INTRODUCTION

The mammalian H1 histone family contains seven known subtypes and represents the most divergent histone class [1, 2]. The H1 histone gene subtypes exhibit differences in expression patterns during development and differentiation, and the H1 subtypes exhibit differences in binding affinity to chromatin [3]. The H1 histones are involved in chromatin remodeling and in formation of higher order chromatin structure by facilitating the transition from the 10-nm filament to the 30-nm fiber [4]. Through chromatin binding and remodeling, histone H1 acts as a global gene regulator. Studies show that H1 histones can interact with components of the transcription initiation complex to block transcription [3, 5]. Our laboratory has examined the testis-specific histone H1t variant gene to determine mechanisms of regulation of expression of this gene [6–9].

The proximal promoter of the testis-specific histone H1t gene contains all of the consensus elements common to the somatic H1 promoters including an AC box, a GC box, a CCAAT box, and a TATA box [10–15]. The proximal promoter of the testis-specific histone H1t contains an enhancer element designated TE that binds factors responsible for activation of H1t gene transcription in the testis [16–19]. The H1t promoter also contains an upstream silencer element [6] and a poly C element within the proximal promoter termed the GC box 2 [20, 21] that appear to contribute to repression of H1t gene transcription in somatic cells. Studies with transgenic animals have revealed regions of DNA that are needed for proper testis-specific transcription. All sequences necessary and sufficient for proper developmental and spermatocyte-specific transcription of the H1t gene are present within a genomic fragment containing 2.5 kilobases (kb) of upstream and 3.8 kb of downstream flanking DNA [22]. Transgenic animals bearing a deletion of the TE element in the rat transgene fail to express the rat H1t gene in any tissue [8]. Therefore, several mechanisms may act synergistically to enhance transcription of the testis-specific histone H1t gene in primary spermatocytes and to repress transcription in nongenomic cells.

Methylation of the DNA of eukaryotic genes is the most common DNA modification [23] and is required to be important for regulation of gene expression [24, 25]. However, absence of detectable DNA methylation in some eukaryotes such as Drosophila [26] and Saccharomyces cerevisiae [27] has led to doubts about its physiological significance in development and tissue-specific gene expression. Recent studies show abnormal development and embryonic lethality in transgenic mice expressing decreased DNA methyltransferase (MTase) activity after DNA-MTase gene knockout and therefore reveal the importance of DNA methylation in developmental gene regulation [28]. Frequently, there is a correlation between DNA hypomethylation and active transcription of a gene and conversely between hypermethylation and transcriptional repression. In general there appears to be a mechanistic link between DNA methylation, histone deacetylation, and transcriptional silencing [29, 30].

The DNA methylation status of only a few testis-specific genes has been reported. For example, methylation of the lactate dehydrogenase (LDH-C) gene was found to be low in testis compared to somatic tissues [31]. Likewise, the testis-specific histone H2B gene was found to be hypomethylated in testis but not in somatic tissues [32]. Several male germ line-specific genes (MAGE-type genes) belong to a unique subset of germ line-specific genes that use DNA methylation as a primary silencing mechanism [33, 34]. Most MAGE-type genes have promoters that are highly methylated in somatic tissues, but they are largely unmethylated in male germ cells where they are transcribed [33, 34].

ABSTRACT

The testis-specific histone H1t gene is expressed only in pachytene primary spermatocytes during spermatogenesis. There is a correlation between the specific binding of testis nuclear proteins to a rat histone H1t promoter sequence, designated the H1t/TE element, and the onset of transcription in primary spermatocytes. Our laboratory has shown that mice bearing the rat gene with a deletion of the TE promoter element and replacement with a heterologous stuffer DNA fragment fail to express the rat H1t transgene in any tissue. In this study we report that five CpGs located within the H1t proximal promoter, including two CpGs located within the essential TE promoter element, contain unmethylated cytosines in vivo in genomic DNA derived from primary spermatocytes where the H1t gene is expressed. All seven CpGs are hypermethylated in vivo in genomic DNA derived from liver cells where gene expression is repressed. Further, in vitro methylation of an H1t promoter-driven reporter plasmid markedly reduced expression in a transient transfection assay system. These results suggest that cytosine methylation may contribute to the transcriptional silencing of the testis-specific histone H1t gene in nonexpressing tissues such as liver.

