Testis-Specific Expression of the Rat Histone H1t Gene in Transgenic Mice†

Jane M. vanWert, Steven A. Wolfe, and Sidney R. Grimes*

Research Service, VA Medical Center, and Department of Biochemistry and Molecular Biology, LSU Medical Center, Shreveport, Louisiana 71101-4295

Received March 6, 1995; Revised Manuscript Received April 26, 1995®

ABSTRACT: The testis-specific histone H1t gene is transcribed only in testis. The appearance of testis-specific nuclear proteins that bind to a unique promoter sequence element designated H1t/TE located between the H1/AC box and the H1/CCAAT box correlates with the onset of transcription of the H1t gene during the meiotic cell cycle. In order to determine whether sequences flanking the rat H1t gene are sufficient to confer tissue-specific expression *in vivo*, a 6859 bp *Eco*RI restriction fragment of genomic DNA containing the rat histone H1t gene has been microinjected into mouse embryos. S1 nuclease protection analysis has shown that the descendants of the resulting transgenic mice express the rat gene in the proper tissue and at the proper meiotic cell cycle stage. Furthermore, when populations of mouse testis cells were prepared by centrifugal elutriation, only the fraction enriched in pachytene primary spermatocytes had a significant steady-state level of rat H1t mRNA. Although the copy-number of the transgene was variable in these animals, rat H1t mRNA levels in high copy-number animals never exceeded 2.6 times the level in normal rat testes. The appearance of appropriate meiotic cell cycle-specific transcription indicates the importance of the conserved promoter sequence elements between the two species.

Transgenic mouse technology is important in the study of regulation of gene expression (Simoni, 1994). This technique has become an indispensable tool for the *in vivo* analysis of functionally important regions of promoters for tissue-specific mammalian genes. In this respect, the technique has been utilized in the study of testis-specific genes such as lactate dehydrogenase [$LDHC_4$; (*S*)-lactate:NAD^+ oxidoreductase, EC 1.1.1.27] and protamine (Salehi-Ashtiani et al.; 1993, Peschon et al., 1987). $LDHC_4$ is a single-copy gene and is expressed solely in testis under stringent developmental and temporal regulation (Blanco, 1991). Studies of the $LDHC_4$ gene have led to the identification of *cis*-acting regulatory elements important for testis-specific expression (Zhou et al., 1994). Likewise, the study of the spermatid-specific transcription of the protamine genes has led to the identification of shared sequences that possibly contribute to regulation of expression of these haploid-specific genes (Johnson et al., 1988). More importantly, these studies allow investigators to correlate *in vivo* promoter function with *in vitro* DNA−protein interactions (Tamura et al., 1992; Johnson et al., 1991; Zambrowicz & Palmiter, 1994).

Our studies have focused upon mechanisms contributing to the regulation of expression of the testis-specific histone H1t gene. Most histone genes are expressed during S-phase of the cell cycle, and it is likely that their transcription is controlled by cell cycle regulatory factors possibly including CDC2, cyclin A, and RB-related proteins (vanWijnen, 1994). However, histone H1t is testis-specific and expressed only in cells in prophase of the meiotic cell cycle, cells designated pachytene primary spermatocytes (Kistler et al., 1973; Branson et al., 1974; Grimes et al., 1986).

Histone H1t, like other H1 family members, has a tripartite organization consisting of an N-terminal domain, a highly conserved central globular domain, and an extremely basic C-terminal domain. The central globular domains of histones H1 and H5, the H1 variant found in nucleated erythrocytes, are evolutionarily conserved. The isolated globular domain of H5 has been shown to bind to DNA (Krylov et al., 1994), and X-ray diffraction studies of crystals formed from this region reveal two probable DNA binding sites in that area (Ramakrishnan et al., 1993). It is possible that the H1t globular domain has similar DNA binding sites.

In general, histones are small, basic proteins found in abundance in most eukaryotic cells. The histone H1 family variants bind where the DNA enters and leaves the nucleosome (Finch & Klug, 1976). H1 protects an additional 20 base pairs of DNA from nuclease digestion and mediates the packing of chromatin into the 30-nm filament (Thoma et al., 1979; Hayes & Wolffe, 1993) most likely being located in the interior of the filament (Graziano et al., 1994). The presence or absence of nucleosomes and H1 molecules appears to regulate transcription by sequestering or exposing DNA to transcriptional activators (Wolffe, 1994). Thus, one role of H1 histones appears to be to maintain chromatin in the condensed state. It has been shown that, when compared to somatic histone H1 variants, germinal H1t binds DNA with lower affinity, rendering it more sensitive to nucleases. It has been suggested that the special role of H1t is to maintain meiotic chromatin in a relatively decondensed state, permitting such events as recombination (De Lucia et al., 1994; Khadake et al., 1994).

Previous work revealed that histone H1t synthesis occurs only in prophase of the meiotic cell cycle (Bucci et al., 1982) and is retained in haploid early spermatids until it is replaced by transition nuclear proteins TP1 and TP2 (Grimes et al.,

† This research was supported by the Veterans Administration and by NIH Grant RO1 HD29381. Transgenic mice were produced by DNX Corp., Princeton, NJ, contracted by the National Institute of Child Health and Human Development Transgenic Mouse Development Facility (NTDF) under Contract NO1-HD-0-2911 in support of DNX, Inc.

® Abstract published in *Advance ACS Abstracts,* July 1, 1995.

1977). Accumulation of H1t mRNA is maximal in pachytene primary spermatocytes (Grimes et al., 1992a; Smith et al., 1992), but it is absent or present at very low steady-state levels in earlier germinal cell types and in early spermatids. Therefore, there is a direct correlation between H1t mRNA accumulation and histone H1t synthesis in pachytene primary spermatocytes.

More recent studies of the histone H1t gene revealed a unique promoter sequence located between the H1/AC box and the H1/CCAAT box (Grimes et al., 1990; Doenecke et al., 1994; Wolfe & Grimes, 1993; Wolfe et al., unpublished results). Testis-specific binding of nuclear proteins to this sequence element, designated TE, has been found (Grimes et al., 1992a). The appearance of the testis-specific binding proteins correlates with the onset of transcription of the H1t gene during spermatogenesis (Grimes et al., 1992b). Consistent with these observations, a conserved sequence within the promoters of a number of mammalian testis-expressed genes has been identified by comparison of published gene sequences (Queralt & Oliva, 1993), and the rat H1t/TE element is located within this conserved potential regulatory sequence. Furthermore, a portion of the H1t TE element is shared with the testis-specific $LDHC_4$ promoter and has been shown in an *in vitro* transcription assay to be necessary and sufficient for directing testis-specific transcription (Zhou et al., 1994).

A genomic DNA fragment containing the rat histone H1t gene, 2.5 kb of upstream sequence, and 3.8 kb of downstream sequence was used to generate transgenic mice in order to determine whether the fragment contained functionally important sequence elements and especially those essential for the appropriate testis-specific expression of the H1t gene. In this paper, we show that this rat genomic DNA fragment is sufficient to confer testis-specific expression of the rat H1t gene in transgenic mice. Since mouse transcription factors recognize and regulate rat H1t gene expression in the expected tissue-specific manner, this is a dramatic demonstration that functionally important promoter sequence elements essential for tissue-specific transcription are present in the cloned rat DNA fragment.

MATERIALS AND METHODS

Reagents and Supplies. Radiochemicals were purchased from New England Nuclear. An oligolabeling kit was obtained from Pharmacia. The fmol DNA sequencing kit was ordered from Promega, and DNA sequencing was performed with universal primers end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. S1 nuclease and calf intestinal alkaline phosphatase were purchased from Boehringer-Mannheim. Restriction enzymes were obtained from New England Biolabs and Boehringer-Mannheim. Low-melting agarose was purchased from FMC Corp. Deoxynucleotides and Ampli-Taq for polymerase chain reaction (PCR)[1] were purchased from Perkin-Elmer. X-ray film was purchased from Eastman Kodak (X-OMAT XAR-5) and DuPont (Cronex-7). Oligonucleotides were obtained from Biosynthesis, Inc., Denton, TX, and from Oligos Etc., Wilsonville, OR. These were used 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. Oligonucleotides and the numbering system to identify base numbers are described in Table 1 and Figure 1B.

Isolation of Normal Tissues and Enriched Populations of Testis Cells. Male C57-Black mice were obtained from Harlan (Dallas, TX), and male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Houston, TX). Animals were maintained in an AAALAC-accredited facility. Normal animals and transgenic mice (described in the next section) were sacrificed, and tissues were removed for preparation of DNA and RNA. Enriched populations of mouse testis germinal cell types were prepared by centrifugal elutriation. Trypsin was used to prepare single-cell suspensions from 6−10 mice, and elutriation was conducted as described previously (Grabske et al., 1975; Grimes et al., 1990; Meistrich et al., 1981). Five fractions were eluted using the following rotor speeds and flow rates: fraction 1, 3000 rpm, 13.5 mL/min; fraction 2, 3000 rpm, 17.9 mL/min; fraction 3, 3000 rpm, 31.3 mL/min; fraction 4, 2000 rpm, 23.2 mL/min; fraction 5, 2000 rpm, 40 mL/min.

