Expression of the Testis-Specific Histone H1t Gene: Evidence for Involvement of Multiple Cis-Acting Promoter Elements†

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ABSTRACT: The histone H1t gene is expressed exclusively in testis primary spermatocytes. Previous studies indicate that accumulation of H1t mRNA occurs only in primary spermatocytes in normal rats and in transgenic mice bearing the rat H1t transgene. In this study, DNA sequences of human, monkey, mouse, and rat H1t genes were compared and found to be almost identical in the proximal promoter region extending from the H1/AC box through the TATAA box. In addition to conserved elements common to replication-dependent H1 promoters, the H1t promoter contains a unique TE element, and sequences within this element may contribute to enhanced expression of the gene in primary spermatocytes. Two imperfect inverted repeat sequences designated TE1 and TE2, that are located within the larger TE element, overlap a central GC-rich region and bind specifically to nuclear proteins derived from primary spermatocytes. Protein interactions characterized by methylation interference and UV cross-linking experiments indicate that a complex of proteins with a molecular mass of approximately 180 kDa binds TE1. The GC-rich region in H1t and in some replication dependent histone H1 promoters contains an Sp1 consensus sequence. Although the H1t/TE element that contains the GC-rich region binds nuclear proteins, it does not appear to bind Sp1 obtained from cell populations enriched in primary spermatocytes as determined by electrophoretic mobility supershift assays using polyclonal anti-Sp1 antibodies.

Seven mammalian histone H1 variants, designated H1.1, H1.2, H1.3, H1.4, H1.5, H1t, and H1°, have been identified (Albig et al., 1991; Kistler, 1989; Lennox, 1984; Carozzi et al., 1984; Doenecke & Toenjes, 1986; Drabent et al., 1991; Eick et al., 1984; Meistrich, 1989; Ohe et al., 1989). In testis, H1.1 and H1t are the most abundant H1 variants (Meistrich, 1989). H1t is first detected during spermatogenesis in primary spermatocytes, cells in meiotic prophase of the cell cycle, where it replaces other H1 subtypes and comprises the major portion of the H1 complement. H1t binds DNA with lower affinity (De Lucia et al., 1994; Khadake et al., 1994); and it may have a role in recombination (De Lucia et al., 1994). It persists until the midspermatid stage of spermiogenesis, when it is replaced by transition proteins TP1 and TP2 (Bucci et al., 1982; Grimes et al., 1977; Grimes, 1986) when nuclear condensation occurs (Meistrich et al., 1985).

Tissue-specific expression of the histone H1t gene appears to be regulated primarily at the transcriptional level (Grimes et al., 1990, 1992a; Wolfe & Grimes, 1993). Cis-acting elements sufficient for regulating testis-specific transcription are found within 2.45 kb upstream and 3.78 kb downstream of the H1t gene (Kremer & Kistler, 1992; vanWert et al., 1995a,b). The H1t promoter contains all of the conserved sequence elements typical of replication-dependent H1 promoters. These include the H1/AC box, a GC-rich region containing an Sp1 consensus element, an H1/CCAAT box, and a TATA box (Dalton & Wells, 1988; van Wijnen et al., 1988; Osley, 1991). The H1/AC box and H1/CCAAT box are involved with elevated transcription of the replication-dependent H1 genes during the S-phase of the cell cycle (Dalton & Wells, 1988; Gallinari et al., 1989; La Bella et al., 1990). Previous work revealed that appearance of testis-specific binding proteins which recognize a sequence element within the proximal promoter correlates with onset of transcription of the H1t gene in primary spermatocytes (Grimes et al., 1992a,b).

Histone genes are typically divided into two types, replication-dependent variants and replacement variants. The distinction between the two is based upon cell cycle expression, structural characteristics of the gene, and steady-state level of its mRNA during the mitotic cell cycle. The S-phase rise in histone synthesis is caused by increased histone gene transcription and mRNA stability in early S-phase, leading to increased translation. At the end of S-phase selective decreased transcription and destabilization of histone mRNA result in decreased histone synthesis. Replication-dependent histone genes lack introns and yield mRNAs containing a conserved 3′ terminal stem-loop sequence rather than polyadenylation. Additionally, replacement variant genes may contain introns and have polyadenylated mRNAs. Conversely, replacement variants do not exhibit increased S-phase synthesis. Interestingly, the H1t gene encoding a tissue-specific replacement variant has neither introns nor polyadenylated mRNA and can be expressed like a replication-dependent histone gene when transfected into somatic cell lines (Grimes et al., 1990; Kremer & Kistler, 1992).