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Therefore, methylation may be important for regulating transcription of many testis-specific genes. However, the difficulties of examining DNA methylation of specific regions of DNA by methylation-sensitive restriction enzymes has hampered efforts to extend these results. Recently, a new powerful bisulfite genomic sequencing method was developed that allows examination of individual strands of almost any region of the genome [23, 35]. Because many testis-specific genes appear to be methylated differentially in testis cells compared to nontestis cells and because the methylation status of the H1t gene has not been established, we decided to use bisulfite genomic sequencing to examine the methylation of the H1t gene in primary spermatocytes where the gene is expressed and in nongermlinal cells where transcription of the gene is repressed.

**MATERIALS AND METHODS**

**Reagents and Supplies**

Radiochemicals were purchased from New England Nuclear (Boston, MA), X-OMAT XAR-5 x-ray film (Eastman Kodak) and Cronex-7 x-ray film (DuPont) were purchased from Sigma (St. Louis, MO) and Sterling Diagnostic Imaging (Greenville, SC). Nytran membrane for Northern blots was obtained from Schleicher & Schuell (Keene, NH). Deoxynucleotides and Ampli-Taq for polymerase chain reaction (PCR) were purchased from Perkin-Elmer (Foster City, CA). Random primer labeling was conducted using a kit obtained from Pharmacia (Piscataway, NJ). The DNA sequencing was performed using a kit obtained from Schleicher & Schuell (Keene, NH). Kodak) and Cronex-7 x-ray film (DuPont) were purchased to allow photography of the stained rRNA bands with UV light.

The DNA was extracted from tissues using RNA Stat-60 (Tel-Test Inc.) according to the manufacturer’s protocol. The RNA was dissolved in H2O and quantity was determined by measuring absorbance at 260 nm in a Beckman UV-visible spectrophotometer. For Northern blot analysis, RNA samples, precipitated in desired quantities, were dissolved in 20 μl of sample loading buffer (50% formamide, 1X MOPS [40 mM morpholino propanesulfonic acid, pH 7, 10 mM sodium acetate, 1 mM EDTA], 6% formaldehyde, 0.02% bromophenol blue), incubated at 56°C, and electrophoresed on a 1.5% agarose gel containing 1X MOPS and 6% formaldehyde as described previously [19]. Following electrophoresis in a minisube gel device at 75 mA for 2 h in buffer containing 1X MOPS and 3.7% formaldehyde, the gel was soaked in H2O to remove formaldehyde, stained with 0.5 μg/ml ethidium bromide in H2O, and destained in H2O to allow photography of the stained rRNA bands with UV light.

**Bisulfite Genomic Sequencing**

The DNA was obtained on a Nytran filter (Schleicher & Schuell) by capillary transfer from the gel and UV crosslinked using a Stratagene 1800 (Stratagene). The H1t mRNA was detected by hybridization to a rat H1t gene probe pH1 oligolabeled with [32P]dCTP using a Pharmacia kit following the manufacturer’s protocol. The Nytran membrane was prehybridized at 43°C for a minimum of 4 h in a solution containing 50% formamide, 5X SSC (standard saline citrate: 0.75 M NaCl, 0.075 M sodium citrate, pH 7.4), 5X Denhardt (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), 0.1% SDS, and 250 μg/ml denatured salmon sperm DNA. Hybridization was conducted for a minimum of 16 h at 47°C in fresh solution of the same composition and containing 1 × 106 dpm/ml of the denatured labeled H1t DNA probe. The filter was washed with 5X SSC, 1X Denhardt at room temperature for 10 min followed by incubation for 30 min at 59°C. Then the blot was washed successively in solutions of 5X SSC, 2X SSC, 1X SSC, and 0.1X SSC solutions all containing 0.1% SDS. The membranes were analyzed by autoradiography by exposing the membrane to Kodak XOMAT XAR-5 film at −70°C.

