THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 266, No. 30, Issue of October 25, pp. 20504-20511, 1991
Printed in U.S.A.

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 spermatogonia 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.*, 1985, 1987). Recently, several testis-specific variant histone genes have been cloned from the testis of rat (Cole *et al.*, 1986; Kim *et al.*, 1987; Wolfe *et al.*, 1989). Although RNA analyses revealed the cell type-specific and stage-specific expression of these genes, little is known about the transcriptional regulation of testis-specific histone genes.

Previously, we have isolated TH2B[1] 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 (ATTTGCAT) 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 concomitant 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-Ariga 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. Transfec-

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[1] The abbreviations used are: TH2B, testis-specific H2B; sH2B, somatic H2B; bp, base pairs; kb, kilobase pairs; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeCP, methyl-CpG-binding protein.

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 same promoters of sH2B and TH2B genes can be regulated in an opposite way in the same cell types. Transcription factors which are available for the transcription of sH2B 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. Spermatozoa were isolated from the caudal epididymis and vas deferens of mature rats (>60 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 were isolated; pachytene spermatocytes, round spermatids, 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 Barberius *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 *Msp*I 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 H_2O. Fifty μg of DNA were then subjected to a partial chemical cleavage at cytosine residues as described by Maxam and Gilbert (1980) and Saluz and Jost (1987). After complete removal of piperidine by repeated lyophilization in 100 μl of H_2O, the sequencing ladder over the promoter region of TH2B gene was specifically amplified with ^{32}P-labeled primer (27 mer) by 30 cycles of PCR reaction. The cycling conditions were 1 min of denaturation at 95 °C, 2 min of annealing at 62 °C, and 3 min of polymerization at 72 °C. The high specific activity sequencing primer was prepared by annealing a short oligonucleotide (9-mer; 5′-AAA-ATA-AGT-3′) to the underlined complementary sequences in template (33-mer; 5′-AGA-CGT-TGG-AGT-GGA-CAA-ACT-TAT-TTT-TCC-ACG-3′), followed by extension of 9-mer with DNA polymerase I large fragment in the presence of [α-^{32}P]dCTP (3,000 Ci/mmol). The radiolabeled, single-stranded sequencing primer (27-mer) was then separated from the template (33-mer) by electrophoresis through a 15% polyacrylamide denaturing gel, and recovered from the gel by elution. The resulting primer is complementary to the region spanning from +61 to +47 nucleotides of TH2B gene (27-mer; 5′-AAA-ATA-AGT-TTG-TCC-ACT-CCA-ACG-TCT-3′, Fig. 1). As an unmethylated control, the pTB plasmid containing TH2B gene (insert; 3.8 kb) was treated as described (Saluz and Jost, 1989). After purification of amplified samples as described (Saluz and Jost, 1989), they were loaded on an 8% polyacrylamide sequencing gel in 7 M urea. The gel was fixed, dried, and autoradiographed for 1–3 days at −80 °C with intensifying screens.

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 Barberius *et al.* (1987), and resuspended at a concentration of 10^8 nuclei/ml in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride. DNase 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 DNase I, deproteinized genomic DNA (50 μg) of liver and testis was digested with 0.1 ng/μg of DNase I at 37 °C for 3 min. DNase I-treated genomic DNA was purified by phenol-chloroform extraction, and precipitation by ethanol, and 50 μg of purified DNA was digested with *Msp*I restriction enzyme. PCR amplification was carried out as described above. Amplified samples were analyzed on a 6% denaturing polyacrylamide gel (38.5 × 31 × 0.08 cm). Fixed and dried gels were exposed to x-ray films in the presence of an intensifying screen 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-methylcytosine phosphoamidite replaced cytosine at four CpG sites indicated in bold type (upper strand, 5′-CAT-CTT-T**CG**-**CG**T-GCT-CAT-**ACG**-TCA-TCC-AAG-GCC-CA**C**-**G**CC-T-3′). Methylated and unmethylated DNA probes were prepared by annealing end-labeled, single-stranded oligonucleotides. Binding reactions (20 μl) were carried out by incubating end-labeled DNA probes (10^4 cpm) with 10–12 μg of nuclear proteins and 2 μg of poly(dI-dC) in a buffer containing 60 mM KCl, 12 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 12% glycerol, 0.3 mM phenylmethylsulfonyl fluoride. After 15 min at 25 °C, samples were loaded on a 4% native polyacrylamide gel (80:1, acrylamide to bisacrylamide) in 0.25 × TBE. The gels were dried and autoradiographed with intensifying screens at −80 °C. Nuclear proteins were extracted from isolated nuclei as described before (Hwang *et al.*, 1990).