In order to gain insight into mechanisms of tissue-specific transcription of the H1t gene, the proximal promoters of four

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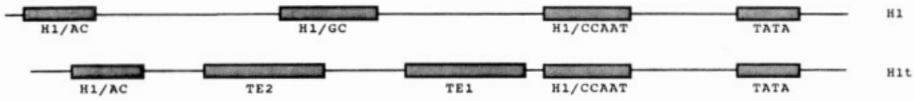

FIGURE 1: Promoters of replication-dependent histone H1 and testis histone H1t genes. This figure shows alignment of the replication-dependent H1 promoter with the testis-specific histone H1t promoter. Conserved elements are boxed. Note the conservation of the positions of the H1/CCAAT box and TATA box region in both types of H1 promoters.

Table 1: Oligonucleotides Used in This Study and Features of the H1t Gene[a]

OLIGONUCLEOTIDES USED FOR PCR AND EMSA		
Name	**Position**	**Sequence**
H1/AC Box	-112 - -93	GGGGGAAAAGAAACACAAAT
H1t/TE Element	-92 - -54	GCCCCTTCCCCAGGGGCGGGGGAGGCGCCTAGGGATGCA
H1t/TE2 Element	-92 - -76	GCCCCTTCCCCAGGGGC
H1t/TE1 Element	-71 - -55	GAGGCGCCTAGGGATGC
H1t/TE1 Element (human)		GCGGTGCCTAGGTGATGCA
Sp1 Consensus[b]		ATTCGATC**GGGGCGGGGG**GAGC
CONSERVED PROMOTER SEQUENCES AND OTHER FEATURES OF THE RAT H1t GENE		
H1/AC Box	-102 - -95	AAACACAA
H1t/TE Element	-92 - -54	GCCCCTTCCCCAGGGGCGGGGGAGGCGCCTAGGGATGCA
H1/GC Sequence	-81 - -71	AGGGGCGGGGG
H1/CCAAT Box	-53 - -40	CCAATCACAGCGCG
TATA Box	-31 - -25	TATATAA
mRNA start	1	ACTCCA
ATG start codon	70 - 72	ATG
Coding region	70 - 693	207 AMINO ACIDS
3' Stem-loop	726 - 741	GGCTCTTTTAAGAGCC
Imperfect inverted repeat		CGCCTAGGGATG (TE1)
		CCCCTGGGGAAG (TE2 inverted)

[a] Numbers refer to rat H1t sequence presented in vanWert et al. (1995a,b). Oligonucleotides are listed in the 5′ to 3′ direction. [b] 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.

cloned mammalian histone H1t genes were compared. In contrast to the variability of nucleotide sequences of replication-dependent histone gene promoters, highly conserved sequences between the H1/AC box and H1/CCAAT box are found among H1t promoters. This conserved region contains an element designated TE1 that binds to testis nuclear proteins derived from primary spermatocytes, cells at a stage of spermatogenesis where maximal transcription of the H1t gene is detected (Grimes et al., 1992a,b). The present study extends the examination of the TE1 element using mobility shift assays, methylation interference assays, and UV cross-linking experiments. Sequence alignments and electrophoretic mobility shift experiments revealed a second imperfect inverted copy of this element, designated TE2, located upstream from TE1. These two elements overlap a GC-rich region containing an Sp1 consensus sequence. However, the GC-rich region, centrally located between the H1/AC box and the H1/CCAAT box, failed to bind testis SP1, as determined by supershift assays.

