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Binding of Nuclear Proteins to a Conserved Histone H1t Promoter Element Suggests an Important Role in Testis-Specific Transcription

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

Research Service, Veterans Administration Medical Center and Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana 71101-4295

Abstract The testis-specific histone H1t gene is transcribed only in primary spermatocytes during spermatogenesis. Recently, expression of the rat gene was shown to be limited to primary spermatocytes in transgenic mice, revealing that promoter elements sufficient for regulating tissue-specific transcription were present in the cloned rat gene. In this study the mouse histone H1t gene has been cloned, and sequenced and its promoter region has been compared to the rat H1t promoter with regard to conserved elements and protein binding activity. The amino acid sequence of each of the three H1t coding region domains is conserved when compared to the homologous domain in H1t derived from other species. H1t mRNA is found only in testis, where it accumulates to a high steady-state level, and examination of enriched testis cell populations shows that expression is limited to primary spermatocytes. Protein binding assays using nuclear extracts from various mouse tissues reveal testis-specific binding to TE1 and TE2, imperfect inverted repeat elements within the larger TE element. Although the H1t promoter contains an Sp1 consensus motif within the H1t/TE element, binding of testis Sp1 to the motif could not be detected using specific anti-Sp1 antibodies.

Key words: histone H1t, gene regulation, tissue-specific gene expression, tissue-specific transcription, TE, DNA binding protein

Histones are small, highly abundant basic nuclear proteins found in eukaryotic cells. DNA wraps twice around the core histone octamer forming a nucleosome that protects 146 base pairs from micrococcal nuclease digestion [Richmond et al., 1984]. Histone H1 variants mediate chromatin condensation, binding to the nucleosome where DNA enters and leaves the octamer [Finch and Klug, 1976], protecting an additional 20 base pairs from nuclease digestion, and packing chromatin into a 30 nm fiber [Thoma et al., 1979; Hayes and Wolffe, 1993]. The presence or absence of histone H1 and nucleosomes may regulate transcription by sequestering or exposing DNA to transcriptional activators [Wolffe, 1994].

H1t, like other H1 variants, has a variable N-terminal domain, a conserved central globular domain, and a basic and highly variable C-terminal domain. Globular domains of H1 histones including H5, a variant found in nucleated erythrocytes, are evolutionarily conserved. This domain binds to DNA [Krylov et al., 1993], and two probable DNA binding sites have been identified [Ramakrishnan et al., 1993]. The H1t globular domain may have similar DNA binding sites. Although assumed to mediate chromatin condensation, H1t binds DNA with lower affinity than other H1 variants, rendering it relatively more sensitive to DNase I. Studies leading to these conclusions [De Lucia et al., 1994; Khadake et al., 1994] provide compelling evidence supporting a suggestion that H1t maintains chromatin in a relatively decondensed state, facilitating events such as recombination [De Lucia et al., 1994].

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

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Address reprint requests to Sidney R. Grimes, Research Service (151), Overton Brooks VA Medical Center, 510 E. Stoner Avenue, Shreveport, LA 71101-4295.

Synthesis of replication-dependent histone variants is coupled to DNA synthesis during the mitotic cell cycle. Increased S-phase transcription is mediated by mitotic cell cycle regulatory factors including CDC2, cyclin A, and RB-related proteins [vanWijnen et al., 1994]. Unlike somatic, replication-dependent H1 histones, histone H1t synthesis occurs outside of the mitotic cell cycle. The H1t gene is transcribed and histone H1t is synthesized during the meiotic cell cycle [Kistler et al., 1973; Branson et al., 1975; Grimes, 1986; Bucci et al., 1982], and H1t mRNA accumulates only in pachytene primary spermatocytes [Cole et al., 1986; Drabent et al., 1991; Grimes et al., 1992a; Smith et al., 1992]. Transcription is likely regulated by meiotic cell cycle factors specific to the testis.

Recent studies revealed an interesting H1t/TE promoter sequence element located between the H1/AC box and H1/CCAAT box of the rat H1t gene [Grimes et al., 1990; Wolfe et al., 1995]. The appearance of testis-specific binding proteins that recognize this sequence correlates with the onset of transcription of the H1t gene during spermatogenesis [Grimes et al., 1992a,b]. Potential regulatory sequences within promoters of some testis-expressed genes [Queralt and Oliva, 1993] contain a copy of the TE element. Additionally, a portion of the TE element is found in the testis-specific $LDHC_4$ promoter and is necessary and sufficient for directing testis-specific transcription of the $LDHC_4$ gene [Zhou et al., 1994]. More recently the rat H1t gene driven by its natural promoter was shown to provide testis-specific expression of rat H1t mRNA in transgenic mice [vanWert et al., 1995], demonstrating the presence of functionally important H1t promoter sequence elements sufficient for tissue-specific transcription of the gene.

To further explore the H1t promoter and to compare the amino acid sequences of mammalian H1t histones, the mouse gene was cloned and sequenced. The amino acid sequence is highly conserved compared to other mammalian H1t histones. Expression of the gene was limited to testis and within the testis to cells enriched in primary spermatocytes. Although the H1t gene shares promoter sequences found in other H1 genes, experiments show testis-specific binding of nuclear proteins to both the H1t/TE2 element and the H1t/TE1 element, imperfect inverted repeats contained within the larger TE element. On the other hand, binding of testis Sp1 to the GC-rich region, which is also located within the TE element, was not detected.

MATERIALS AND METHODS

Reagents and Supplies

Radionuclides were purchased from DuPont NEN (Boston, MA). An oligolabeling kit was obtained from Pharmacia (Piscataway, NJ). The fmol™ sequencing kit was ordered from Promega (Madison, WI), and DNA sequencing was performed with universal primers end-labeled with [γ-^{32}P] ATP using T4 Polynucleotide kinase (Promega). Calf intestinal alkaline phosphatase and S1 nuclease were purchased from Boehringer-Mannheim (Indianapolis, IN). Anti-Sp1 antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Oligonucleotides were obtained from Biosynthesis, Inc. (Denton, TX) and from Oligos Etc. (Wilsonville, OR). X-ray film was purchased from Eastman Kodak (New Haven, CT) (X-OMAT XAR-5) and DuPont (Cronex-7).

Isolation of Tissues and Enriched Populations of Testis Cells

Male C57BL/6 and CD-1 mice were obtained from Harlan (Dallas, TX), and Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Houston, TX) and maintained in a fully AAALAC accredited facility. Populations of mouse testis cell types were enriched by centrifugal elutriation using trypsin to prepare single-cell suspensions from six to ten mice [Grimes et al., 1990; Grabske et al., 1975; Meistrich et al., 1981]. Fractions were obtained at the following rotor speeds and flow rates: fraction 1—3,000 rpm, 13.5 ml/min; fraction 2—3,000 rpm, 17.9 ml/min; fraction 3—3,000 rpm, 31.3 ml/min; fraction 4—2,000 rpm, 23.2 ml/min; and fraction 5—2,000 rpm, 40 ml/min.

PCR Amplification

Genomic DNA templates used for PCR amplification were isolated from CD-1 mouse testis. In some experiments the template was the mouse H1t gene insert from the plasmid pMH1t. Amplification was performed for 30 cycles in 100 µl of solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM concentration of each dNTP, 100 pmol of each primer, 1 µg of genomic DNA template, and 4 units of *Taq* DNA polymerase. Prior to addition of *Taq* (Parkin-Elmer, Roche, Branchburg, NJ) polymer-

ase, samples were heated to 94°C for 5 min. Each cycle reaction consisted of a 1 min 94°C denaturation step, a 1 min 55°C annealing step, and a 1 min 72°C extension step. Reaction products were separated electrophoretically on 1% agarose gels, and ethidium bromide–stained bands were recorded by photography.