Preparation of Transgenic Mice. A 6859 bp *Eco*RI restriction fragment of genomic DNA containing the rat histone H1t and H4t genes (Figure 1A) was cut from the plasmid pPS3 (Grimes et al., 1987, 1990; GenBank Accession No. M28409) and was microinjected into mouse embryos by DNX Corp., Princeton, NJ. Animals were prepared under contract by the National Institute of Child Health and Human Development Transgenic Mouse Development Facility (NTDF) (Contract NO1-HD-0-2911 in support of DNX, Inc.). Although rat H1t and the mouse H1t gene sequences are conserved, the sequence differences allowed the design of species-specific primers that could be used to identify animals bearing the H1t transgene. Nine mice with the rat H1t transgene were bred to C57-Black mice. Genomic template DNA from tail snips of F1 generation mice was prepared (Enrietto et al., 1983) and examined for the presence of the transgene by PCR amplification using a rat-specific 5′ primer (5′-ATCACTGTTGGCTCTCCTTTAG-3′) and a 3′ primer designated the post stem−loop primer (5′-AGTGTTTGGCCATTTTAAGA-3′). This and other PCR primers referred to in this paper are listed in Table 1 and marked in Figure 1B. Transgenic animals detected in this way were verified by digesting the PCR products with *Pst*I. Figure 1B shows the location of the *Pst*I site in the rat gene; the mouse H1t gene lacks this site.

F1 generation mice bearing the transgene were bred to each other, and offspring were screened. Positive F1 and F2 generation mice were sacrificed, tissues were removed, and total cellular RNA was isolated from the tissues. Expression of the rat H1t transgene was examined by measuring steady-state levels of rat H1t mRNA in total cellular RNA samples derived from various tissues by S1 nuclease protection analysis. Expression of rat H1t mRNA during spermatogenesis also was examined by S1 nuclease protection analysis of RNA from enriched populations of mouse testis cells.

PCR Amplification. Genomic DNA templates used for amplification in these studies were isolated from normal rat

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4); SDS, sodium dodecyl sulfate; dpm, disintegrations per minute; UV, ultraviolet light; TBE, Tris/borate/EDTA (0.1 M Tris base, 0.1 M boric acid, 10 mM EDTA).

testis, normal mouse testis, and tail snips from transgenic animals. In some experiments, templates used were purified insert DNA from plasmids. The amplification was performed for 30 cycles, in 100 μL of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each dNTP at 200 μM, 100 pmol of each primer, 1 μg of genomic DNA, and 4 units of *AmpliTaq* DNA polymerase. The samples were heated to 94 °C for 5 min prior to the addition of *Taq* polymerase. Each cycle of the polymerase chain reaction consisted of a 1 min denaturation step at 94 °C, a 1 min annealing step at 55 °C, and a 1 min extension step at 72 °C. The products were electrophoretically separated on a 1% low-melting agarose gel, and the gel was photographed to record the ethidium bromide stained bands.

RNA Isolation and Analysis. RNA was extracted from mouse tissues using RNA Stat-60 (Tel-Test Inc.) following the manufacturer's protocol with the additional steps of a phenol (pH 4.5) extraction and ethanol precipitation. RNA was dissolved in H_2O, and the quantity was determined by measuring the absorbance at 260 nm in a Beckman UV−vis spectrophotometer. For Northern blot analysis, RNA samples, precipitated in desired quantities, were dissolved in 20 μL of sample loading buffer [50% formamide, 1 × MOPS (40 mM morpholinopropanesulfonic acid, pH 7, 10 mM sodium acetate, and 1 mM EDTA), 6% formaldehyde, and 0.02% bromophenol blue], incubated at 56 °C, and electrophoresed on a 1.5% agarose gel containing 1 × MOPS and 6% formaldehyde. Following electrophoresis in a mini-sub cell gel device at 75 mA for 2 h in buffer containing 1 × MOPS and 3.7% formaldehyde, the gel was soaked in H_2O to remove formaldehyde, stained with 0.5 μg/mL ethidium bromide in H_2O, and destained in H_2O to allow photography of the stained ribosomal RNA bands with UV light.

Following visualization, RNA was immobilized on a Nytran filter (Schleicher & Schuell) by capillary transfer from the gel and UV cross-linking with 2.4×10^5 μJ of 254 nm light in a Stratalinker 1800 (Stratagene). H1t mRNA was detected by hybridization to a mouse H1t gene probe oligolabeled with [α-^{32}P]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, 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.4), 5 × Denhardt's (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 1×10^6 dpm/mL of the single-stranded labeled H1t DNA probe. The filter was washed with 5 × SSC, 1 × Denhardt's at room temperature for 10 min followed by incubation for 30 min at 59 °C. Then the blot was washed successively in solutions of 5 × SSC, 2 × SSC, 1 × SSC, and 0.1 × SSC solutions all containing 0.1% SDS.

Southern Blot Analysis. Genomic DNA was prepared from testes of normal mice, normal rats, and several transgenic mice (Enrietto et al., 1983). Each 10 μg sample was digested with the restriction endonuclease *Eco*RI, to release the 6859 bp DNA fragment containing the single-copy H1t gene from rat DNA. Digested DNA samples were electrophoresed on a 0.7% agarose gel, and following denaturation and neutralization, the DNA was immobilized on a NYTRAN membrane by capillary transfer and UV cross-linking. Prehybridization and hybridization conditions were similar to those described for Northern blots. The H1t gene was detected by hybridization to a ^{32}P-oligolabeled probe [the 967 bp *Mae*III DNA insert excised from plasmid pJA19 containing the rat H1t gene (Grimes et al., 1990)].

Probes. For Northern blots to detect mouse H1t mRNA, the mouse H1t gene (bases −109 to 742 in Figure 1B; GenBank Accession No. L28753) was cloned into the *Sma*I site of pUC19. This plasmid, designated pMH1t (vanWert et al., unpublished results) was cut with *Bam*HI and *Eco*RI, yielding the full-length mouse H1t gene insert (851 bases) plus 25 bases of plasmid DNA sequence from the polylinker region. The digested fragments were resolved on a 1% low-melting agarose gel, the appropriate band was excised, and the DNA was recovered essentially by the hot phenol extraction procedure (Guo et al., 1983). The quantity of the purified fragment was estimated and oligolabeled with [α-^{32}P]dCTP using a Pharmacia kit, following the manufacturer's protocol. A spin-column procedure using Sephadex G-50 was utilized for separating radioactive DNA from unincorporated dNTP precursors (Sambrook et al., 1989).

For S1 nuclease protection analysis to detect rat H1t mRNA, a 1140 bp *Ava*I−*Xba*I fragment was cut from the plasmid pHD1, a clone containing the 1340 bp *Pvu*II−*Hin*dIII rat genomic DNA fragment derived from plasmid pPS3 (Grimes et al., 1987). Rat H1t mRNA protects approximately 181 bases of DNA derived from this probe derived from the 5′ end of the H1t gene. The probe was dephosphorylated with calf intestine alkaline phosphatase and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

For S1 nuclease protection analysis to detect mouse H1t mRNA, a DNA fragment corresponding to nucleotides −109 to 191 of the mouse H1t gene (Figure 1B) was amplified using a ^{32}P end-labeled 3′ primer (Table 1 and Figure 1B, nucleotides 172−191) and an unlabeled H1/AC box primer (Table 1 and Figure 1B, nucleotides −109 to −90) and using the linearized plasmid pMH1t as a template. The same conditions described earlier for PCR were used for amplification of this DNA fragment. The product was electrophoresed on a 1% low-melting agarose gel, and the 300 bp band was recovered.

S1 Nuclease Protection Analysis of Messenger RNA. S1 nuclease analysis to detect H1t mRNA was conducted essentially by the method of Favaloro et al. (1980). For each reaction, 50 μg of RNA was coprecipitated with 50 000−100 000 dpm of probe, resuspended in 20 μL of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, and 0.4 M NaCl), denatured 10 min at 65 °C, and hybridized overnight at 30 °C. The resulting DNA/RNA hybrids were digested with 300 units of S1 nuclease for 15, 30, 45, or 60 min in S1 nuclease buffer (0.28 M NaCl, 50 mM sodium acetate, pH 4.5, 4.5 mM $ZnSO_4$, 20 μg/mL single-stranded calf thymus DNA, and 300 units/mL nuclease S1). Reactions were stopped by adding 80 μL of S1 nuclease stop buffer (4 M ammonium acetate, 20 mM EDTA, and 40 μg/mL yeast tRNA). The products were ethanol-precipitated, resuspended in 3 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) to which was added 4 μL of formamide loading solution [1 × TBE (0.1 M Tris base, 0.1 M boric acid, and 10 mM EDTA), 90% formamide, and 0.5% bromophenol blue], and loaded and electrophoresed on a 6% polyacrylamide gel containing 7 M urea and 1 × TBE. Adjacent lanes contained a mouse H1t sequencing ladder produced by using

a primer 124 bases into the coding region and extending 5′ through the promoter region. After electrophoresis, the gel was soaked to remove urea, dried, and exposed to X-ray film.