**Plasmids**

Methods for construction of recombinant DNA followed standard procedures [36]. Construction of the histone H1t-promoted luciferase reporter plasmid Pst-pGL3 and of the Northern blot probe pHD1 used in this study were described previously [6]. Plasmid DNA was harvested from cultures of Escherichia coli strain HB101 using alkaline lysis [37] followed by passage through a Wizard DNA clean up minicolumn (Promega, Madison, WI). Plasmid DNA concentration was determined by reading absorbance at 260 nm on a Beckman DU 64 spectrophotometer and by comparison of ethidium bromide-stained plasmid DNA restriction fragments to DNA standards of known concentration electrophoresed on agarose gels. In the transient transfection experiment, one plasmid sample for transfection was unmethylated and one plasmid sample was methylated. Methylation was accomplished using SssI methylase (New England Biolabs), and the extent of methylation was determined by digestion with a mixture of the methylation-sensitive restriction enzymes AcII, HhaI, and HpaII.
Transient Transfections

Mouse C127I mammary cells, obtained from American Type Culture Collection, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with an atmosphere of 5% CO₂. Cells were transfected using LipofectAMINE (GibcoBRL, Gaithersburg, MD) as described previously [6] by the supplier’s protocol. Mouse C127I cells from a single cell suspension were plated on 60-mm dishes and grown to a density of 40–60% before transfection. Transfections were performed in triplicate to help control for variation in cell number and culture conditions. Cells in serum free medium were cotransfected using 2 µg of a specific plasmid construct plus 200 ng of pRL TK (as a control for transfection efficiency) complexed with 20 µl of LipofectAMINE per dish. The plasmid used was histone H1t-promoted luciferase reporter plasmid Pst-pGL3 which was either mock methylated (unmethylated) or fully methylated by SssI methylase. The mock methylase used was identical to the methylation procedure except for the omission of S-adenosylmethionine. The LipofectAMINE/DNA complexes were incubated with cells 5 h before feeding with an equal volume of DMEM containing 20% fetal bovine serum. Lysates were made from transfected cells 48 h after feeding.

Luciferase Assays

Cell lysates and luciferase assays were performed using the dual-luciferase reporter assay system protocols manual as supplied by Promega. Following transfection, the growth medium was removed and 4 ml of PBS was added to each 60-mm dish of cells. The dishes were gently swirled to wash the cell surfaces, followed by removal of the rinse solution and addition of 400 µl of 1× passive lysis buffer (Promega) to each dish. The dishes were incubated for 15 min at room temperature before harvesting the cell lysates by scraping the bottom of the dishes with disposable plastic scrapers. Each lysate was pipetted several times to obtain a homogenous solution and transferred to a microfuge tube. The lysates were cleared by centrifugation in the microfuge at 4°C for 1–2 min. and transferred to fresh tubes and stored at −70°C.

Luciferase assays were performed in a Beckman LS6000SC scintillation counter with the coincidence counter disabled. Firefly luciferase and the Renilla luciferase activities were sequentially measured for 2 min as directed by the protocol set forth for manual luminometers in the dual-luciferase reporter assay system technical manual. Results obtained were normalized for firefly luciferase activity. Protein concentrations were measured spectrophotometrically using a program contained in a Soft-Pac module on a Beckman DU series 64 spectrophotometer based upon the Warburg and Christian coefficients [39].

RESULTS

The Testis-Specific Histone H1t Gene Is Transcribed Only in Primary Spermatocytes

The expression pattern of core and linker histone genes changes dramatically during spermatogenesis. The pattern in immature prepubertal germinal cell types resembles the pattern in somatic cell types. However, during sexual maturation, testis-specific and testis-enriched histone genes are preferentially expressed. For example, the testis-specific histone H1t, not seen in prepubertal animals [40], is first observed when primary spermatocytes appear. In adult animals the H1t gene is the predominant H1 linker histone variant gene expressed in primary spermatocytes [41] and H1t is the predominant H1 synthesized in primary spermatocytes.