In Vitro Methylation of the pTHAB Plasmid—The pTHAB plasmid was constructed by cloning about 1.7-kb *Nco*I DNA fragment containing TH2B gene (Fig. 1*a*) into pSP72 vector. The plasmid was methylated at all cytosine residues in CpG dinucleotide sequences *in vitro* by using *Sss*I methylase (New England Biolabs). The reaction mixture contained 10 μg of DNA and 20 units of *Sss*I methylase in 100 μl of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 160 μM *S*-adenosylmethionine, 1 mM DTT), and incubated for 16 h at 37 °C. As a control, mock-methylation reactions were done in the absence of *S*-adenosylmethionine. The methylated DNA was purified by phenol extraction, recovered by ethanol precipitation, and resuspended in H_2O prior to transfection. The methylation of plasmid DNAs was confirmed by incubation with methylation-sensitive *Hpa*II enzyme and analysis of restriction patterns by blot analysis.

DNA Transfections—Methylated and mock-methylated TH2B constructs were transfected into mouse embryo fibroblast C3H 10T1/2 cells as described previously (Hwang and Chae, 1989). For transient transfections, 7 μg of test DNAs was cotransfected with 5 μg of RSVneo using the calcium phosphate method (Gorman, 1985). After 12 h, transfected cells were synchronized by the treatment of aphidicolin (2 μg/ml) for 20 h. Cells were then released from the aphidicolin block by several washes in Hank's balanced salt solution lacking Ca^{2+} and Mg^{2+} and media replacement. Cells were harvested 4 h later, and the total RNA was prepared by guanidine-thiocyanate extraction followed by centrifugation through a cesium chloride cushion (Chirgwin *et al.*, 1979). The non-S phase cells were maintained in the aphidicolin-containing medium for 24 h before harvested. To isolate stable transformants, 2 μg of methylated or mock-methylated TH2B constructs were cotransfected with 0.2 μg of RSVneo plasmid (Gorman *et al.*, 1983). Cells expressing the neo gene were selected with geneticin (G418, GIBCO) at 500 μg/ml for 10 days. About 200–400 clones were pooled together and grown for 3 days, before cells were synchronized and harvested at 4 h after the release from the aphidicolin block. Total cellular RNA was isolated by using the guanidine isothiocyanate-cesium chloride method (Chirgwin *et al.*, 1979), and DNA was purified from isolated nuclei as described previously (Weintraub and Groudine, 1976).

Blot Experiments—RNA samples were size-fractionated on a 1.5% agarose/formaldehyde gel and transferred to GeneScreen™ membrane (Du Pont-New England Nuclear). The filters were hybridized to a ^{32}P-labeled probe for 20 h at 68 °C in 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM $NaPO_4$ and 1 mM EDTA) containing 5 × 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 × SSC (standard saline citrate), 0.1% SDS and twice at 65 °C for 1 h in 1 × SSC, 0.1% SDS. The probe which is a 180 bp *Eco*RI/*Msp*I DNA fragment containing the 5′-

untranslated region of TH2B gene (Fig. 1*a*) was labeled with [α-^{32}P] dCTP (3000 Ci/mmol) by random-primer extention to a specific activity of 5×10^8 cpm/μg of DNA (Feinberg and Vogelstein, 1983). For DNA blot analysis, genomic DNA was digested with restriction enzymes (10 units/μg) for 16 h. The digested DNA was then electrophoresed on a 1% agarose gel and transferred to GeneScreen™ nylon membrane. Pre-hybridization, hybridization, and washing were done under the same conditions as Northern blots. The probe was a 310-bp *Hin*fI-*Msp*I 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. 1*a*). Since no *Msp*I and *Hpa*II 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. 1*b*. 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 methylated 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 spermatogenic differentiation using a Celsep apparatus. The analyzed cell types include spermatogonia type A (~30% purity), Sertoli cells (~95%), pachytene spermatocytes (~90%), early spermatid (~90%), late spermatid (~85%), and spermatozoa. As shown in Fig. 3, all five CpG sites in the promoter region of TH2B gene were unmethylated 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 promoter region 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