RESULTS

Detection of the Rat H1t Gene in Transgenic Mice. In previous studies, we described the cloning and sequencing of a 6859 bp *Eco*RI fragment of rat genomic DNA containing the testis histone H1t and H4t genes (Grimes et al., 1987, 1990; GenBank Accession No. M28409). The two genes are separated by approximately 1800 bp and oriented with their 3′ ends toward each other as noted in Figure 1A. The promoter of the testis-specific histone H1t gene has a unique sequence located between the H1/AC box and the H1/CCAAT box (Grimes et al., 1990; Wolfe & Grimes, 1993; Wolfe et al., unpublished results). Testis proteins bind specifically to this sequence (Grimes et al., 1992a), and a correlation exits between the time of appearance of the testis-specific binding proteins and the onset of transcription of the H1t gene during spermatogenesis (Grimes et al., 1992b).

A 6859 bp *Eco*RI restriction fragment of rat genomic DNA (Figure 1A) containing the histone H1t gene was microinjected into mouse embryos by the DNX Corp. Since the transgenic mice retain their own testis-specific histone H1t gene, a method was needed to unambiguously identify the rat gene in the mouse background. In recent studies, we cloned and sequenced the mouse H1t gene (vanWert et al., unpublished results; GenBank Accession No. L28753). It is apparent that the rat H1t and mouse H1t gene sequences are highly conserved with 91.1% identity from the AC/Box in the promoter through the post-hairpin element (Figure 1B). Note that a 6 base gap in the mouse sequence in the mRNA leader region starting at base 36 is necessary for alignment of the rat and mouse sequences (Figure 1B). Nevertheless, important sequence differences allowed the design of species-specific 5′ primers that could be used with a common 3′ primer for the specific amplification of the rat and mouse H1t genes. The unique 5′ primers (5′-ACTCCAGCGCT-GTTCGCTCT-3′ for the rat and 5′-ATCACTGTTGGCTCTC-CTTTAG-3′ for the mouse) near the mRNA start site are listed in Table 1 and marked in Figure 1B. The 3′ primer corresponding to the post-hairpin region of the H1t gene (5′-AGTGTTTGGCCATTTTAAGA-3′) was used for amplification of both the rat and mouse H1t genes (Table 1 and Figure 1B).

Nine mice were shown by PCR analysis to have the rat H1t transgene, and these founders were bred to C57-Black mice. DNA from tail snips of F1 generation mice was examined by PCR to detect the transgene as shown in Figure 2A (top panel). Normal mouse (lane 2) and rat (lane 3) genomic DNA samples were used as negative and positive controls as shown in the figure, and only rat DNA was amplified with the rat-specific 5′ primer. A DNA product about 772 bp long was amplified as seen by comparison with the 100 bp size standard ladder in lane 1 (the darker band is 600 bp). Lanes 4 through 11 represent amplified products from DNA samples from potential transgenic mice. Samples in lanes 4, 6, 7, 8, 9, 10, and 11 appear to be positive for the rat transgene. Lane 12 is a negative control with no primer DNA. Note that some samples from potential transgenics produced a very dark band (lanes 4, 6, 7, 8, and 9) compared to normal rat DNA (lane 3) , one sample (lane 10) produced a band with an intensity only slightly greater than the intensity of the band from normal rat DNA (lane 3), and one sample (lane 11) produced a band roughly equivalent to normal rat DNA. The variable intensity of the band reflects the variability in copy-number.

However, to assure that the amplified band being detected was the rat H1t gene and that the mouse H1t gene was not being amplified inadvertently, the DNA products produced by PCR amplification were digested with *Pst*I, cleaving a 89 bp fragment. The diagnostic *Pst*I site in the rat H1t gene is indicated in Figure 1B. As seen in this figure, the mouse H1t gene lacks this *Pst*I restriction site. Data in Figure 2B (bottom panel) reveal that the size shift of the restricted PCR product is sufficient for detection by agarose gel electrophoresis. Upon digestion, there is a perceptible shift of the major band to a higher mobility (approximately 80 bp shorter) when compared to the bands in the 100 bp standard ladder (lane 1). Full-length and shortened bands are marked in the figure. Lanes 2 and 3 show undigested and digested products respectively produced from normal rat genomic DNA template. The remaining lanes (4−17) are pairs of products from seven different transgenic mice. Even lanes (4−16) show undigested products; odd lanes (5−17) show digested products from the same animals. Note that all seven of these animals were positive. All mice born from matings between a transgenic mouse and a normal mouse or between two transgenic mice appeared to be normal. There was no observable phenotype from the transgene. Approximately 30% of the F1 generation mice born to one transgenic parent were transgene-positive.

Southern blot analysis was conducted to estimate the number of copies of the rat transgene in positive mice. The H1t gene is present in a single copy in rats (Cole et al., 1986), and therefore genomic DNA from a normal rat was used as a control to establish the intensity of the hybridization signal of the single-copy 6859 bp *Eco*RI genomic DNA fragment. Data from this analysis are presented in Figure 3. Lanes 1 and 2 are *Eco*RI-digested genomic DNA samples from testes of a normal mouse and normal rat, respectively. Lanes 3, 4, and 5 represent *Eco*RI-digested DNA from three different transgenic mice. The photograph shows that approximately equal amounts of genomic DNA were loaded in each lane.

The autoradiogram in the right panel of Figure 3 shows the hybridization signal of the blot of the gel probed with a ^{32}P-labeled probe for the rat H1t gene. The rat probe cross-hybridizes with mouse H1 genes as shown in lane 1, and the mouse sample appears to produce at least two major positive bands, one with a mobility slightly greater than or equal to that of the rat 6859 bp *Eco*RI fragment and one with a lower mobility with a size estimated to be at least 14 kb. *Eco*RI-digested DNA from transgenic mice shown in lanes 3 through 5 produces two major bands in the autoradiogram compared to the one band seen in DNA from normal rats (lanes 2). The higher mobility band is the expected size (approximately 7 kb), and the second band has a lower mobility (estimated to be at least 14 kb). We also determined the mobility and intensity of the hybridization signal of the purified, cloned 6859 bp *Eco*RI rat genomic DNA fragment representing different genomic copy-numbers as a hybridization size standard and as a control for copy-number (data not shown). The intensities of the bands positive for the H1t gene in the samples from transgenic mice are variable (lanes 3−5), and all have intensities greater than that of the

