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The TE Promoter Element of the Histone H1t Gene Is Essential for Transcription in Transgenic Mouse Primary Spermatocytes[1]

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ABSTRACT

Transcriptional activation of the testis-specific histone H1t gene occurs in pachytene primary spermatocytes during spermatogenesis. Specific binding of testis nuclear proteins to a rat histone H1t promoter sequence, designated the H1t/TE element, correlates with the onset of transcription. This element, located between the H1t/AC box and the H1t/CCAAT box, contains inverted repeats of a shorter element. When the native rat H1t gene along with flanking sequences, including 2453 base pairs (bp) upstream and 3784 bp downstream from the coding region, was microinjected into mouse embryos, the offspring of the resulting transgenic mice transcribed the transgene in a tissue-specific manner and only in primary spermatocytes. In the present study the TE promoter element was deleted and replaced with a heterologous stuffer DNA fragment. When the mutant rat DNA fragment was used to create transgenic mice, offspring of the mice bearing the promoter mutation did not transcribe the rat H1t gene in any tissue. On the other hand, transcription of the rat H4t transgene, which is located approximately 1.5 kilobases downstream from the H1t gene, occurred in these animals. Therefore, these studies support the hypothesis that the TE element is essential for enhanced testis-specific transcription of the H1t gene in primary spermatocytes.

INTRODUCTION

Many genes are transcribed in a tissue-specific manner. Some, such as members of the globin gene family, are expressed at different times during development; others are expressed in specific differentiated cell types, such as albumin in liver, renin (Ren2) in kidney, insulin in pancreas, and collagen in connective tissue. *Cis*-acting sequence elements responsible for such differential gene regulation have relevance to gene therapy treatment in that the elements can be used to drive the transcription of therapeutic genes in a specific target cell type [1].

Transgenic animal technology has proved to be a useful tool in localizing *cis*-acting regulatory elements instrumental in directing tissue-specific gene transcription [2]. Transcription of an exogenous gene in an otherwise normal animal permits manipulation and analysis of the regulation of the transgene without necessarily disrupting the physiological functioning of the developing or adult host animal. Injection of the foreign DNA into early embryos results in integration of the transgene into the host genome, and the transgene is stably transmitted to offspring [3, 4]. Use of transgenic mice has helped define *cis*-acting regulatory elements of a number of genes, including the rabbit β-globin gene [5], the human transthyretin gene [6], the human aldolase A gene [7], the mouse protamine 1 gene [8], and the rat H1t gene [9].

Cis-acting regulatory elements themselves are highly variable in position and number. Normal transcription of the β-globin gene requires an enhancer located many kilobases (kb) upstream [5], while the elements necessary and sufficient for tissue-specific transcription of mouse protamine gene [8] and rat histone H1t gene [9–11] are contained within restriction fragments of 2.4 kb and 141 bp, respectively. The human aldolase A gene utilizes different combinations of three promoters and two enhancers for transcription in specific muscle types [7]. Tissue specificity of human transthyretin gene transcription requires elements contained within 6 kb upstream of the gene [6].

H1t is a testis-specific histone H1 variant gene that is transcribed only in pachytene spermatocytes [12]. The H1t promoter shares several elements with promoters of somatic H1 variants, including the H1/AC box, the H1/CCAAT box, and the TATA box. Additionally, H1t promoters have an element designated the TE element that is common to several mammalian species [13–15]. Nuclear proteins from testis, but not from other tissues, bind the TE element with high affinity [11, 16, 17]. In addition to containing similar TE elements, mouse and rat H1t genes exhibit identical patterns of transcriptional regulation [13, 14, 18]. Furthermore, a 6.85-kb *Eco*RI fragment of rat DNA containing the H1t and the H4t genes [13, 19] also contains all the elements necessary for tissue-specific transcription of the rat H1t in transgenic mice [9, 10].

In order to test the hypothesis that the histone H1t TE element is involved in enhanced spermatocyte-specific expression, we replaced the TE element with heterologous stuffer DNA. The stuffer DNA was created by annealing two oligonucleotides representing a sequence from the coding region of the H1t gene that had been shown previously not to bind testis nuclear proteins [17]. In this study a 5.4-kb *Pvu* II-*Eco*RI fragment containing this replacement mutation was used to create a second strain of transgenic mice, and tissues from mice containing the mutant rat H1t promoter were examined for expression of the transgene. Data from another laboratory [10] reveal that the *Pvu* II-*Eco*RI fragment promotes enhanced testis-specific H1t transcription in transgenic mice at a level comparable to that provided by the 6.85-kb *Eco*RI fragment. Removal of the upstream region does not alter tissue-specific transcription in transgenic mice [10].