Cloning the Mouse H1t Gene

The mouse H1t gene was amplified by PCR from a CD-1 mouse testis DNA template, using the rat H1/AC box primer (Table 1; Fig. 1B) and 3′ stem-loop primer (Table I; Fig. 1B). Following amplification, PCR product was filled using the Klenow fragment of DNA polymerase, phosphorylated using T4 kinase, and electrophoresed on a 1% low melting agarose gel. The 851 bp band was excised and cloned into the *Sma* I site of plasmid pUC 19. The resulting recombinant plasmid containing the mouse H1t gene designated pMH1t (GenBank accession number L28753) [vanWert et al., 1995] was sequenced using universal primers for pUC plasmids supplied by Promega. The promoter region and part of the coding region were directly sequenced from genomic DNA template using the same primers to confirm the sequence of the cloned PCR product.

RNA Isolation and Analysis

RNA was extracted from mouse tissues and testis cell types using RNA Stat-60 (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol with additional steps of a phenol (pH 4.5) extraction and ethanol precipitation. RNA was dissolved in water, and the quantity was estimated by measuring absorbance at 260 nm. Desired quantities of RNA were precipitated and dissolved in 20 μl of sample loading buffer (50% formamide, 1× MOPS buffer (40 mM Morpholinopropanesulfonic acid, pH 7, 10 mM sodium acetate, 1 mM EDTA), 6% formaldehyde, 0.02% bromophenol blue), incubated 5 min at 56°C, and electrophoresed at 75 mA for 2 h on a 1.5% agarose gel containing 1× MOPS and 6% formaldehyde [Grimes et al., 1990]. After electrophoresis, the gel was soaked in water for 30 min to remove formaldehyde, stained with 0.5 μg/ml ethidium bromide in water, and destained in water to allow photography of the stained ribosomal RNA bands. RNA was blotted, immobilized to Nytran (Schleicher and Schuell, Keene, NH) by capillary transfer from the gel, and UV cross-linked with 2.4×10^5 μJoules of 254 nm light in a Stratalinker 1800 (Stratagene, LaJolla, CA).

To detect H1t mRNA, the blot was hybridized using the insert from pMH1t oligolabeled with [α^{32}P] dCTP. The Nytran membrane was prehybridized at 43°C for 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 at 47°C for 16 h in fresh solution of the same composition and containing 1×10^6 DPM/ml of the single-stranded labeled H1t probe. The filter was washed in a solution containing 5× SSC, 1× Denhardt's at room temperature for 10 min followed by a 59°C incubation for 30 min. The blot was washed successively in solutions of 5× SSC, 2× SSC, 1× SSC, and 0.1× SSC, each containing 0.1% SDS.

S1 nuclease protection analysis was conducted [Favaloro et al., 1980] to determine the H1t mRNA start site. Testis RNA samples were coprecipitated with probe DNA, resuspended in hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, 0.4 M NaCl), denatured 10 min at 65°C, and hybridized overnight at 30°C. Resulting hybrids were digested with S1 nuclease. The products were ethanol-precipitated and analyzed on a sequencing gel.

Probes

For northern blots to detect mouse H1t mRNA, plasmid pMH1t containing the mouse H1t gene insert was digested with BamH1 and EcoR1 to yield the full-length mouse H1t gene (851 bases) plus 25 bases of polylinker sequence from the plasmid. Restriction fragments were resolved on a 1% low-melting agarose gel, the appropriate band was excised, and the DNA recovered by hot phenol extraction [Guo et al., 1983]. The quantity of the purified fragment was estimated, the fragment was oligolabeled with [α-^{32}P] dCTP, and unincorporated label was removed from the probe by gel filtration on Sephadex G-50 [Sambrook et al., 1989].

For electrophoretic mobility shift assays, complementary oligonucleotides corresponding to regions of the H1t promoter were annealed and end-labeled with [γ-^{32}P] ATP using T4 kinase. The labeled DNA was separated from reaction mixture components and unincorporated label by purification on an 8% polyacrylamide gel.

TABLE I. Oligonucleotides Used for Amplification, S1 Nuclease Protection Analysis, and EMSA and Features of the H1t Gene*

Oligonucleotides used for PCR, S1 nuclease protection analysis, and EMSA

Name	Position	Sequence
H1/AC box	−109 to −90	GGGGGAAAAGAAACACAAAT
Primer extension	172 to 191	ACTGAGAAACCGCGAGGTTT
Stem-loop	723 to 742	AAGTGGCTCTTAAAAGAGCC
H1t/TE2 element	−89 to −73	G**T**CCC**T**C**T** CC**G**GGGGC
H1t/TE2 element (rat)		GCCCCTTCCCCAGGGGC
H1t/TE1 element	−68 to −51	GAGGC**A**CCT**G**GGGATGC
H1t/TE1 element (rat)		GAGGCGCCTAGGGATGC
Sp1 consensus[a]		ATTCGATC**GGGGCGGGGG**GAGC

Features of the H1t gene

Name	Position	Sequence
H1/AC box	−99 to −93	AAACACA
H1t/TE element	−89 to −51	G**T**CCC**T**C**T** CC**G**GGGGGCGGGGGAGGC**A**CCT **G**GGGATGCA
H1t/TE2 element	−89 to −73	G**T**CCC**T**C**T** CC**G**GGGGC
H1t/TE1 element	−68 to −51	GAGGC**A**CCT**G**GGGATGC
H1/GC box	−69 to −77	GGGGGCGGGGG
H1/CCAAT box	−50 to −37	CCAATCACAGCGCG
TATA box	−28 to −22	TATATAA
mRNA start	1	ACTACCC
ATG start codon	67	ATG
Coding region	67 to 690	207 amino acids
Stem-loop	723 to 742	GGCTCTTTTAAGAGCC
Imperfect inverted repeat		CGCCTAGGGATG (rat TE1)
		C**C**CCT**G**GGGAA**G** (rat TE2 inverted)

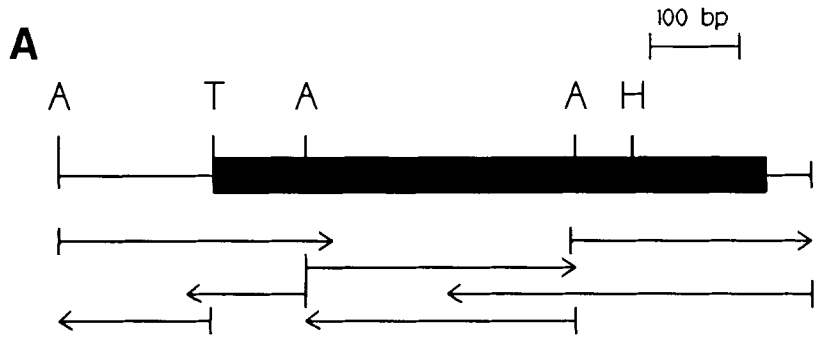
r GGGGGAAAAGAAACACAAATG**C**CCCTTC**C**CC**A**GGGGCGGGGGAGGC**G**CCT**A**GGGATGCACCAATCACAGCGCGCCCTGCTCTATATAA

m GGGGGAAAAGAAACACAAATGTCCCTCCTCCGGGGGCGGGGGAGGCACCTGGGGATGCACCAATCACAGCGCGCCCTGCTCTATATAA

H1/AC H1t/TE2 H1t/TE1 H1/CCAAT TATA

*Numbered sequences are presented in Fig. 1B. Oligonucleotides are listed in the 5′ to 3′ direction.

[a]The 21 bp Sp1 consensus oligonucleotide was purchased from Santa Cruz Biotechnology, Inc. The region identical to the H1/GC box is underlined and in bold letters.