To examine testis-specific transcription of the histone H1t gene we prepared total cellular RNA from various testis cell types and from various rat tissues. Figure 1A depicts germinal cell types found in the testis and a time line of spermatogenesis in the rat. The enriched cell types prepared by centrifugal elutriation are shown as elutriation fraction 1 (late spermatids), fraction 3 (early spermatids), and fraction 5 (pachyteme primary spermatocytes). Steady-state levels of H1t mRNA in these and nongerminatal cells were estimated by conducting a Northern blot analysis. Figure 1B shows a blot of total cellular RNA derived from enriched primary spermatocytes and liver. The intensities of the ethidium bromide-stained RNA bands in the upper panel reveal relatively equal loading of RNA samples on the denaturing agarose gel. A photograph of the ethidium bromide-stained gel after blotting revealed essentially complete transfer of RNA. The autoradiogram in the lower panel reveals that the rat H1t gene probe hybridizes only to RNA from cells enriched in primary spermatocytes (lane 5). The low level hybridization in lane 4 is due to cross contamination of this fraction with primary spermatocytes. This pattern of H1t mRNA accumulation corresponds to expression of the histone in primary spermatocytes. No H1t mRNA is seen in liver showing the tissue-specific transcription pattern of the gene.

The Testis-Specific Histone H1t Promoter Is Hypomethylated in Primary Spermatocytes and Hypermethylated in Liver

Several potential cis-acting regulatory elements have been identified that may be involved in transcriptional regulation of the H1t gene, but the methylation status of the H1t promoter has not been examined in expressing and nonexpressing cells. Therefore in this study we conducted an examination of the H1t promoter using the relatively new bisulfite genomic sequencing technique. Figure 2A shows a low-resolution map of CpGs located within the H1t promoter and within the H1t gene. This diagram shows that CpGs are clustered in the proximal promoter and within the coding region of the gene. It is clear that CpGs are less abundant in the distal promoter and in the downstream non-coding DNA.

Five CpGs within the proximal promoter of the rat histone H1t gene are located between the H1/AC box and mRNA start site (Fig. 2B). Note that one CpG is located within each of the essential TE subelements, TE1 and TE2. Also note that the CpG within TE2 is located within the core of the consensus SPI binding site. The same figure shows two additional CpGs that are located between the mRNA start site and the ATG start codon. In the in vivo bisulfite genomic sequencing procedure unmethylated cytosines in genomic DNA are converted to uracils that are then PCR amplified as thymidines. Because methylated cytosines (5-methylcytosines) within genomic DNA are protected from conversion to uracil, they are PCR amplified as cytosines. Figure 3 shows the results of bisulfite genomic sequencing of DNA from rat testis pachyteme primary spermatocytes and from rat liver. Note that cyto-
FIG. 1. Expression of the histone H1t gene during spermatogenesis. A) This figure shows the germinal cell types present during spermatogenesis in the rat. The time line shows that the 60-day process is divided into roughly three equal periods: spermatogonia, spermatocytes, and spermatids. The testis-specific histone H1t gene is transcribed only in pachytene primary spermatocytes as indicated by the closed solid rectangle. The three major cell types enriched by centrifugal elutriation are pachytene primary spermatocytes, early spermatids, and elongated late spermatids. B) Northern blot analysis was used to detect histone H1t mRNA from various rat testis germinal cell types and rat liver. Total cellular RNA samples were prepared from testicular cell populations enriched in pachytene primary spermatocytes, early spermatids, and late spermatids and from rat liver as described in Materials and Methods. Approximately 40 μg of each total cellular RNA sample was electrophoresed on a denaturing agarose gel. The photograph in the top panel shows the ethidium bromide-stained 18S and 28S rRNA banding pattern, which reveals that the RNA samples were not degraded and that approximately equal amounts of RNA were loaded on the gel. The RNA was transferred to a Nytran membrane and hybridized to a labeled histone H1t probe designed to detect H1t mRNA. The lower panel shows the hybridization pattern of the probe, revealing that H1t mRNA accumulates to a high level in primary spermatocytes (lane 5) but is not present in liver.