MATERIALS AND METHODS

Reagents and Supplies

Radiochemicals were purchased from New England Nuclear (Boston, MA). X-OMAT XAR-5 x-ray film (Eastman

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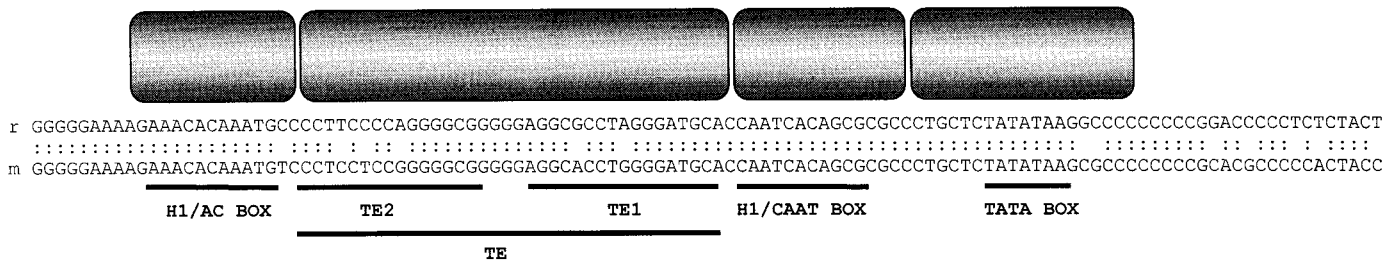

FIG. 1. Model of the histone H1t promoter. The H1/AC box, the H1/CAAT box, and the TATA box are common to all H1 promoters. The TE element, located between the H1t/AC box and H1t/CAAT box and shown subdivided into TE1 and TE2, is conserved across species. TE1, TE2, and the larger complete TE element bind specifically to testis nuclear proteins. Rat (r) and mouse (m) sequences are shown in this figure with aligned conserved bases indicated by dots. Shaded boxes represent potential DNA-binding proteins or protein complexes. The TE element was the focus of this study.

Kodak, Rochester, NY) and Cronex-7 x-ray film (DuPont, Boston, MA) were purchased from Sigma (St. Louis, MO) and Sterling Diagnostic Imaging (Greenville, SC). Nytran membrane for Southern blots and 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). DNA sequencing was performed with reagents from the fmol DNA sequencing kit from Promega (Madison, WI) with universal primers end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclease S1 and calf intestinal alkaline phosphatase were purchased from Boehringer-Mannheim (Indianapolis, IN). Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and Boehringer-Mannheim. Low-melting SeaPlaque GTG agarose was purchased from FMC Corp. (Rockland, ME). Oligonucleotides were obtained from Biosynthesis, Inc. (Denton, TX) and from Oligos Etc. (Wilsonville, OR). These were used as primers for DNA sequence analysis, for PCR amplification of segments of the rat and mouse H1t gene, and for preparation of probes for S1 nuclease protection analysis. Universal forward and reverse sequencing primers were obtained from New England Biolabs. RNA Stat-60 was ordered from Tel-Test, Inc. (Friendswood, TX).

Probes and Primers

All radiolabeled probes and primers were end-labeled using T4 polynucleotide kinase with [γ-^{32}P]ATP. Construction of a rat H1t-specific probe for S1 nuclease protection analysis has been described [9]. Briefly, the probe consists of a 1140-base pair (bp) *Xba* I-*Ava* I fragment cut from plasmid pHD1, containing the 5′ end of the rat H1t gene, which hybridizes to approximately 181 bases of rat H1t mRNA.

The probe for S1 nuclease protection analysis of rat H4t mRNA was derived from plasmid pPS7 by digestion with *Sau*3A I and gel purification of the 1.7-kb fragment containing the 5′ end of the rat H4t coding sequence and approximately 1.48 kb of upstream sequence [19]. Primers designated rat N-term (5′-ACTCCAGCGCTGTTCGCTCT-3′), rat N-term (−) (5′-GTACAGGAGAGCGAACAGCG-3′), and H1/AC box (5′-GGGGGAAAAGAAACA-CAAAT-3′) were obtained for sequencing.