B

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-109                GGGGGAAAA GAAACACAAA TGTCCCTCCT CCGGGGGCGG GGGAGGCACC
                                        5                4
-60  TGGGGATGCA CCAATCACAG CGCGCCCTGC TCTATATAAG CGCCCCCCCC GCACGCCCCC
                3       2                       1
  1  ACTACCCCAT CACTGTTGGC TCTCCTTTAG TTTTCACTTG GTGTAGTTCT TGTCCAGCTC

 61  TTGACC          ATG TCG GAA ACG GCT CCT GCG GCC TCA AGT ACC
                         Ser Glu Thr Ala Pro Ala Ala Ser Ser Thr    10

100  CTT GTT CCA GCT CCT GTG GAG AAA CCT TCA TCT AAA AGG CGA GGG
     Leu Val Pro Ala Pro Val Glu Lys Pro Ser Ser Lys Arg Arg Gly    25

145  AAA AAG CCT GGC CTG GCC CCT GCT CGC AAA CCT CGG GGT TTC TCA
     Lys Lys Pro Gly Leu Ala Pro Ala Arg Lys Pro Arg Gly Phe Ser    40
                                                    7
190  GTT TCC AAG CTG ATT CCT GAG GCC CTT TCC ACA TCG CAG GAA CGG
     Val Ser Lys Leu Ile Pro Glu Ala Leu Ser Thr Ser Gln Glu Arg    55

235  GCA GGA ATG TCC CTT GCT GCC CTG AAG AAA GCC CTG GCT GCG GCT
     Ala Gly Met Ser Leu Ala Ala Leu Lys Lys Ala Leu Ala Ala Ala    70

280  GGT TAC GAC GTG GAG AAG AAC AAC AGC CGC ATC AAG CTG GCC CTC
     Gly Tyr Asp Val Glu Lys Asn Asn Ser Arg Ile Lys Leu Ala Leu    85

325  AAG AGA CTT GTG AAT AAA GGA GTC CTG GTG CAG ACC AAG GGC ACT
     Lys Arg Leu Val Asn Lys Gly Val Leu Val Gln Thr Lys Gly Thr   100

370  GGC GCC TCA GGC TCC TTC AAG CTC AGT AAG AAG GCG GCT TCT GGG
     Gly Ala Ser Gly Ser Phe Lys Leu Ser Lys Lys Ala Ala Ser Gly   115

415  AAC GAC AAG GGC AAG GGC AAG AAA TCT GCT TCT GCC AAG GCT AAG
     Asn Asp Lys Gly Lys Gly Lys Lys Ser Ala Ser Ala Lys Ala Lys   130

460  AAG ATG GGC TTG CCC CGG GCC TCC AGA TCT CCC AAG AGT AGC AAG
     Lys Met Gly Leu Pro Arg Ala Ser Arg Ser Pro Lys Ser Ser Lys   145

505  ACC AAG GCT GTC AAG AAG CCA AAG GCT ACG CCC ACA AAA GCT TCT
     Thr Lys Ala Val Lys Lys Pro Lys Ala Thr Pro Thr Lys Ala Ser   160

550  GGG AGC AGA AGG AAG ACC AAA GGG GCC AAG GGC GTG CAG CAA CGT
     Gly Ser Arg Arg Lys Thr Lys Gly Ala Lys Gly Val Gln Gln Arg   175

595  AAA AGC CCC GCC AAA GCC AGG GCA GCA AAC CCC AAT TCT GGG AAG
     Lys Ser Pro Ala Lys Ala Arg Ala Ala Asn Pro Asn Ser Gly Lys   190

640  GCA AAG ATG GTC ATG CAG AAG ACC GAT CTG AGG AAG GCA GCA GGG
     Ala Lys Met Val Met Gln Lys Thr Asp Leu Arg Lys Ala Ala Gly   205

685  AGG AAG TGA          GTTTCAAAGC CAGTTTTCAA AAACCCAAAG GCTGTTTTAA
     Arg Lys ***                                                 6

734  GAGCCACTT
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Fig. 1. Nucleotide sequencing strategy, partial restriction map, and nucleotide sequence of the mouse testis-specific histone H1t gene. **A:** Nucleotide sequencing strategy and partial restriction map of the H1t gene. *Arrows* indicate the actual areas and direction sequenced. The three Ava I fragments were subcloned to facilitate sequencing using pUC universal primers. The restriction enzymes marked on the map include T, TthIII-1; A, AvaI; and H, HindIII. The coding region of the mouse H1t gene is marked as a black-filled box. **B:** Nucleotide sequence of the H1t gene with the deduced amino acid sequence. GenBank accession number L28753 has been assigned to the mouse histone H1t gene described in this paper. The sequence shown includes the primers (lowercase underlined regions 5 and 6) used to amplify the fragment during PCR. The oligonucleotide primer at 5 is derived from the rat H1t sequence (accession number M28409), and the primer at 6 is derived from the human H1t sequence (accession number M60094). Numbering on the left side refers to the nucleotide sequence relative to the transcription start site at +1. The italic numbering on the right refers to the amino acid residues. Other underlined conserved sequence elements are 1) TATA box, 2) H1/CCAAT box, 3) H1t/TE1 element, 4) H1/TE2 element, and 7) a 3′ primer used in conjunction with the 5′ H1/AC box primer to amplify a 300 bp DNA fragment corresponding to the 5′ end of the H1t gene that was used as an S1 nuclease probe to map the start site of the mature mouse H1t mRNA.

For S1 nuclease protection analysis, a fragment composed of nucleotides −109 to 191 of the mouse H1t gene was amplified using an end-labeled 3′ primer (Table I; Fig. 1B; nucleotides 172–191) and the H1/AC box primer (Table I; Fig. 1B; nucleotides −109 to −90) and using plasmid pMH1t template DNA. The product was electrophoresed on a 1% low melting agarose gel, the 300 bp band was excised, and the probe was recovered by hot phenol extraction [Guo et al., 1983].

Electrophoretic Mobility Shift Assays

Nuclear extracts [Dignam et al., 1983] were prepared from crude nuclei as described previously [Grimes et al., 1992a,b]. Reactions contained 10 μg of nuclear extract, 500 ng of nonspecific competitor (poly dGdC), and labeled probe representing 10,000 DPM in a final volume of 20 μl. Samples were incubated on ice for 30 min. In competition assays, specific unlabeled competitor DNA was added to the nuclear extract after the nonspecific competitor DNA, and the mixture was incubated on ice for 15 min before adding labeled probe. In Sp1 supershift experiments anti-Sp1 (Santa Cruz Biotechnology, Inc.) was added at the end of the normal binding reaction, and the sample was incubated on ice for an additional hour. Samples were electrophoresed on a 4% polyacrylamide gel (60:1, acrylamide:bis-acrylamide [wt/wt]) for 3.5 h.

RESULTS

Cloning and Sequencing the Testis Histone H1t Gene

The histone H1t gene was amplified from mouse genomic DNA using oligonucleotide primers designed from the rat H1t gene sequence [Grimes et al., 1990]. The forward primer was identical to the rat H1/AC box, and the reverse primer was identical to the complement of the rat 3′ stem loop located in the 3′ untranslated region of the H1t gene. The sequences of these primers, presented in Table I, are underlined in Figure 1B. The validity of this method was confirmed by cloning and sequencing the known Sprague-Dawley rat H1t gene.

Both strands of the testis histone H1t gene were sequenced using the strategy illustrated in Figure 1A. Template DNA used for sequencing had been cloned from a DNA fragment amplified by *Taq* polymerase. To further verify that sequence artifacts had not been generated by *Taq* polymerase, portions of the mouse gene were sequenced using mouse genomic DNA as a template. Direct sequencing of mouse as well as of rat histone H1t genes from genomic DNA has not revealed differences compared to the sequences obtained from templates that were amplified and cloned. However, two differences were noted in this sequence from CD-1 mice compared to the sequence from BALB/c mice [Drabent et al., 1993]. The CD-1 mouse H1t gene sequence is two bases shorter (missing a CC dinucleotide sequence) in the region located between the TATA box and the mRNA start site (Fig. 1B). The CD-1 mouse H1t sequence is **A**GA (bases 556–558) encoding the amino acid Arginine (amino acid 163) (Fig. 1B), whereas the BALB/c mouse H1t sequence is **C**GA encoding Glycine. This difference is located in the hypervariable C-terminal domain of histone H1t (Fig. 2A,B). Note that the amino acid at this position in the rat is also Arginine, while in the human and monkey it is Glycine (Fig. 2A).