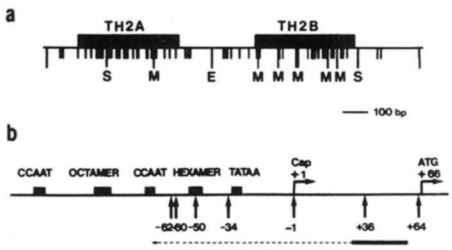

FIG. 1. **Maps showing the distribution of CpG dinucleotides in the TH2A-TH2B gene cluster and the promoter region of TH2B gene.** *Panel a*, coding sequences of TH2A and TH2B genes are indicated by *black boxes*. The location of each CpG dinucleotide in the TH2A-TH2B cluster is indicated by a *vertical bar* below the map. There are 10 CpG dinucleotides in the 5′ upstream region shared by the TH2A and TH2B genes, of which seven and three CpG sites are located in the 5′ upstream region of TH2B and TH2A genes, respectively. The coding sequences of TH2A and TH2B genes are CpG-rich; they contain high G + C content (>50%) and high observed/expected CpG ratio like somatic histone genes (Gardiner-Garden and Frommer, 1987). The map also shows the sites of the restriction enzymes *Eco*RI (*E*), *Sac*I (*S*), and *Msp*I (*M*). *Panel b*, a blowup of 5′ upstream region of TH2B gene. *Small filled boxes* represent the location of regulatory sequences. *Numbers* indicate the distance from the transcription initiation site of TH2B gene in base pairs. The *arrows* above the map show the initiation sites as well as the direction of transcription and translation. An *arrow* below the map shows the location of a sequencing primer and the direction of genomic sequencing reaction.

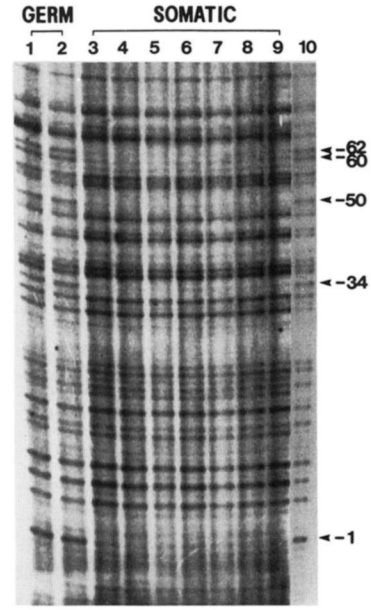

FIG. 2. **Methylation patterns of five CpG sites in the promoter region of TH2B gene.** The promoter region of TH2B gene was analyzed for cytosine methylation by genomic sequencing. DNA from various tissues was restricted with *Msp*I restriction enzyme and subjected to the genomic sequencing reaction as described under "Experimental Procedures." *Arrowheads* mark bands corresponding to cytosines contained in CpG dinucleotides. Germ cells: *lane 1*, the enriched fraction of pachytene spermatocytes; *lane 2*, the whole testis of 28-day-old rat. Male somatic tissues: *lane 3*, liver; *lane 4*, thymus; *lane 5*, kidney; *lane 6*, spleen; *lane 7*, lung. Female somatic tissues: *lane 8*, liver; *lane 9*, kidney. *Numbers* refer to the positions of cytosine residues in CpG dinucleotide with respect to the cap site of TH2B gene (Fig. 1). As an unmethylated control DNA, the pTB plasmid containing TH2B gene was genomically sequenced (*lane 10*).