Construction of the TE Replacement Promoter Mutation

Two oligonucleotides that represented the *Hin*dIII site within the coding region of a human histone gene and that had a 10-bp overlap were annealed. The ends were filled using the Klenow fragment of *Escherchia coli* DNA polymerase I to generate a double-stranded DNA fragment that was used to replace the rat H1t TE element. The normal H1t promoter (Fig. 1) contained in the plasmid pHD1 [19] was digested with *Eco*N I and *Avr* II (Fig. 2A), and the ends were blunted by digestion with S1 nuclease. The double-stranded replacement oligonucleotide was cloned into the opened pHD1 to form pHD1m (Fig. 2B). Plasmid pPS3 [13, 19] was digested with *Eco*RI, and the released 6.85-kb rat genomic insert was moved into the *Eco*RI site of pUC18, creating pPS3–18. The plasmids pPS3–18 and pHD1m were digested with *Sca* I, the *Sca* I fragment from pPS3–18 was eliminated, and the *Sca* I fragment from pHD1m was ligated into the vacant *Sca* I site of pPS3–18, creating pPI3 (Fig. 2B).

A fragment approximately 5.4 kb in length was excised from pPI3 by digestion with *Bam*HI and *Eco*RI. This fragment, which contained rat genomic DNA extending from the *Pvu* II site to the *Eco*RI site, was sent to DNX Corporation (Princeton, NJ) for injection into mouse embryos to create transgenic mice (Fig. 2, B and C).

Animal studies were conducted in an AAALAC, Inc. accredited facility in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Detection of the Transgene in Mice

PCR amplification of the transgene from genomic DNA purified from tail snips was performed as previously described [9]. For PCR amplification, 1 μg of DNA template was used with primers corresponding to the rat H1t transcription start site (5′-ACTCCAGCGCTGTTCGCTCT-3′) and the rat post-hairpin 5′-AGTGTTTGGCCATTTTAA-GA-3′), which amplify a 771-bp product. Amplified PCR products were visualized on 1% agarose gels, and the source of any 771-bp product was verified by digestion with *Pst* I, which produces 90- and 681-bp fragments from the rat H1t but does not digest the mouse H1t [9].

S1 Nuclease Protection Assays

RNA was harvested from various tissues from transgenic animals using RNA Stat-60 according to the manufacturer's protocol. For each S1 nuclease protection assay, 50 μg of total cellular RNA was coprecipitated with DNA probe representing 100 000 dpm. Hybridization, digestion, and electrophoresis were performed as described previously [9]. After electrophoresis, the gel was soaked in a 10% methanol, 10% acetic acid solution, dried, and exposed to Kodak XAR-5 film to detect labeled bands representing protected probe.