FIG. 2. Location of CpGs within the H1t gene. A) This panel shows a map of CpGs within the rat testis-specific histone H1t gene. Note the clustering of CpGs in the proximal promoter region, in the leader region, and in the coding region (filled rectangle) of the gene. The heavy bar below the map shows the position of the proximal promoter region that is shown in more detail in B of this figure. B) This figure shows the sequence of the H1t proximal promoter and leader region with each CpG presented in bold uppercase letters and marked with an asterisk. The numbering is relative to the mRNA start site. The major H1 consensus promoter elements are underlined and include the H11/AC box, the H11/CCAAT box, and the TATA box. The H11/TE1 and TE2 elements, unique to the H1t variant, are located between the AC box and the CCAAT box. One CpG is located within each of the TE elements, and two CpGs are located between the CCAAT box and TATA box. One additional CpG is found between the TATA box and mRNA start site, and a tandem pair of CpGs is located between the mRNA start site and ATG start codon.
Methylation of the Testis-Specific Histone H1t Gene

A correlation between promoter CpG methylation and transcriptional repression has long been known. In this study we examined transcription by using a histone H1t promoted reporter vector $Pst$-pGL3 in transient expression assays. This expression vector, containing a short histone H1t promoter that extends 141 base pairs (bp) upstream from the transcription initiation site to the $Pst$ site and lacking the upstream silencer element, is active in initiating transcription in several nongerminal cell lines [7].

In this experiment we compared promoter activity of fully methylated to unmethylated vector. Methylation was conducted using $Sss$I methylase. In order to examine the extent of CpG methylation we digested both methylated and unmethylated constructs with a mixture of three methylation-sensitive restriction enzymes $Acl$1, $Hha$I, and $Hpa$II. As shown in lane 3 of Figure 4A, the unmethylated DNA sample was digested at multiple sites by the enzyme mixture yielding high mobility short DNA fragments of various sizes. However, methylation by $Sss$I methylase blocks digestion by these enzymes (lane 2 of Fig. 4A) indicating complete methylation. When we compared the transcriptional activity of the fully methylated construct to the unmethylated construct, we found that transcription of the methylated template was repressed approximately 30-fold (Fig. 4B). Thus, in this transient expression assay, unmethylated plasmid was transcriptionally active while hypermethylation resulted in transcriptional repression.

**DISCUSSION**

The Methylation Status of Testis-Specific Genes

Methylation of DNA and chromatin modification are global mechanisms involved in regulating gene expression. Methylation of DNA in eukaryotes involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA MTase in the context of the sequence 5'-CG-3', which is referred to as a CpG dinucleotide [42]. It is the most common eukaryotic DNA modification and is one of the many epigenetic (alterations in gene expression without a change in nucleotide sequence) phenomena [43]. Although extensive in plants and mammals, the absence of detectable DNA methylation in some eukaryotes such as *Drosophila* and *S. cerevisiae* has raised doubts about its significance in normal development and tissue-specific gene expression [26, 27]. However, recent studies showing abnormal development and embryonic lethality in transgenic mice expressing decreased but not completely absent DNA MTase activity after DNA MTase gene knockout lends support to a critical role for DNA methylation in developmental gene regulation [28].

There are a number of testis-specific and testis-enriched proteins expressed during spermatogenesis. Among these there are core and linker histone variant genes that are expressed specifically or preferentially in the testis. Testis core histone variants include TH2A, TH2B, and TH3 [44]. In addition, an H4t variant gene is expressed at a high level in testis primary spermatocytes, although the peptide sequence is identical to a somatic H4 histone expressed from a gene that is not expressed in testis [41, 45]. It is possible that these testis core histone genes have no special function, but it seems more likely that they play special roles in the altered chromatin structure and the changing pattern of gene expression seen during maturation of germinal cells.

The methylation status of the TH2B-TH2A gene pair has been examined and it has been reported that the TH2B gene is hypomethylated in testis where the gene is expressed compared to other tissues where transcription of the gene is repressed [32]. 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 sper-
produces a 30-fold reduction in expression. The error bars represent the standard error of the mean for measurements of three different samples.

The H1t is the only one of the seven known linker histone gene variants that is expressed exclusively in testis [47]. Although a specific role for the variant histone H1t in gametogenesis has not been assigned, linker histones are essential for higher order chromatin structure [4] and they are involved in regulation of transcription of some genes [3, 5]. Therefore, it is likely that the testis-specific histone H1t is essential for the dramatic changes in chromatin structure and the changing patterns of gene transcription seen during spermatogenesis.