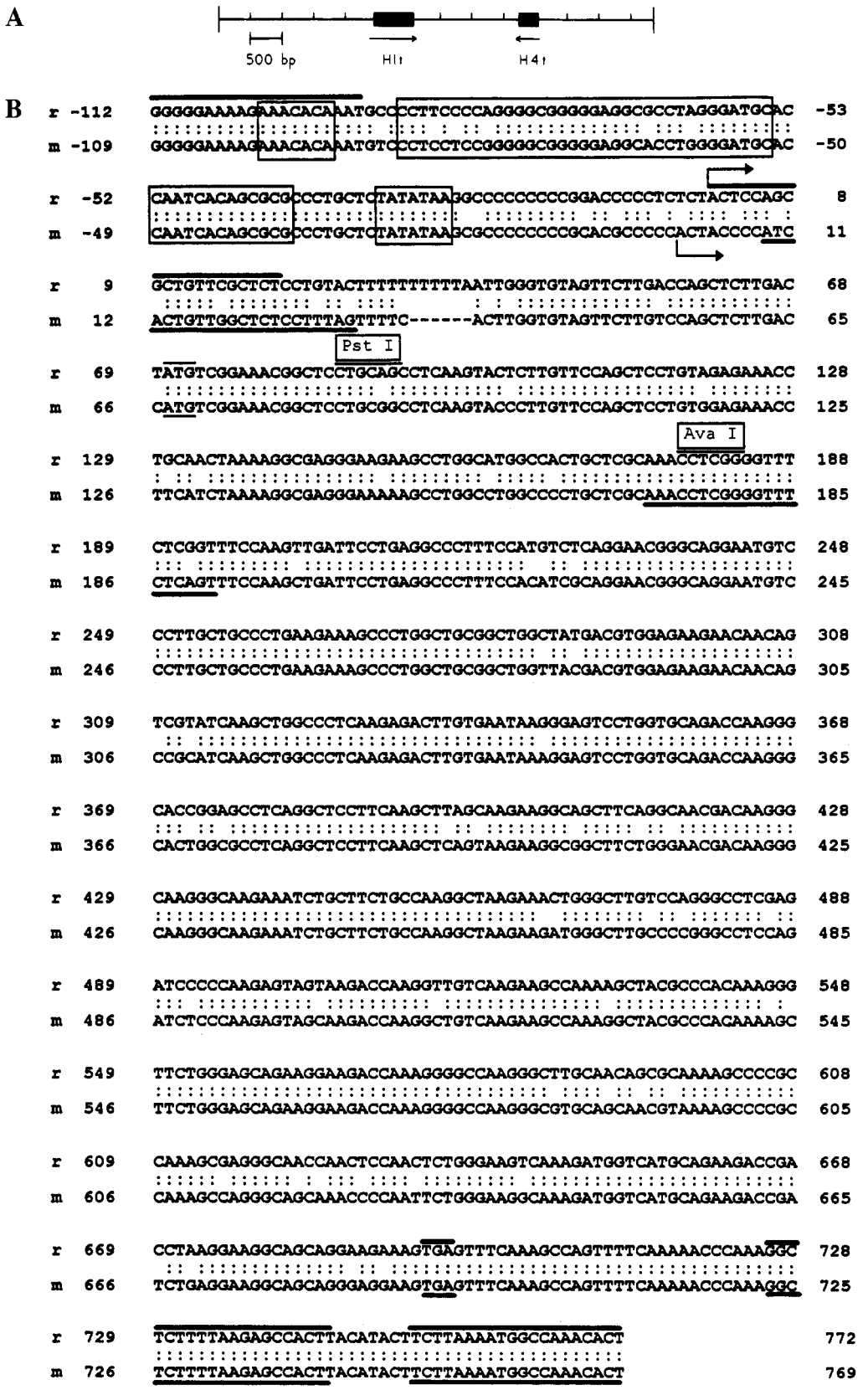

FIGURE 1: Genomic DNA used to prepare transgenic mice and comparison of rat and mouse H1t gene nucleotide sequences. (A) Map of the rat H1t gene. Map of the cloned 6859 bp *Eco*RI genomic fragment of rat DNA that was introduced into mouse embryos, and the locations of the H1t and H4t genes on the fragment. Note that the genes are transcribed convergently. (B) Alignment of the rat and mouse H1t genes. Note the high degree of identity between the two sequences (91%). The primers and restriction sites used to differentiate between the two sequences are marked. The experimentally determined mRNA start site is marked as number 1 in both genes, and numbering in the text and in Table 1 refers to the numbering system shown in this figure.

faint band produced by digestion of normal rat DNA (lane 2) and greater than those of the bands produced by digestion of normal mouse DNA (lane 1). The copy-number of the H1t gene in these three transgenic animals was estimated to

Table 1: Oligonucleotides Used in PCR Amplification and S1 Nuclease Protection Analysis and Features of the H1t Gene[a]

Oligonucleotides Used in PCR and S1 Nuclease Protection Analysis			
name	position	sequence	length
H1/AC box	−109 to −90 (m)	GGGGGAAAAGAAACACAAAT	20
H1/AC box	−112 to −93 (r)	GGGGGAAAAGAAACACAAAT	20
5′ primer	9 to 30 (m)	ATCACTGTTGGCTCTCCTTTAG	22
5′ primer	1 to 20 (r)	ACTCCAGCGCTGTTCGCTCT	20
primer extension	172 to 191 (m)	ACTGAGAAACCGCGAGGTTT	20
stem−loop	723 to 742 (m)	AAGTGGCTCTTAAAAGAGCC	20
post-stem−loop	750 to 769 (m)	AGTGTTTGGCCATTTTAAGA	20
post-stem−loop	753 to 772 (r)	AGTGTTTGGCCATTTTAAGA	20
Features of the H1t Gene			
name	position	sequence	
H1/AC box	−99 to −93 (m)	AAACACA	
H1/AC box	-102 to −96 (r)	AAACACA	
H1t/TE element	−86 to −52 (m)	CCTCCTCCGGGGGCGGGGGAGGCACCTGGGGATGC	
H1t/TE element	−89 to −55 (r)	CCTTCCCCAGGGGCGGGGGAGGCGCCTAGGGATGC	
H1/CAAT box	−49 to −37 (m)	CAATCACAGCGCG	
H1/CAAT box	−52 to −40 (r)	CAATCACAGCGCG	
TATA/box	−28 to −22 (m)	TATATAA	
TATA/box	−31 to −25 (r)	TATATAA	
mRNA start	1 (m)	ACTACCC	
mRNA start	1 (r)	ACTCCAG	
ATG start codon	67 (m)	ATG	
ATG start codon	70 (r)	ATG	
coding region	67 to 690 (m)	207 amino acids	
coding region	70 to 693 (r)	207 amino acids	
stem−loop	723 to 742 (m)	GGCTCTTTTAAGAGCC	
stem−loop	726 to 745 (r)	GGCTCTTTTAAGAGCC	
post-stem−loop	750 to 769 (m)	TCTTAAAATGGCCAAACACT	
post-stem−loop	753 to 772 (r)	TCTTAAAATGGCCAAACACT	

[a] See Figure 1B for sequences and numbering. The(r) and (m) refer to rat or mouse sequence, and oligonucleotides are listed in the 5′ to 3′ direction.

be 2, 10, and 20, respectively, by scanning the autoradiograms from the same blot that had been developed for different time periods.

Testis-Specific Accumulation of Rat Histone H1t mRNA in Transgenic Mice. In previous studies, our laboratory and other laboratories have shown by Northern blot analysis that high steady-state levels of histone H1t mRNA accumulate in testis of rats (Cole et al., 1986; Grimes et al., 1987), mice (Drabent et al., 1993, vanWert et al., unpublished results), and humans (Drabent et al., 1991), but that H1t mRNA was absent or present at undetectably low levels in other tissues. Furthermore, we have demonstrated the high steady-state level of the rat testis-specific H1t mRNA in primary spermatocytes by Northern blot analysis and by the more sensitive and more stringent technique of S1 nuclease protection (Grimes et al., 1987). Because of the high degree of conservation of the rat and mouse H1t genes (Figure 1B) and the known cross-hybridization of the rat H1t probe with both rat and mouse H1t mRNA seen in our experiments (data not shown), it was imperative to use a method such as S1 nuclease protection analysis to specifically detect rat H1t mRNA in a mouse background in the present study.

Members of F1 generation mice carrying the transgene were bred to each other, and the offspring were subjected to screening by the same type of PCR and restriction digest analysis shown in Figure 2. Selected positive F1 and F2 generation mice were sacrificed, and total cellular RNA samples were analyzed by S1 nuclease protection to determine rat H1t mRNA steady-state levels in several tissues. Of the five tissues analyzed, only testis had detectable steady-state levels of rat histone H1t mRNA (Figure 4). Lanes 1 and 2 (Figure 4) are normal rat testis and rat brain control samples. Note the protected band in testis (lane 1) but the absence of this band in brain (lane 2). Lanes 3 and 4 show normal mouse testis and brain control samples. No protected band corresponding to the fully protected rat H1t mRNA probe is seen with the mouse tissues. However, note that there is a higher mobility band in lane 3 representing a shorter protected DNA fragment in mouse testis. This may be due to partial protection of the rat probe with an unidentified RNA generating a shorter protected DNA fragment. Lanes 5 through 14 show tissues from transgenic mice with approximately equal copy-numbers of the rat H1t gene. Lanes 5 through 9 represent testis, liver, brain, spleen, and kidney, respectively, from transgenic mouse 55, and lanes 10 through 14 represent the same tissues from mouse 72. We have observed this testis-specific rat H1t gene expression pattern in at least six mice. Furthermore, we have not observed expression of the rat H1t gene in other tissue but testis in these mice.

Expression of the Rat Histone H1t Gene in Testis Pachytene Primary Spermatocytes of Transgenic Mice. Although it was clear from the previous experiments that the rat histone H1t gene was being transcribed in a testis-specific manner, it was important to determine whether it was being transcribed appropriately in the meiotic prophase stage of the cell cycle. Therefore, we examined the pattern of expression of rat H1t mRNA in enriched populations of mouse testis cells prepared by centrifugal elutriation.