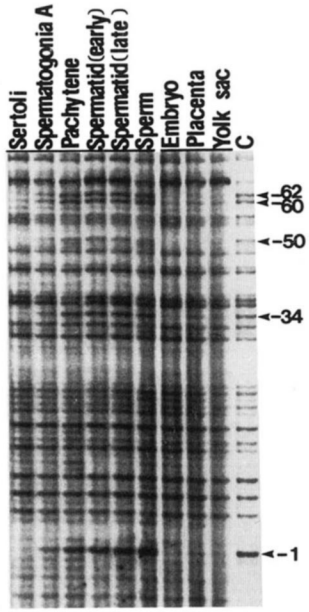

FIG. 3. **Methylation patterns of five CpG sites in the promoter region of TH2B gene in testicular, embryonic, and extraembryonic cells.** Testicular cells in different stages of spermatogenesis were fractionated as described under "Experimental Procedures." Embryo, placenta, and yolk sac were prepared 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).

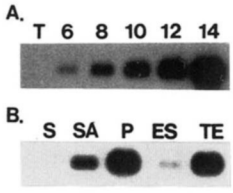

FIG. 4. **Changes in the TH2B mRNA during the prepubertal development of rat testis.** *Panel A*, total RNA (15 μg) isolated from thymus (*lane T*) and the testes of rats at 6, 8, 10, 12, and 14 days after birth (age matches *numbers* above each *lane*) was analyzed by RNA blot hybridization as described 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.

gene expression at pre- and post-meiotic stages. To see whether TH2B gene is expressed at such a low level at pre-meiotic stages that could not be detected by *in situ* cytohybridization technique, we have carried out Northern analyses on RNAs from testes at different ages. Fig. 4*A* shows that TH2B mRNA is detected in RNA from testis of 6-day-old rat and the mRNA level is markedly elevated by Day 14, coincident with the appearance of pachytene spermatocytes (Bellvé *et al.*, 1977). Since the testis of 6-day-old rat contains mainly spermatogonia (primitive type A) as germ cells (Bellvé *et al.*, 1977) this result indicates that TH2B gene is expressed in pre-meiotic germ cells, although at a low level. To further demonstrate the pre-meiotic expression of TH2B gene, RNA was purified from fractionated germ cells, and TH2B mRNA was analyzed by Northern blot analysis. As shown in Fig. 4*B*, a significant amount of TH2B transcript was detected in RNA from the enriched fraction of spermatogonial cells, whereas no TH2B transcript was found in Sertoli cells. Thus, TH2B gene appears to be expressed in both mitotic and meiotic germ cells in which the promoter region of TH2B gene is unmethylated. Also, these results show that there is a drastic increase in the level of TH2B mRNA in pachytene spermatocytes, whereas very weak signal was detected in elongated spermatids. The expression of TH2B gene appears to be almost completely repressed in somatic tissues (*lane T* and *S*).

Correlation between DNA Hypomethylation, Gene Expression, and the Binding of Transcription Factors to the Promoter in Vivo—To test if the DNA methylation in somatic cells inhibits the TH2B gene activity by preventing the binding of transcription factors to the promoter of TH2B gene, we have carried out *in vivo* footprinting experiments. For this analysis, nuclei were isolated from liver and testis of adult rats and digested with DNase I for increasing times.

The cleavage products were amplified by 30 cycles of PCR reaction and resolved on a 6% sequencing gel to single-nucleotide resolution. To monitor the sequence specificity of DNase I, protein-free genomic DNA was digested with DNase I (Fig. 5, *lane F*). Two features of DNase I digestion patterns were used to define the sites of stable protein-DNA interactions: enhanced cleavages and protected regions compared to DNase I digestion patterns of protein-free genomic DNA (*lane F*). Fig. 5 shows that several sites of enhanced cleavages and protected regions are evident only in samples from testis. Major differences between footprints from liver and testis were observed at CCAAT (−71 to −75), hexamer (−46 to −51), and TATAA (−26 to −30) sequences. (i) These regions exhibit lower levels of DNase I cleavage when compared to the digestion patterns of protein-free genomic DNA, and (ii) markedly enhanced DNase I cleavages were observed at the