Other testis basic nuclear proteins examined in some detail include the transition nuclear proteins TP1 and TP2 that replace histones in mid-spermatids during spermiogenesis [48]. These transition proteins bind to DNA during the time period when the DNA fiber changes from a beads on a string structure to a smoother fiber, indicating loss of nucleosomes, and the nucleus begins to elongate and condense. The protamines then replace the transition nuclear proteins in mid and late spermatids [48]. The spermatid nucleus becomes more condensed and protamines are responsible for the extreme packaging and stabilization of DNA within the spermatid nucleus. Genes encoding three different mammalian testis-specific nuclear chromatin proteins, mouse transition protein 1, mouse protamine 1, and mouse protamine 2, all of which are expressed postmeiotically, are methylated at early time periods during spermatogenesis in the mouse [47]. Transition protein 1 became less methylated during spermatogenesis, while the two protamines became progressively more methylated. In contrast, the methylation of β-actin, a gene expressed throughout spermatogenesis, does not change. Therefore, both de novo methylation and demethylation events occur after the completion of DNA replication, during meiotic prophase in the mouse testis. Protamine 2, TP2, and approximately 2.8 kb of a CpG island, called CpG island-dTP2, are clustered in a small region surrounding the CpG islands of the TH2A and TH2B genes [46]. Unlike most other tissue-specific genes, the protamine 1, protamine 2, and TP2 genes are located within a large methylated domain in round spermatids, the cell type where they are transcribed [49]. The protamine 1 gene is only partially methylated in somatic cells and in testes from 7-day-old mice. Furthermore, the approximately 2 kb upstream and downstream of the CpG island-dTP2 are only partially methylated in somatic tissues [49].

Methylation patterns of DNA and mRNA expression of a somatic form of LDH, LDH-A, were compared with those of the testis-specific form, LDH-C, in testes from prepubertal and sexually mature mice, from isolated testicular cells, and from somatic tissues [31]. At specific sites, LDH-A was less methylated in adult testis than in spleen DNA with decreased testicular DNA methylation occurring as early as type A spermatogonia. In contrast, DNA methylation patterns for the LDH-C gene did not differ between spleen and testis DNAs. The levels of LDH-A transcripts were low in total testis RNA obtained from 6- to 12-day-old mice, and in type A and B spermatogonia from 8-day-old mice.

FIG. 4. A methylated H1t promoter reporter vector is silenced. A) The H1t promoted expression vector Pst-pGL3 used to test the effects of cytosine methylation on transcription was fully methylated with SssI methylase. To determine whether the methylation reaction was complete, a portion of the unmethylated and methylated samples was digested with a mixture of three methylation-sensitive restriction enzymes AciI, HhaI, and HpaII. The fully methylated sample in lane 2 was essentially undigested compared to the control undigested sample in lane 4. The unmethylated sample in lane 2 was digested extensively as shown by production of several small fragments compared to the undigested sample. B) Transient expression assays were conducted with the unmethylated and fully methylated Pst-pGL3 expression vectors. Cells were transfected with 2 μg of either the unmethylated or the methylated expression vector. After 48 h growth in complete medium, cell lysates were prepared and assayed for firefly luciferase activity as described in Materials and Methods. This figure shows the relative luciferase activity of the unmethylated and methylated vectors and reveals that methylation produces a 30-fold reduction in expression. The error bars represent the standard error of the mean for measurements of three different samples.
old mice. The LDH-A mRNA levels increased gradually in testes from 16- to 45-day-old mice, and LDH-C transcripts were first detectable in the testes of 12-day-old mice and increased as spermatogenesis proceeded. Both LDH-A and LDH-C mRNA levels were low in preleptotene spermatocytes and leptotene/zygotene spermatocytes and increased substantially in pachytene spermatocytes and round spermatids [31].

Several germ line-specific genes, the MAGE-type genes, belong to a unique subset of germ line-specific genes that use DNA methylation as a primary silencing mechanism [33, 34]. Most MAGE-type genes have promoters with a high CpG content, and although CpG-rich promoters are typically unmethylated in all normal tissues, those of MAGE are highly methylated in somatic tissues. In contrast, they are largely unmethylated in male germ cells where they are transcribed [33, 34].