Preliminary Northern blot analyses revealed the pattern of expression of the mouse H1t gene in the enriched populations of normal mouse testis cells. Enriched populations of spermatogenic cells were prepared by centrifugal elutriation of a single cell suspension prepared from testes

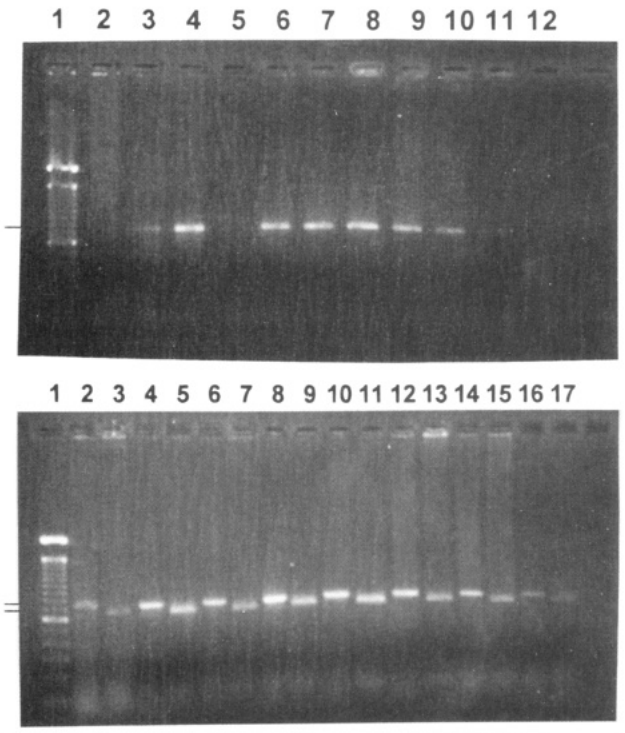

FIGURE 2: Detection of the rat H1t transgene by PCR. (A, top panel) Amplification of the H1t gene. Samples of genomic DNA from mouse tail snips were incubated with dNTP's, Taq polymerase, and primers specific for the rat H1t gene. The expected amplified rat H1t product of about 800 bp is seen in lane 3. (Lane 1) 100 bp size ladder; (lane 2) normal mouse template DNA; (lane 3) normal rat DNA; (lanes 4−11) eight different transgenic mice; (lane 12) a negative control containing no template. (B, bottom panel) Screening the amplified products for the rat gene. The rat H1t gene has a *Pst*I site that is absent in the mouse (Figure 1B). PCR products were purified and digested with *Pst*I for 2 h at 37 °C. (Lane 1) 100 bp ladder; (lanes 2 and 3) normal rat, undigested and digested, respectively. The remaining lanes are the transgenics, undigested and digested samples of each positive animal.

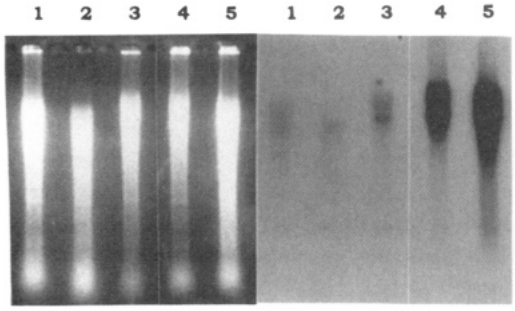

FIGURE 3: Rat H1t gene copy-number is variable in transgenic mice. Samples of genomic DNA containing 10 μg of DNA from normal mice, normal rats, and several transgenic mice were digested with the restriction endonuclease *Eco*RI. The left panel shows ethidium-stained bands produced when digested DNA samples were electrophoresed on a 0.7% agarose gel, and the intensities of the stained samples reveal that the quantities of each were approximately equal. The right panel shows the autoradiogram of the blotted DNA samples hybridized using the ^{32}P-oligolabeled DNA probe for the rat H1t gene. (Lanes 1 and 2) digested genomic DNA samples from testes of a normal mouse and normal rat. (Lanes 3−5) Digested genomic DNA samples from three different transgenic mice. Note the band in lane 2 of the autoradiogram showing the low intensity of the approximately 7 kb single copy H1t gene in rats. Note in lane 1 that the rat H1t probe cross-hybridizes to H1 genes in normal mouse DNA.

of 10 normal mice. Total cellular RNA was isolated from each of five elutriator fractions as previously described

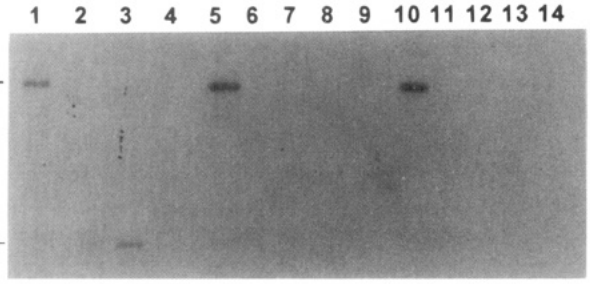

FIGURE 4: Rat H1t gene is expressed only in testes of transgenic mice. This experiment reveals relative steady-state levels of rat H1t mRNA in transgenic mice. Note the expected size band, a 181 base DNA fragment marked at the left of the photograph. Of the tissues examined, only testis accumulated detectable steady-state levels of H1t mRNA (lanes 1, 5, and 10). (Lanes 1 and 2) Rat testis and brain; (lanes 3 and 4) normal mouse testis and brain; (lanes 5−9) testis, liver, brain, spleen, and kidney from transgenic mouse 55; (lanes 10−14) testis, liver, brain, spleen, and kidney from transgenic mouse 72. Note the higher mobility band in lane 3 representing a protected fragment estimated to be 145 bases in length. The identity of the RNA leading to protection of this probe fragment has not been identified, but the intensity of the band is variable and it is seen frequently in testes of rats and normal and transgenic mice (see Figures 6 and 7).

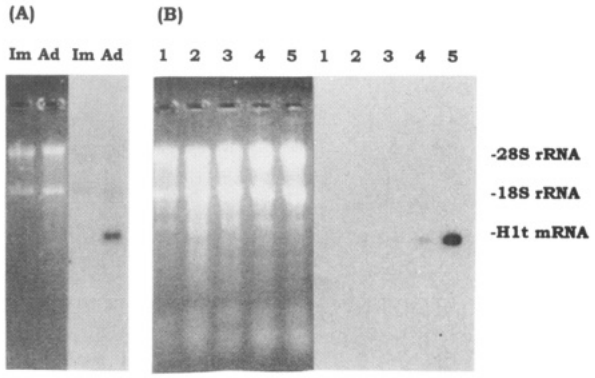

FIGURE 5: Mouse H1t mRNA accumulates in pachytene primary spermatocytes. (A) H1t mRNA accumulates only in testis of sexually mature mice. Lanes marked Im and Ad represent total cellular RNA samples from testes of sexually immature and adult mice, respectively. The photograph in the left panel is of ethidium bromide stained 18S and 28S rRNA bands showing approximately equal loading of the gel. The autoradiogram in the right panel shows the hybridization signal when the gel blot is probed for the H1t mRNA. Note that equal loads of RNA are present in each lane, but only the adult animal has the expected H1t mRNA signal. Faint signals from nonspecific cross-hybridization of the probe to 18S and 28S rRNA species can be seen. (B) H1t mRNA accumulates in mouse testis primary spermatocytes. Cells pooled from the testes of 10 normal mice were separated into 5 fractions by centrifugal elutriation. The left panel is a photograph of ethidium bromide stained RNA bands showing approximately equal loading of the gel with RNA from each of the five fractions (lanes 1−5 represent fractions 1−5, respectively). The right panel is the autoradiogram showing the hybridization signal using a mouse H1t probe. Note that although equal loads of RNA are present in each of the five populations of mouse testis cells, a significant steady-state level of H1t mRNA accumulates only in cellular fraction 5, which is most enriched for pachytene primary spermatocytes. Very little signal is detected in fractions 1 and 3 which are most enriched in late spermatids and early spermatids, respectively.

(Grimes et al., 1990). Mouse histone H1t mRNA accumulated to a high level only in cells in elutriator fraction 5, the fraction enriched in meiotic prophase pachytene primary spermatocytes, as shown in the autoradiogram presented in the right panel of Figure 5B. Roughly equal levels of RNA were present in each lane of the gel as seen

in the photograph of the ethidium bromide stained RNA in the left panel of Figure 5B. The faint H1t mRNA band in lane 4 is most likely due to cross-contamination of cells in elutriator fraction 4 with primary spermatocytes which are most concentrated in fraction 5. It is clear from this experiment that the pattern of expression of the mouse H1t gene during spermatogenesis is similar to that of the rat H1t gene.

An additional experiment was conducted to determine when H1t mRNA accumulates during spermatogenesis in mice. Testes were isolated from sexually immature 12 day old mice which have spermatogonial cells but do not have significant numbers of germinal cells in the spermatocyte or spermatid stages of spermatogenesis. Northern blot analysis was conducted to compare steady-state levels of testis H1t mRNA from the 12 day old mice and adult mice in order to determine whether H1t mRNA accumulates in testes deficient in spermatocytes. The results of this experiment, shown in the autoradiogram in the right panel of Figure 5A, reveal that H1t mRNA does not accumulate in testes of 12 day old mice (lane marked Im for immature mice) compared to adult mice (lane marked Ad for adult mice). Faint bands in both lanes marked Im and Ad in the autoradiogram (right panel of Figure 5A) at the positions of the major ethidium bromide stained bands (left panel of Figure 5A) are due to nonspecific hybridization to the abundant 18S rRNA and 28S rRNA species, and they reveal the equal transfer of RNA from both lanes to the membrane. The photograph (left panel of Figure 5A) shows that approximately equal loads of total cellular RNA from testes of immature and adult mice were present in the agarose gel before transfer to the membrane.