The length of the mouse H1t gene sequence is 851 bp (Fig. 1B). The open reading frame encoded 207 amino acids as shown in the same figure. The entire mouse H1t gene sequence shown is 91% identical to the rat H1t gene sequence. The data presented in Table I reveal that only six nucleotide differences exist between the mouse and rat H1t sequences in the promoter region located between the H1/AC box and the TATA box. The lengths of the two promoters are identical in this region. Although there are 23 nucleotide differences located between the TATA box and ATG start codon, six of these are caused by insertion of extra T residues between positions 34 and 35 in the rat sequence. This high degree of conservation extends to the human and monkey H1t sequences [Koppel et al., 1994].

The mouse H1t promoter has all of the recognized functionally important consensus elements found in replication-dependent histone H1 genes (Fig. 1B; Table I). The H1/AC box, underlined in the Figure 1B, is identical to the rat sequence and is conserved in all H1 genes. There is a GC-rich element centered at nucleotide −73 (Fig. 1B; Table I) that is identical to a consensus Sp1 binding motif. However, this site is covered by the H1t/TE element composed of the subelement TE1 that overlaps the 3′ side of the GC-rich site and of the subelement TE2 that overlaps the 5′ side of the site. TE1 and TE2 are imperfect inverted repeats within the larger H1t/TE element. This is seen most clearly by

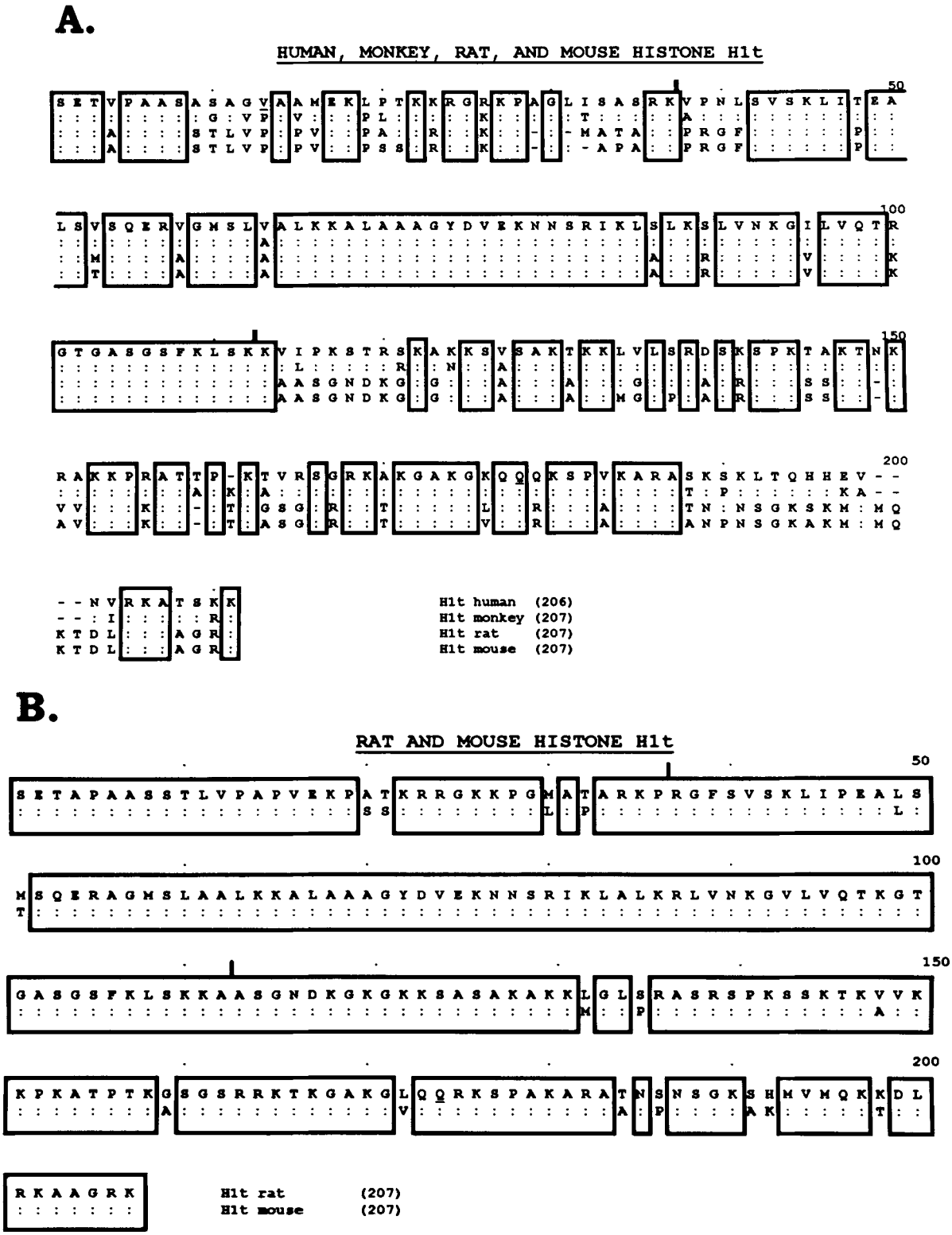

Fig. 2. Comparison of amino acid sequences of human, monkey, rat, and mouse histone H1t. **A:** Alignment of mouse, rat, human, and rhesus monkey H1t amino acid sequences. The top line is the human H1t sequence [Koppel et al., 1994]. Heavy marks at 37 and 112 indicate the domain boundaries separating the N-terminal domain, central globular domain, and the C-terminal domain (amino acids 1–37, 38–112, and 113–207, respectively). For the other sequences a colon signifies identity to the human sequence, and hyphens define gaps in each sequence necessary for alignment. Numbers shown above and to the right refer to a general reference frame, and the parentheses that follow each sequence indicate the protein's presumed length. The boxes are drawn around aligned identical amino acids to emphasize conserved regions such as the central globular domain. **B:** Alignment of rat and mouse H1t amino acid sequences. The mouse H1t amino acid sequence is aligned with the Sprague-Dawley rat sequence to emphasize conservation of the two rodent sequences. Note that no gaps are necessary to align these two sequences. Heavy marks indicate boundaries separating the N-terminal, central globular, and the C-terminal domains, as in Fig. 2A.

comparison of the rat TE1 sequence to the inverted TE2 sequences in Table I. The H1/CCAAT box is found in all known mammalian H1 genes except $H1^0$. The TATA box is found in all vertebrate H1 genes.

To map the 5′ end of the mature testis histone H1t mRNA, S1 nuclease protection was conducted with a probe amplified by PCR. The 3′ primer used for amplification was end-labeled with [^{32}P]. The 5′ and 3′ primers were the 20 base H1/AC box primer (bases −109 to −90) and the complement to bases 172 to 191 as underlined and labeled 5 and 7, respectively, in Figure 1B. The 5′ end of the mature testis H1t mRNA was determined by measuring the size of the protected DNA fragment compared to a DNA sequencing ladder run as a marker. The start site of the mRNA is labeled as base number 1 in Figure 1B and Table I.

The strategy for cloning the mouse H1t gene depended in part upon the identity of the 3′ untranslated region of rat and mouse H1t genes. The sequence of the 3′ primer used in amplification of the mouse H1t gene was based upon the conserved histone specific dyad symmetry element underlined in Figure 1B and listed in Table I. This region of the gene is transcribed and leads to formation of a stem-loop structure at the 3′ terminus of the mature histone mRNA. Although not an ideal sequence to use as a primer with respect to hairpin formation, a DNA fragment corresponding to the mouse H1t gene was successfully amplified using this primer.