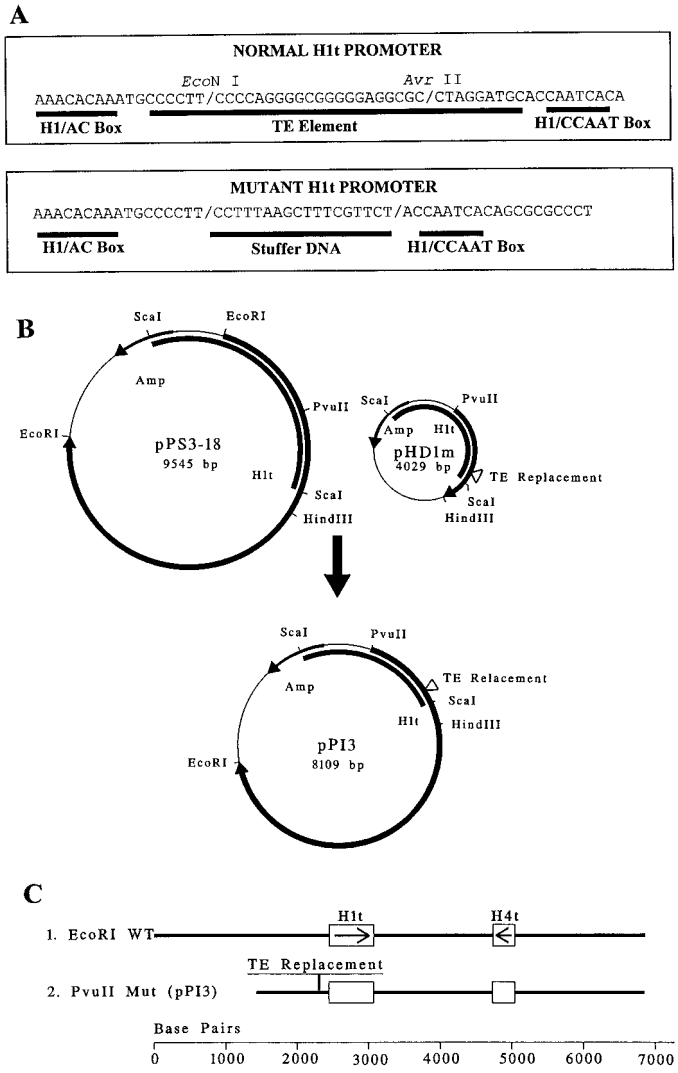

FIG. 2. **A**) Comparison of wild-type and mutant promoter sequences. A mutant H1t promoter was produced by excision of the TE element and replacement with heterologous DNA. This particular replacement sequence was selected in part because it was known that testis proteins do not to bind specifically to the sequence. **Top**) A portion of the native H1t proximal promoter showing the restriction sites *Eco*NI and *Avr* II used to excise the central part of the TE element in order to generate the replacement mutation. **Bottom**) The H1t proximal promoter with the TE sequence substitution obtained as determined by sequencing. Slashes mark the boundaries of insertion of the heterologous stuffer DNA fragment. **B**) Construction of plasmid pPI3 containing the TE replacement mutation. The plasmid pPI3, containing the TE replacement mutation, was constructed by moving the mutant *Sca* I fragment from the plasmid pHD1m (upper-right plasmid in the figure) into the *Sca* I site of pPS3–18, a plasmid containing the normal H1t promoter (upper-left plasmid in the figure). The following steps were taken to construct pPI3 (the restriction fragments referred to are indicated by heavy lines). First, the plasmid pPS3–18 was constructed by moving the entire 6.85-kb rat genomic *Eco*RI insert containing the histone H1t and H4t genes from pPS3 (a pUC9-based plasmid) into pUC18. Next, the plasmid pHD1m was constructed by excising the TE element from the rat promoter in pHD1 (which contains the normal *Pvu* II-*Hin*dIII fragment from pPS3 cloned into the vector pUC18) by digestion with *Eco*NI and *Avr* II and replacing it with the heterologous stuffer fragment shown in panel A, upper sequence. Finally, pHD1m and pPS3–18 were digested with *Sca* I, and the *Sca* I fragment from pHD1m (containing the mutation) was excised and cloned into the vacant *Sca* I site of pPS3–18 to generate the plasmid designated pPI3. **C**) DNA samples used for preparation of transgenic mice and for transient expression assays in this study. Two DNA samples were used for preparation of transgenic mice. The samples are 1) *Eco*RI WT—the histone H1t gene in this 6.85-kb *Eco*RI wild-type genomic fragment was expressed in a tissue-specific manner in transgenic mice (Fig. 5, WT T); 2) *Pvu* II Mut (pPI3)—the TE element in the proximal promoter of the H1t gene was replaced with a stuffer DNA fragment as shown in Figures 1 and 2A (transcription of the rat H1t gene was abolished in transgenic mice bearing this mutant fragment, although the downstream H4t gene was expressed).

PCR Amplification and Sequencing of the Transgene Promoter

Genomic DNA from tail snips of animals positive for the transgene was amplified by PCR as previously described [9] using the H1/AC box (5′-GGGGGAAAA-GAAACACAAAT-3′) and post-hairpin primers (described above), which amplify both rat and mouse H1t [18], producing an 883-bp product. The PCR products were gel purified by the hot phenol extraction procedure [20] and digested with *Pst* I, which releases a 202-bp fragment from the 5′ end of the rat H1t PCR product but does not digest mouse H1t. The 202-base fragment was gel purified as before and sequenced using the fmol kit with the rat N-term (−) (described above) as sequencing primer. The resulting sequence was identical to the sequence of the pPI3 insert, which verified the presence of the transgene.

Southern Blotting

Genomic DNA was isolated from the testes of normal rats and normal mice and from tail snips of transgenic mice [21]. Each 10-μg sample was digested with *Bam*HI and *Eco*RI to release the mutant transgene or the normal 6.85-kb *Eco*RI fragment, which lacks a *Bam*HI site, from normal rat genomic DNA, which contains the H1t gene. The digested DNA samples were electrophoresed through a 0.6% agarose gel, denatured, neutralized, and blotted to a maximum-strength Nytran membrane by capillary transfer. DNA was immobilized on the membrane by cross-linking with 2.4×10^5 μJ of 254 nm light in a Stratalinker 1800 (Stratagene, LaJolla, CA). Prehybridization, hybridization, and washing conditions have been described [9]. The probe used was the *Mae* III fragment excised from pJA19 with *Eco*RI and *Bam*HI, followed by random-primed labeling with [α-^{32}P]dCTP [13].