To examine expression of the rat H1t gene during spermatogenesis in transgenic mice, single cell suspensions prepared from the testes of 10 positive transgenic mice were separated by centrifugal elutriation using the same separation conditions as with the normal mice. S1 nuclease protection analysis was used to detect and quantitate rat H1t mRNA in the mouse background in this experiment. Rat H1t mRNA accumulates to a significant level only in elutriator fraction 5 as shown in Figure 6. As seen in the previous S1 analyses (Figure 4), a higher mobility band representing a shorter protected DNA fragment is seen (Figure 6, fraction 5). As mentioned before, this most likely represents partial protection of the probe due to an unidentified RNA present in this fraction. Therefore, the pattern of expression of the rat H1t transgene during spermatogenesis appears to be identical to the pattern of expression of the H1t gene in the normal mouse. These data reveal that the transgene retains both its tissue and cell type specificity when expressed in the mouse.

Portions of total cellular RNA samples were examined by S1 nuclease protection analysis with the rat H1t specific probe to measure steady-state levels of rat H1t mRNA in mice with high copy-number (lane 7, Figure 2A) and low copy-number (lane 11, Figure 2A) of the transgene. Lane 1 in Figure 7 shows a protected band that is generated from the 181 bp DNA−RNA hybrid formed between the probe and rat H1t mRNA. The intensity of this band represents the steady-state level of H1t mRNA in normal rat testis. In addition to this band, there was again a higher mobility band representing partial protection of the probe by an unidentified RNA. Estimates of peak areas obtained from scanning bands representing rat H1t messenger RNA in Figure 7 reveal that

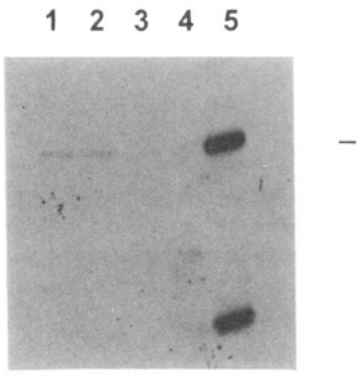

FIGURE 6: Rat H1t mRNA accumulates in primary spermatocytes of transgenic mice. Cells pooled from the testes of 10 transgenic mice were separated into 5 populations by centrifugal elutriation as described for Figure 5B, and total cellular RNA was isolated and purified from each cell type. S1 nuclease protection analysis was conducted with a rat H1t specific probe to detect H1t mRNA generated from the transgene in each of the samples. Lanes 1 through 5 represent S1 nuclease protection using total cellular RNA from fractions 1 through 5, respectively. Fraction 5, the cellular population most enriched in pachytene primary spermatocytes, is the only elutriator fraction to generate the expected band at a significant intensity representing a relatively high steady-state level of rat H1t mRNA (lane 5). The mobility of this 181 base band is marked on the right side of the photograph. A higher mobility band is also seen in fraction 5, but as mentioned in Figure 4, the source of the RNA leading to protection of this probe fragment has not been determined. The pattern of rat H1t mRNA expression of the rat H1t gene during spermatogenesis is similar to the pattern of mouse H1t mRNA expression seen in normal mouse testis cells (Figure 5) and to the pattern of rat H1t mRNA expression seen in previous studies in normal rat testis cells.

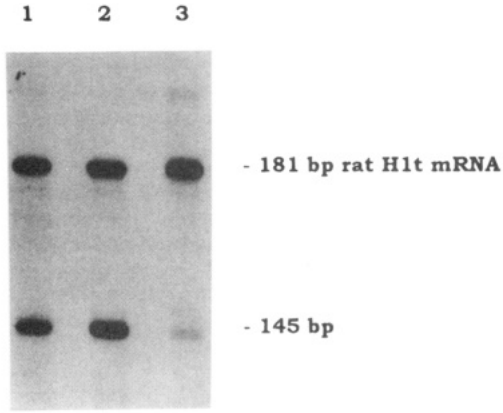

FIGURE 7: Rat H1t mRNA accumulates to similar steady-state levels in testes of rats and transgenic mice. Total testis RNA samples from a rat and from transgenic mice 179 and 183 were probed with the rat H1t mRNA specific S1 nuclease probe to determine the relative steady-state levels of rat H1t mRNA. Lane 1 shows the intensity of the protected labeled DNA fragment from the expected 181 bp DNA−RNA hybrid representing H1t mRNA in rat testis. Again, variable amounts of a higher mobility band, representing DNA from a DNA−RNA hybrid estimated to be approximately 145 base pairs, are produced by both rat and mouse testis RNA samples. Lanes 2 and 3 show the relative equal intensities of bands representing rat H1t mRNA in two different transgenic mice. Notice that the intensities of these bands (lanes 2 and 3) are similar to the intensity of the H1t band in normal rat testis (lane 1).

the steady-state levels of rat H1t message in the two transgenics, one with high copy-number transgene (lane 2) and one with low copy-number (lane 3), are approximately 1.1 and 0.9 times the mRNA level in normal rat testis (lane 1). A similar analysis of bands representing H1t messenger RNA in Figure 4 reveals that steady-state levels of rat H1t

message in two other high-copy transgenics (Figure 4, lanes 5 and 10) are 2.2 and 2.6 times the level in normal rat testis (Figure 4, lane 1). Thus, H1t mRNA levels in some high copy-number animals are higher than in normal animals with a single-copy gene, but measured H1t mRNA levels in transgenic animals have never exceeded 2.6 times the level found in normal animals.

In experiments designed to examine the level of endogenous mouse H1t mRNA in testis of transgenic animals by S1 nuclease protection using a mouse specific probe, the relative steady-state levels of mouse H1t mRNA appeared to be low in some transgenic mice compared to the level of mouse H1t mRNA in normal mice (data not shown). Therefore, it seemed possible that the mouse H1t gene might have been lost in some transgenic animals. An experiment was conducted to confirm that the mouse H1t gene was in fact present in these transgenic mice. Mouse specific and rat specific probes were used to amplify both rat and mouse H1t genes as shown in Figure 8A (top panel). Lane 2 shows the product generated from normal control mouse DNA template using mouse H1t specific primers, and lane 3 shows the product from the same mouse template DNA using rat H1t specific primers. Note that very little product was amplified from mouse template DNA with rat specific primers (lane 3). Amplified products also were generated from normal rat template DNA with both mouse and rat primers (lanes 4 and 5, respectively). It is not clear why the amplified product from rat template DNA using mouse primers (lane 4) was larger than the normal product from rat template DNA using rat primers (lane 5). The products amplified from template DNA from one transgenic mouse using rat and mouse primers are shown in lanes 6 and 7, respectively. Products amplified from a second transgenic mouse using rat and mouse primers are shown in lanes 8 and 9, respectively.

Each of the PCR products was then cut with the restriction enzyme *Pst*I to discriminate between the rat product which has a *Pst*I site and the mouse product which lacks this site. These data are shown in Figure 8B (bottom panel). Even lanes show undigested samples, and odd lanes show samples digested with *Pst*I. The amplified product from normal control mouse template DNA using mouse primers did not digest (lane 3). The product from normal control rat template DNA using rat primers did digest (lane 5); note that the mobility of the band in lane 5 is slightly greater than the undigested band in lane 4, indicating that the band is the rat H1t product. The mouse product shown in lane 7 did not digest (compare the mobilities of the undigested and digested DNA samples in lanes 6 and 7, respectively). This shows that the mouse H1t gene is present in this transgenic mouse. However, the rat product from this transgenic animal did digest as expected (compare the undigested and digested samples in lanes 8 and 9, respectively). Lanes 10 through 12 are pairs of uncut and cut samples from the other transgenic animal, and the results are similar to those shown in lanes 6 through 9. Both the rat H1t gene and the mouse H1t gene were present in all of the transgenic animals tested.

