe, L., and Wells, J. R. E. (1985) Mol. Cell. Biol. 5, 3108-3115

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Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6-13
Fletcher, C., Heintz, N., and Roeder, R. G. (1987) *Cell* 51, 773-781
Gardiner-Garden, M., and Frommer, M. (1987) *J. Mol. Biol.* 196, 261-282
Gorman, C. (1985) in *DNA Cloning: a Practical Approach* (Glover, D. M., ed) Vol. 2, pp. 143-190, IRL Press, Washington, D. C.
Gorman, C., Padmannabhan, P., and Howard, B. H. (1983) *Science* 221, 551-553
Heintz, N., Sive, H. L., and Roeder, R. (1983) *Mol. Cell. Biol.* 3, 539-550
Hermann, R., Hoeveler, A., and Doerfler, W. (1988) *J. Mol. Biol.* 210, 411-415
Hwang, I., and Chae, C.-B. (1989) *Mol. Cell. Biol.* 9, 1005-1013
Hwang, I., Kyu, L., and Chae, C.-B. (1990) *Mol. Cell. Biol.* 10, 585-592
Iguchi-Ariga, S. M. M., and Schaffner, W. (1989) *Genes & Dev.* 3, 612-619
Keshet, I., Yisraeli, J., and Cedar, H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 2560-2564
Keshet, I., Lieman-Hurwitz, J., and Cedar, H. (1986) *Cell* 44, 535-543
Kim, Y.-J., Hwang, I., Tres, L. L., Kierszenbaum, A. L., and Chae, C.-B. (1987) *Dev. Biol.* 124, 23-34
Kovesdi, I., Reichel, R., and Nevin, J. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 2180-2184
LaBella, F., Sive, H. L., Roeder, R. G., and Heintz, N. (1988) *Genes & Dev.* 2, 32-39
Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* 65, 499-560
Maxson, R., Cohn, R., Kedes, L., and Mohun, T. (1983) *Annu. Rev. Genet.* 17, 239-277
Meehan, R. R., Lewis, J. D., McKay, S., Kleiner, E. L., and Bird, A. P. (1989) *Cell* 58, 499-507
Meistrich, M. L. (1987) in *Meiosis* (Moens, P. B., ed) pp. 333-353, Academic Press, Inc., New York
Meistrich, M. L., Bucci, L. R., Tростle-Weige, P. K., and Brock, W. A. (1985) *Dev. Biol.* 112, 230-240
Miller, J. R., Cartwright, E. M., Brownlee, G. G., Fedoroff, N. V., and Brown, D. D. (1978) *Cell* 13, 717-725
Monk, M., Boubelik, M., and Lehner, S. (1987) *Development* 99, 371-382
O'Brien, D. A., and Bellvé, A. R. (1980) *Dev. Biol.* 75, 386-404
Ohmori, H., Tomizawa, J., and Maxam, A. M. (1978) *Nucleic Acids Res.* 6, 1749-1766
Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Navet-Many, T., Scinky-Gallili, N., and Cedar, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 2275-2279
Saluz, H. P., and Jost, J. P. (1987) *A Laboratory Guide to Genomic Sequencing*, Birkhaeuser, Boston
Saluz, H., and Jost, J. P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 2602-2606
Schumperli, D. (1988) *Trends Genet.* 4, 187-191
Shiurba, R., and Nandi, S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 3947-3951
Sittman, D. B., Graves, R. A., and Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 1849-1853
Stauber, C., Luscher, B., Eckner, R., Lotscher, E., and Schumperli, D. (1986) *EMBO J.* 5, 3297-3303
Watt, F., and Molloy, P. L. (1988) *Genes & Dev.* 2, 1136-1143
Weintraub, H., and Groudine, M. (1976) *Science* 93, 848-853
Wolfe, S. A., Anderson, J. V., Grimes, S. R., Stein, G. S., and Stein, J. S. (1989) *Biochem. Biophys. Acta* 1007, 140-150
Wolgemuth, D. J., Gizang-Ginsberg, E., Engelmyer, E., Gavin, B. J., and Ponzetto, C. (1985) *Gamete Res.* 12, 1-10
Young, P. R., and Tilghman, S. M. (1984) *Mol. Cell. Biol.* 4, 898-907