The amino acid sequence of mouse histone H1t is shown as the deduced translation of the nucleotide sequence of the coding region in Figure 1B. The mouse, rat, monkey, and human histone H1t amino acid sequences are compared in Figure 2A. The three domains of H1t are marked in the figure by heavy vertical marks at amino acids 37 and 112 and are known as the N-terminal domain (1–37), central globular domain (38–112), and C-terminal domain (113–207). Overall the mouse and rat sequences are 93% identical, and within the N-terminal, central globular, and C-terminal domains they are 86%, 99%, and 92% identical, respectively, as seen in Figure 2B. For comparison, the human and rhesus monkey sequences are 89% identical overall, and within the three domains they are 78%, 97%, and 86% identical, respectively. The mouse sequence is 61% identical to the human, and within the three domains they are 51%, 84%, and 48% identical, respectively. The homology among mammalian H1t sequences when comparing different species is greater than the homology between H1t and other known H1 variants within a single species.

Testis-Specific Expression of the Histone H1t Gene

Steady-state levels of H1t mRNA in various tissues were examined by northern blot analysis. Total cellular RNA samples from different tissues were electrophoresed on a denaturing agarose gel, as shown by ethidium bromide staining in the left panel of Figure 3. RNA was blotted to NYTRAN and probed for H1t mRNA using the cloned mouse DNA insert cut from the plasmid pMH1t and labeled with [^{32}P]. Histone H1t mRNA accumulated to a high steady-state level only in testis, as seen by the autoradiogram in the right panel of Figure 3.

To determine which testis cell types accumulate histone H1t mRNA, total cellular RNA samples from testis cell types enriched by centrifugal elutriation were examined. The population of cells with the highest steady-state level of histone H1t mRNA was the elutriator fraction most enriched in pachytene primary spermatocytes (Fig. 4B). Elutriator fractions enriched in late spermatids and early spermatids had relatively low steady-state levels of histone H1t mRNA.

To determine whether H1t mRNA accumulates in early germinal cell types that are prede-

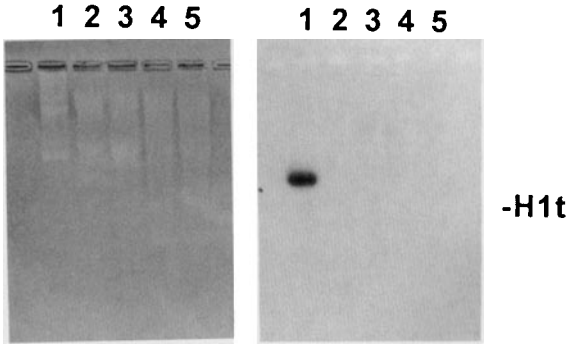

Fig. 3. Testis-specific accumulation of H1t mRNA. In this northern blot analysis, the left panel shows ethidium bromide–stained total cellular RNA from each 10 μg sample indicating approximately equal loading of RNA. The RNA samples are derived from testis, liver, brain, kidney, and spleen (*lanes 1–5*, respectively). The right panel shows the autoradiogram after the samples were transferred to a nylon membrane and hybridized to a [^{32}P] oligolabeled mouse H1t probe (see Methods). Note that H1t mRNA accumulates to a significant steady-state level only in testis (lane 1).

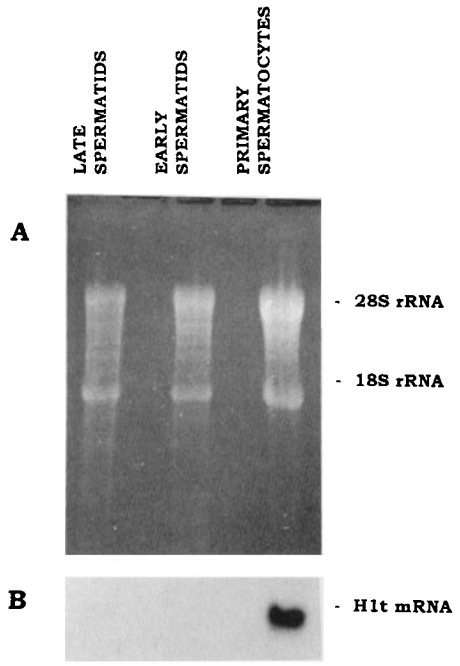

Fig. 4. Accumulation of H1t mRNA in primary spermatocytes. Enriched populations of mouse testis cells were prepared by centrifugal elutriation. RNA was isolated from cells enriched in late spermatids, in early spermatids, and in pachytene primary spermatocytes. Total cellular RNA (10 μg of each sample) from the three elutriator fractions was analyzed by northern blot analysis as in Fig. 3. Note in the autoradiogram in the lower panel (**B**) that H1t mRNA accumulates only in the testis cell population that is most enriched in primary spermatocytes.

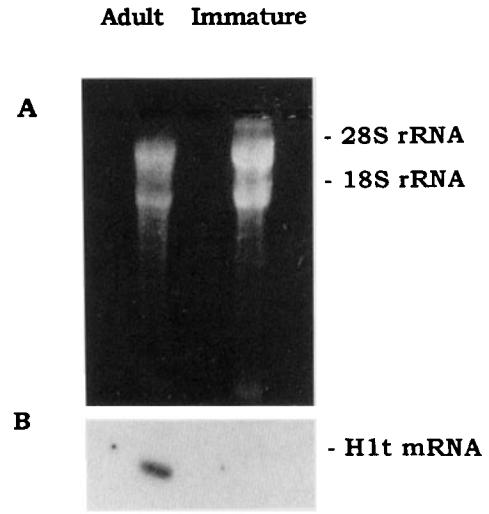

Fig. 5. Testes of sexually immature mice do not accumulate H1t mRNA. Lanes marked Adult and Immature represent total cellular RNA samples from testes of adult and sexually immature 12-day-old mice, respectively. The photograph in the top panel (**A**) shows the ethidium bromide–stained 18S and 28S rRNA bands. The autoradiogram in the lower panel (**B**) shows the hybridization signal when the gel blot is probed for H1t mRNA. Note that only the adult animal has the expected H1t mRNA signal.

cessors to primary spermatocytes, testes were isolated from sexually immature 12-day-old mice which are populated with spermatogonia but not with significant numbers of spermatocytes or spermatids. Northern blot analysis was conducted to compare steady-state levels of testis H1t mRNA from the 12-day-old mice and adult mice. Data presented in the autoradiogram shown in Figure 5B reveal that H1t mRNA does not accumulate in testes of 12-day-old mice. The upper panel shows that RNA samples from testes of both immature and adult mice were present in the agarose gel (Fig. 5A).

Testis Nuclear Proteins Bind to H1t/TE1 and H1t/TE2 Promoter Elements

Studies of the rat H1t gene revealed a sequence element designated H1t/TE1 located between the H1/AC box and the H1/CCAAT box. Proteins in rat testis nuclear extracts bind specifically to the H1t/TE1 element, and the binding proteins appear during spermatogenesis when H1t gene transcription is upregulated in pachytene primary spermatocytes. When mouse testis nuclear proteins were mixed with a labeled DNA probe corresponding to the mouse H1t/TE1 element (Fig. 6; Table I) in a protein-DNA binding reaction and analyzed by electrophoresis on a low ionic strength polyacrylamide gel, a major low mobility shifted band designated T is produced (Fig. 7, lane 2). Higher mobility bands are formed with variable intensities dependent upon the testis nuclear protein and probe preparations. Two faint bands with mobilities higher than band T are seen in the Figure. Although relative intensities of these higher mobility bands vary from preparation to preparation, mobilities of the additional bands are consistent. Nuclear proteins from other tissues do not produce the shifted band T. However, brain proteins consistently produce a faint shifted band with the same mobility as the second fainter band produced by testis (compare lanes 2 and 4 of Fig. 7). It should be noted that the same pattern of testis-specific binding has been observed in previous studies with the rat H1t TE1 element [Grimes et al., 1992a,b].