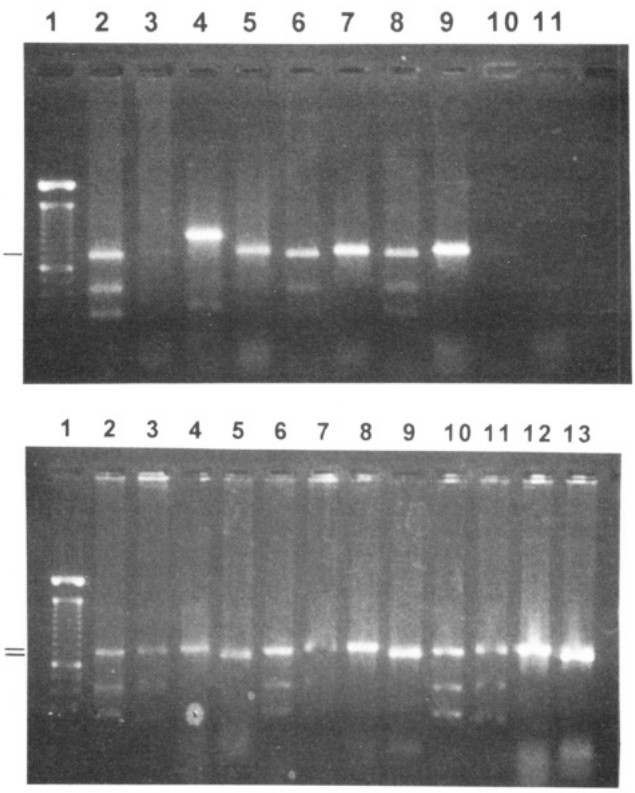

FIGURE 8: Both mouse and rat H1t genes are present in transgenic mice. (A, top panel) Amplification of the H1t gene. The mouse H1t gene was amplified using a 5′ primer complementary to the mouse transcription start site, and the rat gene was amplified using a different 5′ primer that was complementary to the rat transcription start site (see Figure 1B and Table 1). A primer complementary to the rat 3′ post-hairpin sequence was used for both genes. Lane 1 is a 100 bp size ladder. Even-numbered lanes show amplification with the mouse specific primer. Odd-numbered lanes show amplification with the rat specific primer. (Lanes 2 and 3) Normal mouse; (lanes 4 and 5) rat: (lanes 6−9) transgenic mice; (lanes 10 and 11) negative controls containing no template. (B, bottom panel) Screening for mouse and rat PCR amplification products. PCR products shown in Figure 8A were purified and digested 2 h at 37 °C with the restriction enzyme *Pst*I. Lane 1 shows a 100 bp ladder. Even-numbered lanes show undigested amplified products; odd-numbered lanes show *Pst*I digested products. (Lanes 2 and 3) Normal mouse template with mouse 5′ primer; (lanes 4 and 5) rat template with rat 5′ primer; (lanes 6, 7, 10, and 11) transgenic mouse template with mouse 5′ primer; (lanes 8, 9, 12, and 13) transgenic mouse template with rat primer. Note that the rat product amplified using the rat DNA template was cut (compare lane 5 with digested product to lane 4 with undigested product). The H1t products amplified using transgenic mouse DNA templates and the rat 5′ primer were cut with *Pst*I to generate a higher mobility band (compare lanes 9 and 13 with digested products to lanes 8 and 12 with undigested products, respectively). Products amplified with the mouse 5′ primer and normal mouse template DNA (lanes 2 and 3) or with mouse 5′ primer and transgenic mouse template DNA (lanes 6, 7, 10 and 11) did not cut.

DISCUSSION

Transgenic mouse technology is an important tool that can be used to study regulation of gene expression (Simoni, 1994). The technique is indispensable for *in vivo* analysis of regulation of expression of mammalian genes and especially those expressed in a tissue-specific manner. For example, the method has been used to study testis-specific genes such as lactate dehydrogenase and protamine. It has allowed investigators to confirm the importance of promoter elements identified by alignment of conserved sequences or identified by detection of specific binding of nuclear proteins. An example of the latter is the study of spermatid-specific transcription of protamine genes, which led to the identification of sequences that contribute to regulation of expression of haploid-specific genes (Johnson et al., 1988).

Histone H1t, one of seven known histone H1 variants found in mammalian cells, is the only one that is testis-specific. Although histone H1a [referred to as H1.1 by Albig et al. (1993) and as H1E by Ohe et al. (1989)] is expressed in testis, it is also expressed in other tissues (Meistrich, 1989; Burfeind et al., 1994). The study of H1 histones is important in part because they are one of the major DNA binding proteins in eucaryotes. H1 histones bind to DNA where it enters and exits nucleosomes. They are involved in generating or stabilizing higher order chromatin structure. In having an effect on the structure of DNA, they also have an effect on gene expression.

Testis histone H1t is expressed only in pachytene primary spermatocytes, which are germinal cells in the meiotic prophase of the cell cycle during spermatogenesis. The H1t gene has been isolated and cloned from human, monkey, rat, and mouse, and promoter elements in these genes are highly conserved (Cole et al., 1986; Grimes et al., 1990; Koppel et al., 1994; Drabent et al., 1991, 1993; vanWert et al., unpublished results). These elements may account for the tissue specificity and meiotic cell-cycle specificity of H1t gene expression. One way to examine promoter function is to introduce the gene into a different species and to observe the effect of the wild-type and mutated promoters on expression of the gene. In experiments described in this paper, the host animal retained its own copy of the gene.

One of the problems in our study was how to unambiguously detect the rat H1t gene in a mouse background. Since rat H1t and mouse H1t gene sequences are conserved (Figure 1B), it was necessary to design species-specific 5′ primers that could be used for specific PCR amplification of the rat gene in a mouse background. Unique 5′ primers near the mRNA start site (Table 1 and Figure 1B) allowed amplification of either gene. The 3′ primer corresponding to the post-hairpin region of the H1t gene (Figure 1B) was used as a common 3′ primer for amplification of both rat and mouse H1t genes.

Since PCR products from amplification of both genes were approximately the same size, prohibiting their identification by mobility on agarose gels, an additional screening step was included. To assure that we were detecting only the rat H1t gene, the PCR products were digested with *Pst*I. This extra step reduced the size of the restricted rat PCR product, and this size difference was sufficient for detection of a shift to a slightly higher electrophoretic mobility on agarose gels. In some experiments, false positive results were generated in which a gene product was amplified, but the amplified DNA products could not be cut with *Pst*I. In these cases, presumably the mouse gene was amplified, yielding the correct size product.

Overall, approximately 30% of the F1 generation mice were transgenic when one parent was transgenic. The number of F2 offspring bearing the rat H1t gene is approximately 66% when two transgenic F1 mice are mated. Positive animals do not have an apparent special phenotype compared to normal animals, and the fertility of the animals appears to be normal.

The copy-number of the rat gene varies considerably in transgenic animals. In one experiment, the relative levels of the rat H1t gene in four out of six positive transgenic animals appeared to be higher than the level of the amplified rat gene from normal rat DNA (data not shown). In an experiment shown in Figure 3, the relative levels of the rat transgene in the three positive mice were estimated to be 2, 10, and 20 copies, respectively. This type of variability in transgene copy-number is not unusual.

It was an equally difficult problem to detect rat H1t mRNA in the presence of the homologous mouse H1t mRNA. Conserved rat and mouse H1t mRNA sequences rendered Northern blot analysis inadequate for unambiguous identification of rat H1t mRNA. In fact, we have successfully conducted Northern blot analyses (data not shown) to measure relative steady-state levels of H1t mRNA in normal mouse tissues based upon the ability of the rat H1t probe to cross-hybridize to the mouse H1t mRNA. Therefore, S1 nuclease protection analysis was utilized to ensure that only rat or mouse H1t mRNA was detected when examining total cellular RNA from various tissues and testis cell types from transgenic animals.

Transgenic mice were bred to each other, and the offspring were subjected to screening by the same PCR and restriction digest analysis used for initial screening of founders. Selected positives from the F1 and F2 generations were sacrificed, and their RNA was analyzed by S1 nuclease protection to determine steady-state levels of H1t mRNA levels in several tissues. Only testis had a detectable steady-state levels of rat histone H1t mRNA. Six individual mice had the testis-specific pattern, and we have not observed expression of the rat H1t gene in any other tissue.

Since the copy-number of the rat H1t gene was high in several transgenic animals, we felt that the gene might not be expressed in a tissue-specific manner, but this was not the case. Therefore, even though the copy-number of the transgene is variable, testis-specific expression of the gene is under very stringent control. Furthermore, although the copy-number of the gene is high is some transgenic animals, the relative steady-state level of rat H1t mRNA never exceeded 2.6 times the level found in testes of normal rats. This is not surprising because both core and linker histones may be able to autoregulate steady-state levels of histone mRNA (McLaren & Ross, 1993).

However, the steady-state level of endogenous mouse H1t mRNA may be lower in some high copy-number transgenic animals (data not shown). In these cases, it is possible that the high copy-number transgene might compete for transcription factors so that the transgene is preferentially transcribed over the endogenous mouse gene. The apparent low level of the mouse H1t mRNA in some animals was not due to the loss of the mouse H1t gene as shown in Figure 8. Both rat and mouse genes have been detected in all animals examined.