RESULTS

Construction of the Replacement Mutant of the Histone H1t/TE Promoter Element

It had been shown previously that the rat histone H1t proximal promoter contains an element designated the TE element in addition to the elements common to somatic, cell cycle-regulated H1 genes. This element, present and conserved in mammalian H1t promoters among all species examined, consists of an imperfect inverted repeat that specifically binds testis nuclear proteins [15, 16]. Figure 1 shows relative positions of the H1/AC box, the testis-specific TE element, the H1/CAAT box, and the TATA box. Appearance of TE-binding proteins in primary spermatocytes was shown to correlate temporally with initiation of enhanced transcription of the H1t gene during spermato-

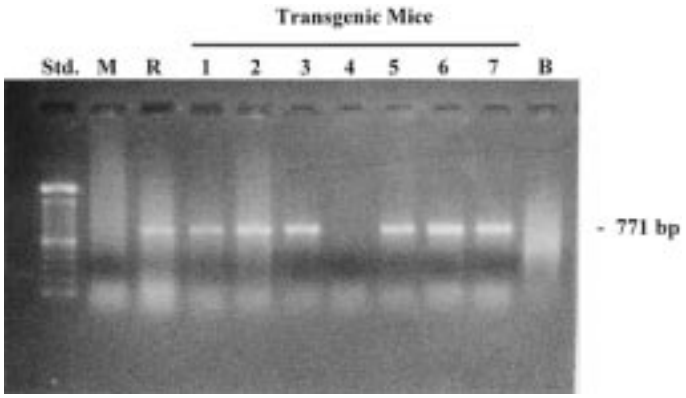

FIG. 3. PCR analysis of transgenic mouse DNA. The standard lane (Std.) contained a 100-bp ladder as a size standard. The lane designated M used mouse DNA as a negative control template with the rat N-term and rat post-hairpin primers (*Materials and Methods*). Lane R contained a 771-bp amplified product obtained using normal rat DNA as a positive control template. Lanes numbered 1–7 contained amplified products obtained using DNA templates from pups from transgenic parents. Lane B contained the amplified product using no template as a negative control.

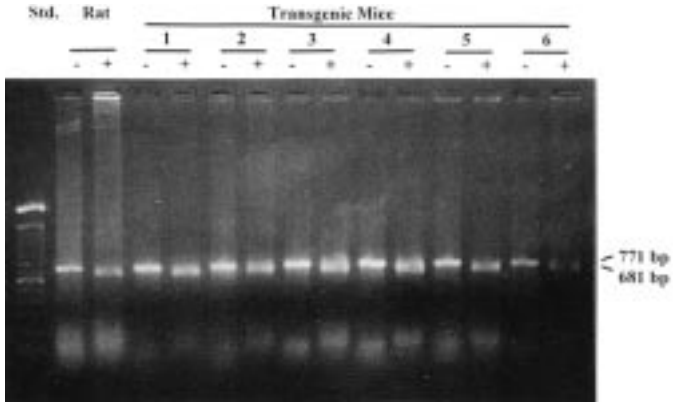

FIG. 4. *Pst* I digestion of PCR products. The first lane (Std.) contained a 100-bp ladder as a size standard. The next two lanes contained the PCR products obtained using rat DNA as a positive control template and using the same primers as in Figure 3. The remaining lanes contained PCR products obtained using DNA templates from six pups. Lanes designated (−) show undigested PCR products, and adjacent lanes to the right designated (+) show the same samples digested with *Pst* I. When the 771-bp product obtained using normal rat DNA as a template was cut with Pst I, two bands with lengths of 681 and 90 bp were produced (the 90-bp bands are not visible in this figure).

genesis [17]. The 6.85-kb *Eco*RI fragment from rat containing both the H1t and H4t genes has all necessary and sufficient *cis*-acting sequences to effect enhanced spermatocyte-specific transcription of the rat H1t in transgenic mice [9].

To determine the importance of the TE element in spermatocyte-specific transcription, the element was deleted and replaced with a stuffer fragment to partially conserve the spacing of the conserved elements. Figure 2A shows the sequence of normal and mutated TE elements. The mutant element was most likely shortened when S1 nuclease was used to form blunt ends from the promoter that had been cut with the restriction enzymes *Eco*NI and *Avr* II. A plasmid designated pHD1m (Fig. 2B) containing the H1t gene with this replacement was constructed first. The *Sca* I fragment containing this mutant sequence was removed from pDH1m and cloned into the plasmid pPS3–18 from which the normal *Sca* I fragment had been excised. The resulting plasmid designated pPI3 is shown in Figure 2B.