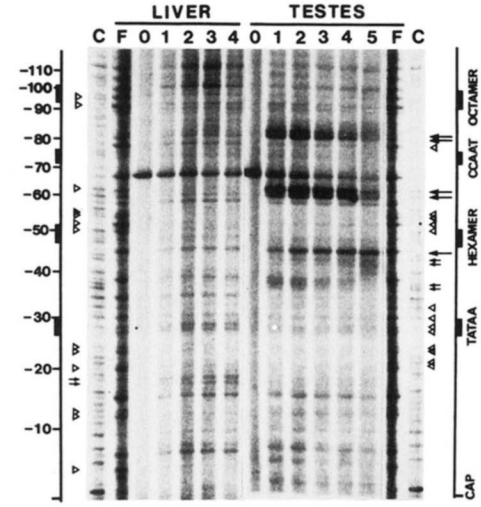

FIG. 5. ***In vivo* footprinting experiments reveal testis-specific protein-DNA interactions in the promoter region of TH2B gene.** Nuclei from liver and testis were digested with DNase I for increasing times (*lanes 1*, 1 min; *2*, 2 min; *3*, 4 min; *4*, 8 min; *5*, 16 min). As a control for the background 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*). As a size marker, the pTB 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.

boundaries of these protected regions only in footprints from testis. Thus, 5′ to CCAAT (−80 to −85), 5′ to hexamer (−60 to −62), and the region between hexamer and TATAA sequences (−36 to −43) showed the increased DNase I sensitivity relative to naked DNA. These results indicate that the binding of transcription factors to the promoter 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),[2] 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).

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 sequence were obtained by specific incorporation of 5-methylcytosine during oligonucleotide synthesis. Unmethylated and methylated DNA probes were incubated with nuclear extracts from liver and testis, and the protein-DNA complex was resolved on a 4% native polyacrylamide gel. Fig. 6 shows that DNA methylation directly inhibits the binding of transcription factors to the hexamer motif. Although little different protein-DNA complexes were obtained between samples from liver and testis, it is clear that DNA methylation can prevent formation of all protein-DNA complexes. Also, hemimethylation on either strand was sufficient to inhibit more than 80% of binding activities; hemimethylation on the bottom (antisense) strand was as effective as the methylation on both strands (data not shown).

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 *Sss*I methylase, and the effect of DNA methylation on TH2B transcription was assayed by both transient and

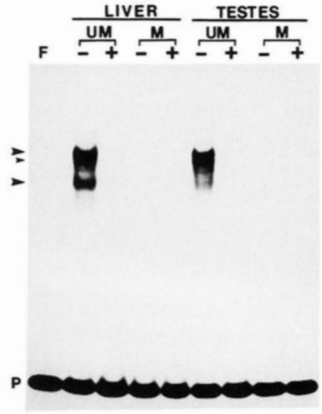

FIG. 6. **Effect of CpG methylation on the binding of transcription factors recognizing hexamer (ACGTCA) motif.** Nuclear extracts from liver and testis were incubated with methylated or unmethylated ^{32}P-labeled, double-stranded oligonucleotides (unmethylated, *UM*; methylated, *M*) with (+) or without (−) 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.

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 *Hpa*II and subsequent blot analysis. Fig. 8*A* shows that DNA from stable clones containing the methylated TH2B construct is poorly cleaved by *Hpa*II enzyme, indicating that the methylation patterns were faithfully maintained in those cells. On the other hand, a 408-bp band was detected following *Hpa*II 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. 8*A* 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

[2] K. Lim and C.-B. Chae, unpublished results.

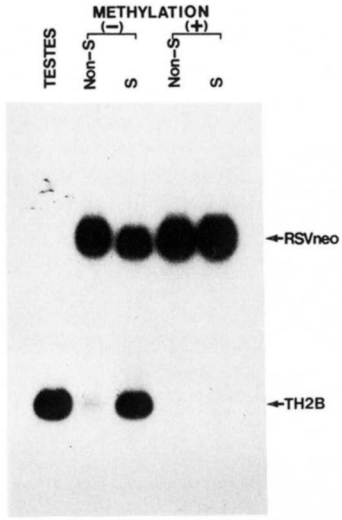

FIG. 7. **Effect of DNA methylation on TH2B transcription in transient assays.** Seven micrograms of methylated (+) and nonmethylated (−) TH2B constructs were transiently introduced into mouse embryo fibroblast C3H 10T1/2 cells with 5 μg of pRSVneo plasmid as a cotransfection control. The cells were 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 ^{32}P-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.