The second promoter element designated H1t/TE2 is located upstream from H1t/TE1 (Table I; Figs. 1B and 6). Since H1t/TE2 is an imperfect inverted copy of H1t/TE1 (Table I), it seemed possible that testis nuclear proteins might also bind to H1t/TE2. The data presented in Figure 8 reveal that testis nuclear proteins do

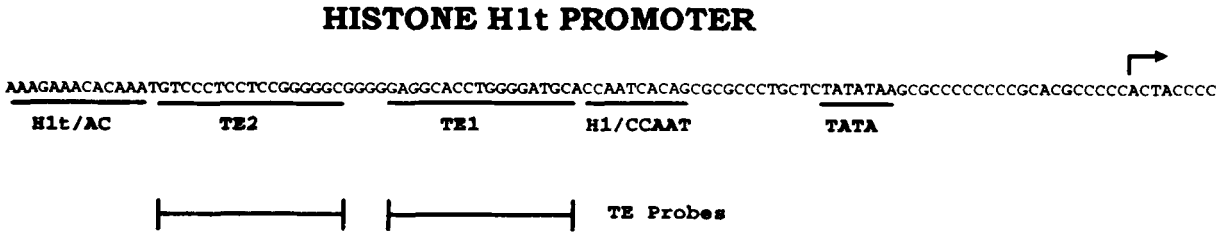

Fig. 6. The H1t promoter sequence showing probes used in electrophoretic mobility shift assays. The underlined histone H1t promoter elements designated TE1 and TE2 were used in mobility shift assays. The figure indicates that TE1 and the imperfect inverted copy designated TE2 overlap a GC-rich region that contains a consensus SP1 motif (Table I).

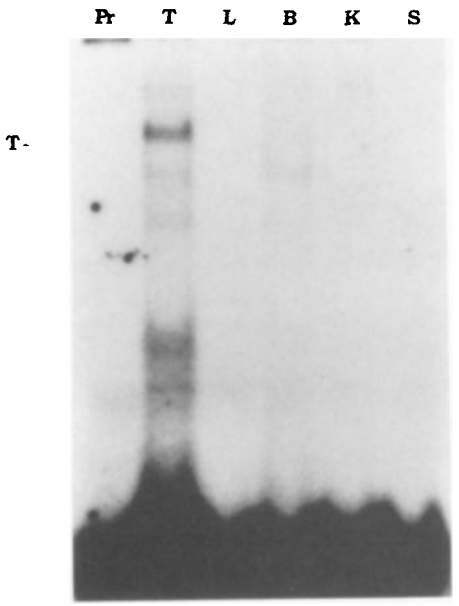

Fig. 7. Testis-specific binding of nuclear proteins to the TE1 element. Electrophoretic mobility shift assays were conducted using the H1t/TE1 probe shown in Fig. 6 and Table 1. Samples were loaded onto a low ionic strength polyacrylamide gel and electrophoresed. Samples in lanes 1–6 represent binding reactions with nuclear proteins as follows. *Lane 1:* Unbound probe (Pr). *Lane 2:* Testis (T). *Lane 3:* Liver (L). *Lane 4:* Brain (B). *Lane 5:* Kidney (K). *Lane 6:* Spleen (S). Note that only testis nuclear proteins bind to give the low mobility shifted band labeled T in the figure. Other higher mobility bands were mostly limited to the testis binding proteins.

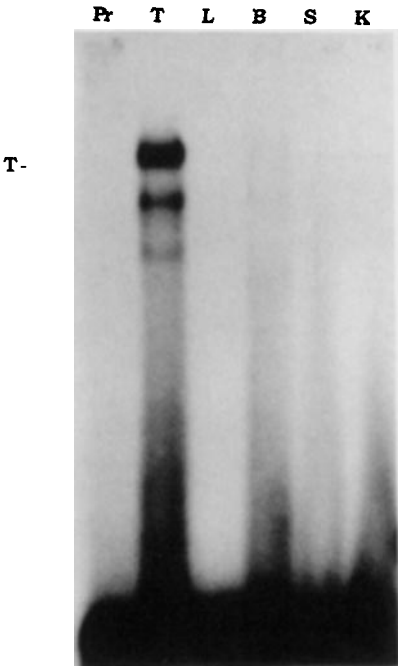

Fig. 8. Testis-specific binding of nuclear proteins to the TE2 element. Electrophoretic mobility shift assays were conducted using the H1t/TE2 probe shown in Fig. 6 and Table I. Samples in lanes 1–6 represent binding reactions with nuclear proteins as follows. *Lane 1:* Unbound probe (Pr). *Lane 2:* Testis (T). *Lane 3:* Liver (L). *Lane 4:* Brain (B). *Lane 5:* Spleen (S). *Lane 6:* Kidney (K). Again note that only testis nuclear proteins bind to TE2 to give the lowest mobility shifted band labeled T in the figure. The two shifted bands with mobilities slightly greater than band T are seen clearly in this figure, and similar but fainter bands are present in Fig. 7 where the TE1 probe was used.

bind to H1t/TE2, and the binding shows the same tissue specificity as binding to the H1t/TE1 element. The low mobility band is labeled T, but note that higher mobility bands are seen with this H1t/TE2 probe as with the H1t/TE1 probe. Two of these bands, as seen with the H1t/TE1 probe in Figure 7, have slightly higher mobilities than band T. In a parallel study of the rat histone H1t gene, TE2 shows the same testis-specific pattern of binding (Wolfe et al., in press).

A GC-rich sequence is located between TE1 and TE2. Since Sp1 potentially might bind to this "GC box" or interact with proteins that bind to the TE elements, we examined the testis nuclear proteins for proteins antigenically similar to Sp1. When a consensus Sp1 element was used in the electrophoretic mobility shift assay, shifted bands were produced with mouse testis nuclear proteins, as shown in lane 5 of Figure 9.

A supershifted band designated αSp1 is produced in lane 6 of Figure 9 when antibodies produced against Sp1 are introduced into the binding reaction after a 15 min binding reaction, indicating that Sp1 is present in our testis nuclear protein extracts. When the antibody was used in a binding reaction containing the TE1 element and testis proteins, no supershifted band was produced and no bands were eliminated, as shown in lane 3 of Figure 9. Therefore, Sp1 does not appear to represent a significant portion of the protein that binds to the TE element that leads to production of band T.

Previous studies of the rat TE1 element showed that testis nuclear proteins bind relatively tightly and specifically [Grimes et al., 1992a]. Binding could be competed with homologous DNA but not with heterologous DNA. Specific binding of mouse testis nuclear proteins to the mouse H1t/TE1 element has been confirmed by competition assays using homologous and heterologous DNA (data not shown). Since the sequence of the mouse H1t/TE2 element differs in five positions from the mouse H1t/TE1 element, it seemed important to test for specific binding of testis proteins to the mouse TE2 element by competition experiments. Data presented in Figure 10 show that the band T formed by binding mouse testis nuclear proteins to mouse H1t/TE2 is specifically competed by an unlabeled homologous TE2 DNA. Note that even though the mouse and rat TE2 elements differ in two positions (Table I), the rat H1t/TE2 also competes binding of proteins to the mouse H1t/TE2 element. More surprisingly, the rat H1t/TE1 element successfully competes the mouse H1t/TE2 element. The binding appears to be sequence-specific, as demonstrated by failure of the GC-rich commercial Sp1 probe to completely compete testis protein binding to the mouse H1t/TE2 element (Fig. 10, lanes 7, 8). The Sp1 oligonucleotide does not compete as well as the TE oligonucleotides, although some lower level of competition is observed. The weak competition using the Sp1 oligonucleotide to compete testis binding to H1t/TE2 is consistent with supershift data which fails to detect Sp1 binding to the H1t/TE2 element (Fig. 9). It should be mentioned that previous studies using mutant oligonucleotides containing substitutions within the recognition motif in TE1 showed the same sequence specificity; the mutant oligonucleotides did not completely compete binding to TE1 [Grimes et al., 1992a,b].