The plasmid pPI3 was digested with *Pvu* II and *Eco*RI to remove the mutant transgene (designated *Pvu* II Mut [pPI3] in Fig. 2C), and the excised fragment was used to prepare transgenic mice for the present study. For comparison, the full-length normal rat *Eco*RI genomic DNA fragment used in our initial transgenic mouse experiment [9] is shown as the first DNA fragment (designated *Eco*RI WT in Fig. 2C). The DNA used for the second transgenic experiment is truncated at the *Pvu* II site and is 1436 bp shorter than the full-length *Eco*RI fragment in addition to containing the TE replacement mutation. The truncation does not significantly alter transcription in transgenic mice [10]. Normal and mutant DNA fragments contained the rat histone H4t gene, which served as an internal positive control in this study.

Wild-Type but Not the Mutant Rat H1t Gene Was Transcribed in Mice Bearing the Transgene

There were at least two possible outcomes in this transgenic mouse study. If the TE element participates in silencing transcription of the H1t gene in nongerminal cells in vivo, we expected to see possibly inappropriate basal or enhanced transcription in many cell types in transgenic mice containing the TE replacement mutation. On the other hand, if the TE element normally participates in enhancing transcription in primary spermatocytes in vivo, we expected to see low basal transcription or no transcription in transgenic mice. Data obtained from this study support the second possibility. The founder generation consisted of six positive animals sent by DNX Corp. One female died before shipping; the remaining five, two females and three males, were bred to normal C57Bl/6 mice.

DNA samples prepared from members of the F1 generation were examined for the presence of the transgene by PCR amplification using the rat N-term and post-hairpin primers (*Materials and Methods*). Variable levels of amplified products from F1 generation animals are shown in lanes 1 through 7 of Figure 3. This figure also shows important positive (R) and negative (M and B) PCR controls, and it shows that animal number 4 did not inherit the transgene. Since the rat but not the mouse gene contains a *Pst* I restriction site between these primers, the amplified products were digested with *Pst* I to confirm the presence of the rat genomic DNA sequence. In Figure 4, lanes marked with a plus, showing digested samples, are compared to lanes marked with a minus, showing undigested samples. Selected sexually mature males containing the mutant transgene were killed, and total cellular RNA was harvested from tissues for S1 nuclease protection analysis (Fig. 5). No rat H1t message was detected in testis, liver, or any other tissue examined in mice containing the mutant transgene. In this figure we show the results from one of five lines examined (lanes marked MUT). On the other hand, rat H1t mRNA was detected in testis but not liver of mice containing the normal transgene. In this figure we show the results from one of six lines examined (lanes marked WT). Normal rat (lanes marked R) and normal mouse (lanes marked M) positive and negative controls, respectively, are also shown in this figure.

Rat H4t Transgene Was Transcribed in Mice Bearing the Transgene

The lack of detectable rat H1t mRNA could be ascribable to the promoter mutation or to local chromatin struc-

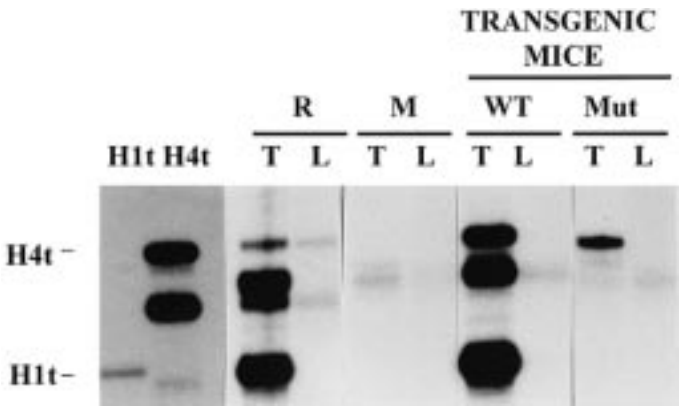

FIG. 5. S1 Nuclease protection analysis to detect rat H1t and H4t mRNA in RNA samples from transgenic mice. The first lane shows the relative electrophoretic mobility of the DNA fragment protected by hybridization to 50 μg of rat testis RNA using the H1t probe as a positive control. The second lane shows the relative mobilities of the DNA fragments protected by hybridization to rat testis RNA using the H4t probe as a positive control; only the upper band (the one with lowest mobility) was used to monitor H4t mRNA levels. R, M, and WT indicate control RNA samples from a normal rat (R), control RNA samples from a normal mouse (M), and control RNA samples from one representative member of six transgenic lines used in this experiment bearing the normal H1t transgene (WT) (the normal transgene is shown in Fig. 2C). Mut indicates RNA samples from one representative member of five transgenic lines used in this experiment bearing the mutant TE element (the mutant transgene shown in Fig. 2C). T and L represent testis RNA and liver RNA samples, respectively.