Additionally, we found that the rat gene was expressed appropriately in every animal examined. Not only was it expressed exclusively in testis but it also appeared to be expressed exclusively in primary spermatocytes. Thus, the rat gene was expressed only in testis in the presence of the active mouse H1t gene. Even though it was present in some animals in a high copy-number, it was expressed only in the meiotic prophase of the cell cycle. There is obviously stringent cell cycle regulation and tissue-specific regulation of expression of the gene. This is due to a combination of regulation of transcription of the gene and regulation of the turnover rate of H1t mRNA, leading to relatively constant steady-state levels of rat H1t mRNA in testes of transgenic mice with low or high copy-number transgene compared to the level in testes of normal rats (Figures 4 and 7).

Concerning transcriptional regulation of the H1t gene, two imperfect inverted copies of the TE element designated TE1 and TE2 are located between the H1/AC box and H1/CAAT box in the promoter. Our laboratory has shown that this region specifically binds testis nuclear proteins (Grimes et al., 1992a,b). Independent studies of the $LDHC_4$ gene have led to identification of a region identical to the TE element which was shown to bind testis nuclear proteins and to be essential for testis-specific transcription (Zhou et al., 1994). One interpretation of our data is that mouse testis-specific proteins bind to the TE elements, leading to tissue-specific expression and meiotic cell cycle specific regulation of both the endogenous mouse H1t gene and the rat H1t transgene. It is interesting that the H1t promoter region extending from the TE2 element through the GC-rich element, TE1 element, and the CCAAT element is reported to contain a sequence element found in many testis genes (Queralt & Oliva, 1993). Clearly, this region of the H1t promoter is unique compared to the promoters of mitotic cell cycle regulated H1 genes, and it seems likely that this region is involved in part in regulating testis-specific expression of the H1t gene.

In future studies, it will be important to examine each H1t promoter element and determine its contributions to testis-specific transcription. These studies should include mutagenesis of the promoter to examine effects on *in vitro* and *in vivo* transcription. Transgenic mice can provide a clear demonstration of the relative importance of specific mutations on tissue-specific transcription *in vivo*, and therefore they may be essential in this type of experiment. It will also be important to examine H1t mRNA stability to determine the contribution of turnover rate to measured steady-state levels of mouse and rat H1t mRNA in the transgenic animals. Furthermore, existing technology should allow the production of mice with an inactivated histone H1t gene. Such "knock-out" mice will be useful for examination of the functional importance of histone H1t to spermatogenesis and should allow expression of rat H1t in mice lacking their own gene.

REFERENCES

Albig, W., Drabent, B., Kunz, J., Kalff-Suske, M., Grzeschik, K.-H., & Doenecke, D. (1993) *Genomics 16*, 649−654.

Blanco, A. (1991) *Acad. Nac. Cienc. (Cordoba Argent.) Misc. 84*, 1−34.

Branson, R. E., Grimes, S. R., Yonuschot, G., & Irvin, J. L. (1975) *Arch. Biochem. Biophys. 168*, 403−412.

Bucci, L. R., Brock, W. A., & Meistrich, M. L. (1982) *Exp. Cell Res. 140*, 111−118.

Burfeind, P., Hoyer-Fender, S., Doenecke, D., Hochhuth, C., & Engel, W. (1994) *Hum. Genet. 94*, 633−639.

Cole, K. D., Kandala, J., & Kistler, W. S. (1986) *J. Biol. Chem. 261*, 7178−7183.

De Lucia, F., Faraone-Mennella, M. R., D'Erme, M., Quesada, P., Caiafa, P., & Farina, B. (1994) *Biochem. Biophys. Res. Commun. 198*, 32−39.

Doenecke, D., Albig, W., Bouterfa, H., & Drabent, B. (1994) *J. Cell. Biochem. 54*, 423−431.

Drabent, B., Kardalinou, E., & Doenecke, D. (1991) *Gene 103*, 263−269.

Drabent, B., Bode, C., & Doenecke, D. (1993) *Biochim. Biophys. Acta 1216*, 311−313.

Enrietto, P. J., Payne, L. N., & Hayman, M. J. (1983) *Cell 35*, 369−379.

Favaloro, J., Treisman, R., & Kamen, R. (1980) *Methods Enzymol. 65*, 718−749.

Finch, J. T., & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1897−1901.

Grabske, R. J., Lake, S., Gledhill, B. L., & Meistrich, M. L. (1975) *J. Cell. Physiol. 86*, 177−190.

Graziano, V., Gerchman, S. E., Schneider, D. K., & Ramakrishnan, V. (1994) *Nature 368*, 351−354.

Grimes, S. R. (1986) *Comp. Biochem. Physiol. B 83*, 495−500.

Grimes, S. R., Platz, R. D., Meistrich, M. L., & Hnilica, L. S. (1977) *Exp. Cell Res. 110*, 31−39.

Grimes, S., Weisz-Carrington, P., Daum, H., Smith, J., Green, L., Wright, K., Stein, G., & Stein, J. (1987) *Exp. Cell Res. 173*, 534−545.

Grimes, S. R., Wolfe, S. A., Anderson, J. V., Stein, G., & Stein, J. L. (1990) *J. Cell. Biochem. 44*, 1−17.

Grimes, S. R., Wolfe, S. A., & Koppel, D. A. (1992a) *Arch. Biochem. Biophys. 296*, 402−409.

Grimes, S. R., Wolfe, S. A., & Koppel, D. A. (1992b) *Exp. Cell Res. 201*, 216−224.

Guo, L., Yang, R. C. A., & Wu, R. (1983) *Nucleic Acids Res. 11*, 5521−5540.

Hayes, J. J., & Wolffe, A. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6415−6419.

Johnson, P. A., Peschon, J. J., Yelick, P. C., Palmiter, R. D., & Hecht, N. B. (1988) *Biochim. Biophys. Acta 950*, 45−53.

Johnson, P. A., Bunick, D., & Hecht, N. B. (1991) *Biochim. Biophys. Acta 44*, 127−134.

Khadake, J. R., Markose, E. R., & Rao, M. R. S. (1994) *Indian J. Biochem. Biophys. 31*, 335−338.

Kistler, W. S., Geroch, M. E., & Williams-Ashman, H. G. (1973) *J. Biol. Chem. 248*, 4532−4543.

Koppel, D. A., Wolfe, S. A., Fogelfeld, L. A., Merchant, P. S., Prouty, L., & Grimes, S. R. (1994) *J. Cell. Biochem. 54*, 219−230.

Krylov, D., Leuba, S., Van Holde, K., & Zlatnova (1993) *Proc. Natl. Acad. Sci. USA 90*, 5052−5056.

McLaren, R. S., & Ross, J. (1993) *J. Biol. Chem. 268*, 14637−14644.

Meistrich, M, (1989) in *Histones and Other Basic Nuclear Proteins* (Hnilica, L. S., Ed.) pp 166−182, CRC Press, Inc., Boca Raton, FL.

Meistrich, M. L., Longtin, J., Brock, W. A., Grimes, S. R., & Mace, M. L. (1981) *Biol. Reprod. 25*, 1065−1077.

Ohe, Y., Hayashi, H., & Iwai, K. (1989) *J. Biochem. 106*, 844−857.

Peschon, J. J., Behringer, R. R., Brinster, R. L., & Palmiter, R. D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5316−5319.

Queralt, R., & Oliva, R. (1993) *Gene 133*, 197−204

Ramakrishnan, V., Finch, J. T., Graziano, V., Lee, P. L., & Sweet, R. M. (1993) *Nature 362*, 219−223.

Salehi-Ashtiani, K., Widrow, R. J., Markert, C. L., & Goldberg, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8886−8890.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Nolan, C., Ed.) 2nd ed., pp E.37−E.38, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Simoni, M. (1994) *Exp. Clin. Endocrinol. 102*, 419−433.

Smith, F. F., Tres, L. L., & Kierszenbaum, A. L. (1992) *Dev. Dyn. 193*, 49−57.

Tamura, T., Makino, Y., Mikoshiba, K., & Muramatsu, M. (1992) *J. Biol. Chem. 7*, 4327−4332.

Thoma, F., Koller, R., & Klug, A. (1979) *J. Cell Biol. 83*, 403−427.

vanWijnen, A. J., Aziz, F., Grana, X., Desai, R. K., Jaarsveld, K., Last, T. J., Soproano, K., Giordano, A., Lian, J. B., Stein, J. L., & Stein, G. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12882−12886.

Wolffe, A. P. (1994) *Trends Biochem. Sci. 19*, 240−244.

Wolfe, S. A., & Grimes, S. R. (1993) *J. Cell. Biochem. 53*, 156−160.

Zambrowicz, B. P., & Palmiter, R. D. (1994) *Biol. Reprod. 50*, 65−72.

Zhou, W., Xu, J., & Goldberg, E. (1994) *Biol. Reprod. 51*, 425−432.

BI950503V