Methylation Status of the Testis-Specific Histone H1t Gene

This is the first study to examine the methylation status of the testis-specific histone H1t gene. To address the question concerning the relationship between DNA methylation and gene expression for this testicular gene, we used bisulfite genomic sequencing to examine CpG methylation within the proximal promoter in cells where the gene is maximally transcribed and in cells where the gene is silenced. This gene is expressed only in primary spermatocytes as demonstrated by the Northern blot data shown in Figure 1B. Although several mechanisms may act synergistically to regulate the transcription pattern of this spermatocyte-specific gene, the contribution of DNA methylation to transcriptional regulation has not been examined previously.

The proximal promoter and coding region of the rat H1t gene have a higher density of CpGs than the surrounding upstream and downstream regions (Fig. 2A). This concentrated pattern of CpGs within the proximal promoter and coding region is common to many genes. Deamination of 5-methylcytosine at a specific site can lead to a heritable change in the DNA at that site (CG to TG transition). This occurs more frequently in noncoding DNA without lethal consequences, and therefore noncoding regions show a scarcity of CpG sequences relative to coding sequences [42]. The CpGs are more abundant in the proximal promoter where transcription factors interact with invariant cis-acting regulatory elements to form the transcription initiation complex. Presumably, mutations within invariant CpGs that are important for protein binding are not tolerated. The proximal promoter of the H1t gene has a cluster of seven CpGs as shown in Figure 2B. Our data indicate that all seven of the CpGs within the H1t proximal promoter are fully unmethylated in vivo in primary spermatocytes where the gene is maximally transcribed. Furthermore, all seven of these CpGs are methylated to some degree in liver where transcription of the gene is repressed. This is the first report of the methylation status of the H1t gene. Thus, the H1t promoter methylation pattern follows the pattern seen in many genes where DNA hypomethylation is found in transcriptionally active genes, and DNA hypermethylation is found in transcriptionally repressed genes [23].

Although there is a strong correlation between H1t proximal promoter hypermethylation and transcription repression in nongerminal cells, these phenomena could be entirely unrelated. In an experiment designed to address this issue, transient expression assays were conducted to compare transcriptional efficiency from fully unmethylated DNA to transcriptional efficiency from fully methylated DNA. The promoter-reporter vector Pst-pGL3 was fully methylated by the enzyme SsI methylase. Failure of methylation-sensitive enzymes to cut the DNA indicated that the reaction was complete (Fig. 4A). When these unmethylated and methylated expression vectors were used in transient expression assays, methylation was found to repress transcription by over 30-fold as seen in Figure 4B. It appears from our data obtained in this transcription assay that there is a link between DNA methylation and transcriptional silencing of the H1t gene.

There are several plausible mechanisms for transcriptional repression by DNA methylation. A methylation-sensitive transcriptional factor may fail to bind tightly to methylated DNA [42, 50, 51]. Failure of transcriptional activators to bind to enhancer elements may silence transcriptional activity. Likewise, failure of repressors to bind to silencer elements may lead to enhanced transcription.

A second potential mechanism for methylation-induced silencing is through the direct binding of specific transcriptional repressors to methylated DNA. Two such factors, MeCP1 and MeCP2 (methyl cytosine binding proteins 1 and 2), bind to methylated CpG residues in any sequence context. Densely methylated DNA associates with transcriptionally repressed chromatin characterized by the presence of underacylated histones. The MeCP2 has been shown to interact with the Sin3/histone deacetylase corepressor complex [52] and this process can be reversed by trichostatin A, a specific inhibitor of histone deacetylase [53]. Other studies have shown that inhibition of histone deacetylases by specific inhibitors can reactivate endogenous genes or reporter constructs previously silenced by DNA methylation [52]. Recently, MeCP1 complex has also been shown to contain histone deacetylase [54]. Thus there appears to be a direct mechanistic link between DNA methylation, histone deacetylation, and transcriptional silencing [52, 53, 55]. Further studies to define the mechanism of methylation-mediated H1t transcriptional repression are presently underway.

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