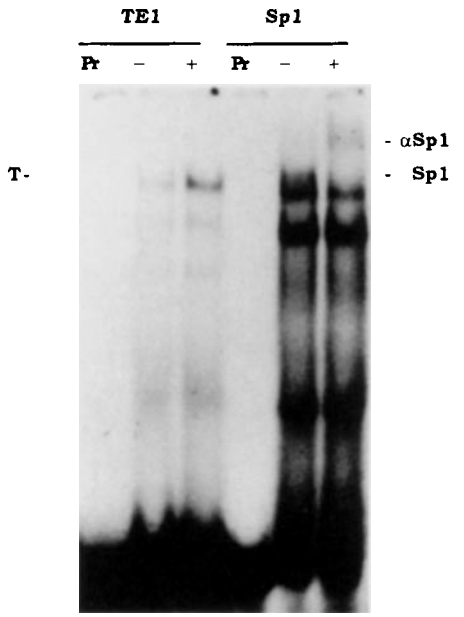

Fig. 9. Testis SP1 does not bind to the TE1 element. Binding reactions were conducted as in Figs. 7 and 8, but two different labeled probes were used. The first was the TE1 probe as in Fig. 7, and the second was the consensus SP1 sequence element listed in Table I. After binding, one sample using each probe (lane 3 [TE1]; lane 6 [Sp1]) was treated with anti-Sp1 antibodies, which causes a supershift if they bind to Sp1 that is present in the bound protein complex. Reactions examined in lanes 1–3 represent reactions with the TE1 probe as follows. *Lane 1:* Probe. *Lane 2:* Testis. *Lane 3:* testis plus anti-Sp1 antibody. Reactions examined in lanes 4–6 represent reaction with the SP1 probe as follows. *Lane 4:* Probe. *Lane 5:* Testis. *Lane 6:* Testis plus anti-Sp1 antibody. A low mobility band in lane 5 using the Sp1 probe approximately comigrates with the band labeled T in lanes 2 and 3. This band supershifts to a lower mobility in lane 6 when the anti-SP1 antibodies are included in the binding reaction, indicating the presence of testis SP1 in the nuclear extracts.

A summary diagram presenting data from sequence analysis, S1 nuclease protection analysis revealing the mRNA start site, and electrophoretic mobility shift assays revealing testis-specific binding to both H1t/TE1 and H1t/TE2 within the larger TE element is shown in Figure 11. The figure incorporates information from an analysis of the homologous rat H1t promoter [Wolfe et al., 1995; Wolfe and Grimes, 1993]. Note that the GC-rich sequence, located between the H1/AC and H1/CCAAT elements (see Fig. 1B), is totally contained within the larger H1t/TE element. Since anti-Sp1 antibodies failed to produce a supershift of the T band produced by the TE1 element, testis Sp1 does not appear to bind to the TE1 element. Note that we have drawn one large TE element spanning both TE1

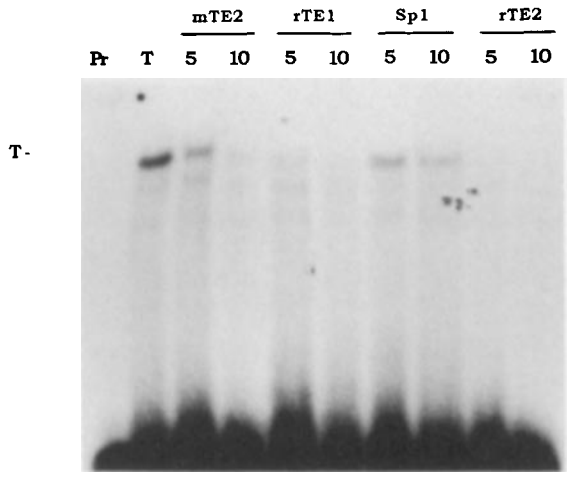

Fig. 10. Specificity of binding of testis nuclear proteins to the TE2 element. Electrophoretic mobility shift competition assays were conducted using the labeled H1t/TE2 probe with various unlabeled homologous and heterologous DNA competitors. Samples in *lanes 1* and *2* represent free probe Pr and a binding reaction with nuclear proteins from testis T, respectively. The competitions were conducted by adding 5 or 10 μg of unlabeled mouse TE2 (mTE2), rat TE1 (rTE1), Sp1, or rat TE2 (rTE2) to the nuclear proteins before the labeled mouse TE2 probe was added. See Table I for the sequences of these competitors.

and TE2, since testis nuclear proteins bind these regions independently.

DISCUSSION

Testis histone H1t is one of seven known mammalian histone H1 subtypes. H1t has been found in many mammalian species, and, although the amino acid sequence of this histone is highly conserved, variable electrophoretic mobilities of H1t from these species reveal sequence differences [Meistrich, 1989]. Expression of each of the histone H1 variants designated H1.1, H1.2, H1.3, H1.4, and H1.5 is replication-dependent [Drabent et al., 1991; Carozzi et al., 1984; Eick et al., 1984; Doenecke and Toenjes, 1986; Ohe et al., 1989; Albig et al., 1991]. The histone variant designated $H1^0$ is expressed constitutively at a low basal level, and its expression is apparently not closely linked to the mitotic cell cycle. The sequence of $H1^0$ is similar to that of histone H5, a replacement histone H1 variant expressed only in nucleated erythrocytes such as avian erythrocytes [Deonecke and Toenjes, 1986].

On the other hand, histone H1t is expressed only in testis pachytene primary spermatocytes and early spermatids [Grimes, 1986; Smith et al., 1992; Meistrich, 1989), accumulating to an estimated level of 60% of the H1 complement in these cells [Meistrich, 1989; Lennox, 1984]. Histone H1a (H1.1 by the nomenclature of Albig [Albig et al., 1991] or H1E by the nomenclature of Ohe [Ohe et al., 1989]) also accumulates in primary spermatocytes to a level of approximately 20% [Bucci et al., 1982; Lennox, 1984; Seyedin and Kistler, 1979]. While H1t is present in both spermatocytes and early spermatids, it is synthesized only in spermatocytes. Previous studies show that H1t mRNA accumulates in primary spermatocytes in rats and humans [Drabent et al., 1991; Grimes et al., 1990]. The present study confirms this pattern of H1t mRNA accumulation during spermatogenesis in mice. These observations indicate that this single copy gene [Cole et al., 1986; Grimes et al., 1990] is transcribed at a significant level only in primary spermatocytes in these organisms.

The high level of accumulation of H1t in meiotic primary spermatocytes and its carryover into postmeiotic haploid early spermatids point to an important role for this linker histone variant in aspects of meiosis such as altered testis chromatin structure or gene expression. The amino acid sequence of histone H1t is conserved among species examined (Fig. 2); rat and mouse H1t are 93% identical, and rhesus monkey and human H1t are 88% identical. The central globular domains have the greatest degree of amino acid sequence identity (99% identical for rat and mouse and 98% identical for monkey and human), but this central region has a lower degree of identity when comparing H1t to each of the other six H1 variants [Koppel et al., 1994]. The N-terminal and C-terminal regions are much more variable.

Variability in the amino acid sequences of H1 variants, especially in H1t and H5, imply important functional differences in the variants, although this has not been proven. For example, thermal denaturation studies reveal that chromatin derived from enriched populations of testis primary spermatocytes and early spermatids is destabilized compared to chromatin from liver which lacks H1t [Grimes, 1986]. Destabilized histone H1t containing chromatin with a less compact DNA-protein complex has been confirmed by circular dichroism studies [De Lucia et al., 1994]. Furthermore, regions of chromatin bound to H1t are more sensitive to DNase I digestion than regions bound to other H1 variants [De Lucia et al., 1994].