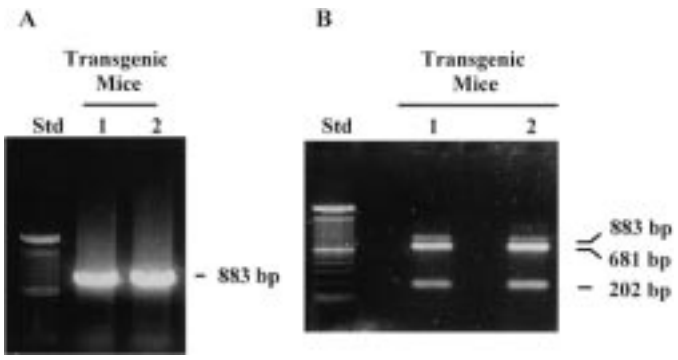

FIG. 6. PCR amplification of rat and mouse H1t genes from transgenic mice. **A**) Product amplified from transgenic mice. The first lane (Std) shows a 100-bp ladder as a size standard. Lanes marked 1 and 2 show the 883-bp PCR products amplified from the rat transgene and the mouse H1t gene when DNA templates from two different transgenic mice were used with the H1/AC box primer and the rat post-hairpin primer (*Materials and Methods*). **B**) *Pst* I digest of PCR products, releasing a 202-bp fragment exclusive to rat. The first lane (Std) shows a 100-bp ladder as a size standard. Lanes marked 1 and 2 show the same samples shown in lanes 1 and 2 of **A**. When these two PCR products were digested with *Pst* I, the products amplified from mouse H1t templates were not cut with *Pst* I and therefore yielded the 883-bp bands. The 681- and 202-bp fragments are *Pst* I restriction fragments of the rat H1t products. The 202-bp band was excised from the gel, purified, and sequenced using the rat N-term (−) primer (*Materials and Methods*) to verify the presence of the TE replacement mutation.

ture that excluded the transcription machinery in the transgenic mice. To determine whether the rat H1t gene was located in a transcriptionally favorable chromatin environment, and to verify loading accuracy, the same RNA samples were subjected to further S1 analysis to detect transcription of the neighboring rat H4t gene. Several mice had relatively high steady-state levels of mRNA that hybridized to the rat H4t probe (Fig. 5), suggesting that environments of the transgenes containing the rat H1t and H4t genes were favorable for transcription of the rat H4t gene, yet no measurable mRNA hybridized to the rat H1t probe contained in the same reaction (Fig. 5). These data suggest that the H1t/TE replacement mutation abolished transcription of the H1t gene in the transgene that presumably was accessible for transcription.

Confirmation That the Expected Rat H1t/TE Mutation Was Present in Transgenic Mice

To verify that the correct mutation was present in the transgene, a fragment of the transgene extending from the H1/AC box downstream to the *Pst* I site that is exclusive to the rat H1t gene was amplified and sequenced, and the sequence was compared to that of the mutant promoter using the plasmid pPI3 as a template. Specifically, a 202-bp fragment was isolated by amplifying the H1t gene from transgenic mouse DNA template using the H1/AC box and post-hairpin primers (*Materials and Methods*) common to both rat and mouse H1t genes (Fig. 6A). The isolated fragment was digested with *Pst* I to yield three products: an uncut 883-bp endogenous mouse H1t fragment and 681- and 202-bp digestion products from the rat transgene (Fig. 6B). The 202-bp fragment was sequenced and found to contain the promoter mutation, and the sequence was identical to the sequence in the promoter of plasmid pPI3.

We had noted previously that the wild-type rat H1t transgene was present in variable copy number in transgenic mice [9]. H1t is normally a single-copy gene, but even in a transgenic animal bearing an estimated 20 copies of the transgene, the rat testis H1t mRNA steady-state level was found to be no more than 2.6 times the normal level. While we feel that copy number is not a major factor in the lack of transcription of the mutant transgene, we were interested in determining whether copy number showed the same variation in this second study. Southern blotting showed that copy number was indeed variable but the levels of transcription of the rat H4t transgene appeared to correlate with copy number (data not shown).

DISCUSSION

Transgenic mouse technology has been used to characterize many tissue-specific promoters. It allows researchers to study regulation of gene transcription in vivo, confirming the importance of conserved regulatory sequences by mutation analysis and revealing novel regulatory sequences by means of serial deletion of flanking regions.