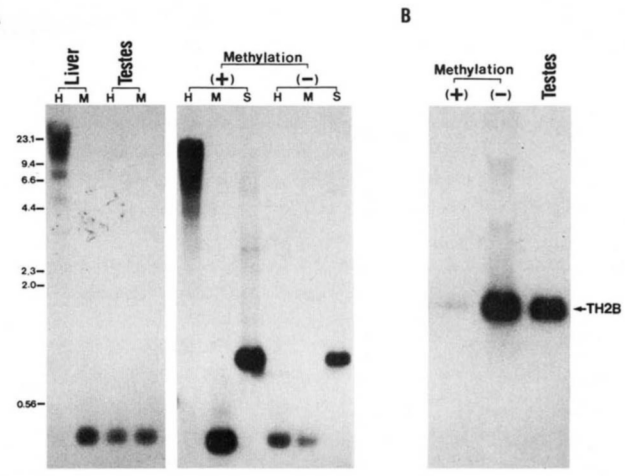

FIG. 8. **Inhibition of TH2B transcription by DNA methylation in stably transfected clones.** Genomic DNA and total cellular RNA were isolated from stably transfected cells containing the methylated (+) or mock-methylated (−) pTHAB construct, and subjected to blot analyses. *Panel A*, methylation patterns of TH2B gene in tissues and stable clones. Methylation status of TH2B gene was determined by using methylation-sensitive enzyme *Hpa*II and its isoschizomer *Msp*I (both *Hpa*II and *Msp*I enzymes recognize the sequence CCGG, but *Hpa*II cannot cleave C^{m}CGG). Total genomic DNA (15 μg) from liver and testis of adult rats or stably transfected clones was digested with *Hpa*II (*H*), *Msp*I (*M*), or *Sac*I (*S*), and restriction patterns were determined by Southern blot analysis. The probe specific for the intergenic sequences between TH2A and TH2B genes detects 408-bp *Msp*I and 980-bp *Sac*I restriction fragments (Fig. 1*a*). Increase in the size of *Hpa*II fragments compared to *Msp*I fragments indicates hypermethylation. The appearance of a 980-bp restriction fragment after *Sac*I digestion indicates that methylated and nonmethylated TH2B copies remain intact during cell divisions without obvious chromosomal rearrangements or deletions. Positions of *Hin*dIII-digested lambda phage DNA are shown as size markers in kilobase pairs (*kb*). *Panel B*, RNA samples (10 μg) from stably transfected clones containing methylated or nonmethylated TH2B construct were electrophoresed in 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.

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. 8*B*).

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 vivo* in gene transfer experiments. *In vivo* 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 (ACGTCA). Such direct effects of DNA methylation on the binding of transcription factors 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 vivo* 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 an CpG island which is characterized by a high G + C content and a high density of CpG dinucleotides (Fig. 1*a*). CpG islands

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), it was observed that DNA methylation was associated with the inactivation of genes containing CpG islands, leading to the suggestion that methylation of CpG islands is incompatible with gene activity. Recently, it was shown that MeCP-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 MeCP-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 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. Thus, TH2B gene appears to be expressed in both mitotic as well as meiotic germ cells. This finding raises an interesting question of whether the regulation of TH2B gene expression in mitotic cells is different from that in meiotic cells. Since our gene transfer experiments have previously shown that the cloned TH2B gene is expressed in an S phase-dependent manner like sH2B 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; 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 pachytene spermatocytes. While it has been shown that the cooperative interaction between the CCAAT sequence element at −127 bp and the octamer at −93 bp is important for the efficient transcription of cloned TH2B gene in mitotically replicating cells after gene transfer (Hwang *et al.*, 1990), it is not yet clear which transcription factors are involved in the expression of TH2B gene in spermatogenic cells. However, *in vivo* footprinting experiments suggest that the interaction between 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 spermatogonial 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.[2] 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 base 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.*, 1983; 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 translocated 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.

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