Probable functional differences in H1t compared to other H1 variants and the need to

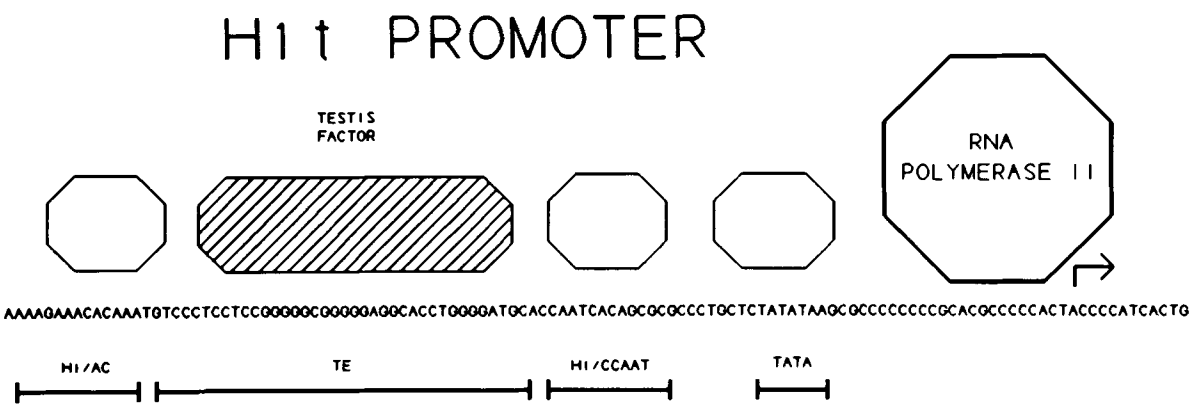

Fig. 11. Model of the testis-specific histone H1t promoter. This model shows proposed DNA-protein binding sites within the H1t promoter based upon published data from other H1 genes and the highly conserved H1 consensus sequence elements, the H1/AC box, H1/CCAAT box, and TATA box. The model emphasizes the testis-specific H1t/TE1 element and the imperfect inverted repeat H1t/TE2 element found in mammalian H1t promoters. The putative binding proteins are drawn showing hypothetical interactions with DNA, with each other, and with RNA polymerase II to form a transcription preinitiation complex. Protein that interacts with TE1 and TE2 individually or that interact and bind as a complex to the larger TE element appear to block binding of testis Sp1 to its cognate GC-rich element.

enhance transcription of this gene in primary spermatocytes is reflected in differences in the H1t promoter compared to promoters of other H1 variants. H1t and H1a promoters are shorter in length between the H1/AC box and the TATA box than the other H1 variant promoters [Koppel et al., 1994; Doenecke et al., 1994]. It is interesting that the H1t and H1a are both expressed in testis and have similar GC-rich sequences. The GC-rich sequence may be important for their expression in spermatocytes. However, only the testis-specific H1t variant has the H1t/TE sequence element [Grimes et al., 1992a,b; Koppel et al., 1994; Wolfe and Grimes, 1993]. Furthermore H1a, which lacks the TE element, is expressed in other tissues [Lennox, 1984; Seyedin and Kistler, 1979].

Mouse histone H1t mRNA accumulates in testis but not in other tissues (Fig. 3). Furthermore, accumulation is limited to primary spermatocytes within the testis (Fig. 4). This strict tissue-specific and cell-specific pattern of expression of H1t is unique for this histone variant and is not observed for mitotic cell-cycle regulated H1 variants. The relative proportions of the replication-dependent H1 variants and $H1^0$ vary in tissues or in cells at different stages of development, but the functional significance of differing proportions of H1 variants is unclear at this time.

Estimation of steady-state levels of H1t mRNA was accomplished by northern blot analysis. This method yields ambiguous results if a DNA probe is used that can cross-hybridize to other H1 variant mRNAs. The degree of cross-hybridization can be minimized but not eliminated. A more definitive method for measuring steady-state levels of specific mRNAs is S1 nuclease protection analysis. Therefore, S1 nuclease protection analyses was used to confirm the results of the northern blot analysis. The results of the analysis shows that mRNA from the H1t gene cloned in this laboratory accumulates only in the testis (data not shown).

Since testis-specific expression of the H1t gene appears to be regulated in large part at the transcriptional level, we initiated an examination of the H1t promoter. A sequence element, designated TE1, is located between the H1t/AC box and the H1t/CCAAT box and is conserved in mammalian H1t promoters. The element binds specifically to testis nuclear proteins but not to nuclear proteins from other tissues (Figs. 6–8). Competitions with homologous and heterologous DNA sequences indicate a relatively high affinity testis-specific binding.

On the other hand, the H1/AC and H1/CCAAT elements most likely bind *to trans*-acting factors found in nuclear proteins from most tissues that express histone H1 genes. The H1/AC box, present in all H1 promoters, is essential for regulation of transcription during S-phase of the mitotic cell cycle. Its function in the H1t gene is not clear at the present time, but it is presumed to be equally important for transcription during the meiotic cell cycle. H1/

CCAAT binding proteins have been identified in replication-dependent histone genes. The CCAAT sequence binds HiNF-B that may be involved in regulating coordinate transcription of H1 and core histone genes [Carozzi et al., 1984]. This sequence element is found in H1t as well as in all other H1 variants. Proteins in nuclear extracts from several different tissue types including testis bind to a probe covering the H1t/CCAAT sequence [Grimes et al., 1992a]. HiNF-D, a multisubunit complex containing CDC2, cyclin A, and RB-related proteins, is a transcription factor shared by histone H1, H3, and H4 genes that may bind to this promoter region [vanWijnen et al., 1994].

The region between the H1/AC box and the H1t/TE1 element is very GC-rich. In some H1 genes this region may bind to the transcription factor Sp1 or may bind to proteins that are members of the Sp1 family. We have examined this region in the H1t gene for binding testis Sp1. Mouse testis and rat testis nuclear protein extracts certainly contain Sp1, as shown when a commercial Sp1 consensus oligonucleotide probe and anti-Sp1 antibodies are used in mobility shift assays (Fig. 9). The complex is supershifted in this assay. Both the H1t/TE2 element (Fig. 9) and the Sp1 probe bind testis proteins, but only the Sp1 complex supershifts when antibodies are used. When a region containing the larger H1t/TE element that spans H1t/TE1 and H1t/TE2 is used as a probe in the assay, there is no supershift with anti-Sp1 antibodies [Wolfe et al., 1995]. Although the Santa Cruz anti-Sp1 antibodies do not shift the proteins bound to the TE1 and TE2 probes, this does not rule out the possibility that other Sp1-related proteins such as Sp3 may bind [Sogawa et al., 1993; Hagen et al., 1994].

A second imperfect inverted copy of the TE1 element designated TE2 is located between the H1/AC box and the GC-rich element (Table I; Figs. 1B, 6, 11). This region also binds testis nuclear proteins (Figs. 8, 9), and the same region in the rat also binds testis nuclear proteins [Wolfe et al., 1995]. One interpretation of these data is that testis-specific proteins bind to both of the two TE elements that overlap the GC-rich sequence region. The testis-specific binding to the TE elements may prevent binding of testis Sp1. It is interesting that the promoter region that extends from the TE2 element through the GC-rich sequence, through the TE1 element, and through the CCAAT element contains sequence elements found in several other testis-specific genes [Queralt and Oliva, 1993; Zhou et al., 1994]. Clearly, this important region of the H1t promoter is unique compared to the promoters of replication-dependent H1 genes, and it is likely that the region is involved at least in part in regulating testis-specific expression of the H1t gene.

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We acknowledge Heather R. Panek and Peggy Carter for their helpful discussions and for the preliminary experiments they conducted related to binding of testis nuclear proteins to the histone promoter. This research was supported by the Veterans Administration (SRG) and by NIH grant R01 HD29381-01A1 (SRG).

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