H1t is one of seven known linker histone H1 variants found in mammals, and the only one that is tissue specific. H1t is expressed only in pachytene primary spermatocytes during prophase I of meiosis. The somatic H1 histones H1a, H1b, H1c, H1d, and H1e are expressed maximally during S-phase of the mitotic cell cycle, while H1° is expressed constitutively in terminally differentiated cells. The nucleated erythrocyte-specific histone H5 is homologous to the mammalian H1° and is the only H1 so far to have been crystallized [22].

Our previous study using the wild-type 6.85-kb *Eco*RI fragment demonstrated that the rat H1t gene and its transcription could be detected in a mouse background, and that enhanced tissue-specific transcription of the transgene occurred in all transgenic mice examined. Therefore, all sequence elements necessary and sufficient for tissue-specific transcription of the rat H1t lie within the 6.85-kb *Eco*RI

restriction fragment. One possible candidate for such an element, a conserved imperfect inverted repeat, lies within the proximal promoter and has been designated the TE element.

The TE element is conserved among H1t promoters from several species, including the rat, mouse, human, and monkey [13, 18, 23–26]. This element specifically binds testis nuclear proteins, whose appearance coincides with initiation of transcription of the H1t gene [11, 16, 17]. With this evidence in mind, the experiments in the present study were designed to determine the importance of the TE element for enhanced tissue-specific transcription.

The TE element was deleted from the proximal promoter of the rat H1t gene (Fig. 2A) and was replaced with a stuffer fragment of DNA to conserve spacing between the other promoter elements. This stuffer fragment had previously been shown not to compete with the TE element for protein binding [17]. The substitution abolished transcription of the rat histone H1t gene in testes of all transgenic mice examined. Furthermore, no H1t gene transcription was detected in any of the other tissues tested, which included brain, liver, kidney, and spleen. In control experiments the histone H4t gene, located 1.5 kb downstream from the H1t gene in the rat transgene, was found to be transcribed in testes of these transgenic mice, indicating that the conditions preventing transcription of the H1t gene do not prevent transcription of the H4t gene.

Another laboratory has shown that the normal rat H1t promoter is able to promote low-level transcription in transiently transfected somatic cells [27]. Our laboratory has found that the normal rat H1t promoter provides low-level transcription of a luciferase reporter gene in transiently transfected mouse C127 cells, but the mutant H1t promoter with the same TE replacement used in the present study provides no transcriptional activity [28] (unpublished results). The mutant histone H1t construct that lacks the TE element but contains all of the conserved promoter elements in common with somatic H1 genes presumably no longer supports even basal transcription. These data suggest that the TE element of the histone H1t gene is essential for enhanced spermatocyte-specific transcription in vivo (in normal animals and in transgenic mice), as well as for low-level transcription of the H1t gene that is mediated by mitotic cell cycle-regulated transcription factors in transient expression assays.

In related studies using CAT-reporter gene transcription driven by the human histone H1t promoter, it was shown that the H1/CCAAT box and its cognate binding protein are essential for the elevated gene transcription seen during S-phase of the cell cycle in transiently transfected HeLa cells [29]. However, our recent experiments have shown that transcription driven by the H1t promoter occurs at a low level in differentiated cells compared to the enhanced transcription normally seen in primary spermatocytes. In exponentially growing cultured HeLa cells and in HeLa cell populations enriched by centrifugal elutriation to a level at which 80% of the cells are located within S-phase of the mitotic cell cycle, human histone H1t-promoted CAT gene transcription was detectable but low [29]. In addition, steady-state levels of histone H1t mRNA in all nongerminal cell types (including HeLa cells) and in all animal tissues that have been examined in our laboratory are below detectable limits using Northern blot analysis and S1 nuclease protection analysis. The normal transcriptional silencing of the histone H1t gene seen in nongerminal cells may be due in part to a sequence between the TATA box and the message start site [30] and in part to sequences upstream from the H1t/AC box (unpublished results).

Taking all of these experimental results into account, the potential role of the histone H1t/TE element in enhanced spermatocyte-specific transcription takes on greater importance. Therefore, in future experiments we will continue to investigate the TE element and its cognate binding proteins in order to determine their role in enhanced transcription in primary spermatocytes. In addition, the possibility of using the testis-specific H1t promoter to transcribe desired therapeutic or cytotoxic genes specifically in primary spermatocytes is attractive, and the tightly regulated histone H1t tissue-specific promoter appears to be ideal for such studies.

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