MATERIALS AND METHODS

DNA Oligonucleotides, Fragments, and Plasmids. Synthetic oligonucleotides were purchased commercially (Oligos etc., Wilsonville, OR). Three oligonucleotide primers were designed for amplification of the histone H1t gene from various mammalian genomic DNA samples using highly conserved sequences within or flanking the histone H1t gene (Figure 1, Table 1). One 5′ amplimer encompassed the "AC box" (5′-GGGGGAAAAGAAACACAAAT-3′, −112 to −93 of the rat promoter) while the 3′ amplimer encompassed the "stem loop" (5′-AAGTGGCTCTTAAAAGAGCC-3′, from rat sequence 30−49 nt downstream from the stop codon) (Table 1). Parameters for PCR thermal-cycling have been described (Koppel et al., 1994; vanWert et al., 1995a,b). Amplified products containing the complete histone H1t gene from human, monkey, and mouse genes were cloned into pUC 19, yielding plasmids pDK 4, pDK 3 (Koppel et al., 1994), and pMH1T (vanWert et al., 1995a,b), respectively. Complete nucleotide sequences of these clones were determined using the fmole™ sequencing procedure (Pharmacia). Plasmids pSW16 and pSW17 containing the human 19 bp TE1 element (Table 1) cloned into the *Sma*I site of pUC19 in opposite orientations were used to provide DNA fragments for methylation interference assays.

Six double-stranded oligonucleotides were used as probes and specific competitors in mobility shift assays. The double-stranded oligonucleotides seen in Table 1 include the following: −92 to −54 (H1t/TE element), −92 to −76 (TE2 subelement), −81 to −71 (GC-rich element), and −71 to −55 (TE1 subelement). The numbering is based upon the number of nucleotides upstream from the rat H1t gene transcriptional start site (vanWert et al., 1995a,b). A fifth probe corresponds to the human TE1 element (5′-GCGGT-GCCTAGGTGATGCA-3′). The sixth probe, containing an Sp1 consensus sequence (5′-ATTCGATCGGGGCGGGGC-GAG-3′), was purchased (Santa Cruz Biotechnology, Inc.). Probes were isotopically labeled using ^{32}P by filling with the Klenow fragment of DNA polymerase or 5′-end labeling with T4 polynucleotide kinase (Maniatis et al., 1982).

Assays for Protein−DNA Interaction. Nuclear and cytoplasmic S100 extracts were prepared from crude nuclei by the method of Dignam et al. (1983). Electrophoretic mobility shift assays were performed essentially as described previously (Grimes et al., 1990; Wolfe & Grimes, 1993). Binding

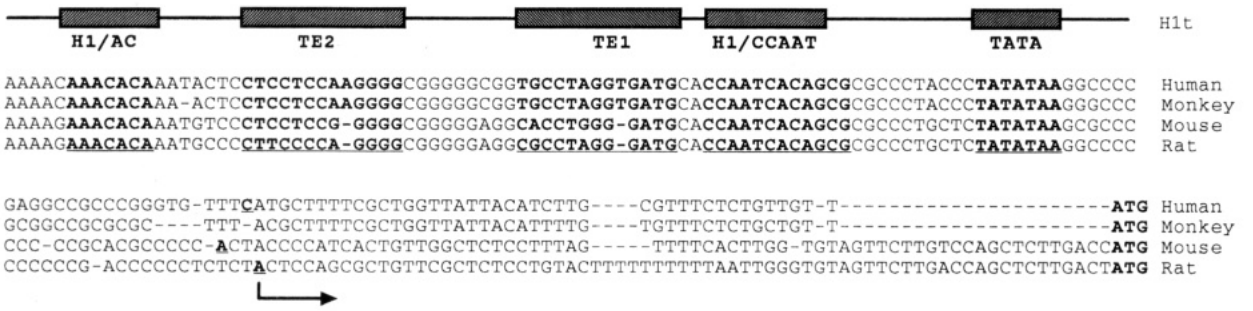

FIGURE 2: Conserved promoter regions of mammalian H1t genes. This figure shows alignment of the promoters of mammalian histone H1t genes amplified, cloned, and sequenced in our laboratory. The H1t/TE region is conserved in all of the promoters. The degree of conservation of the H1t sequences drops dramatically to 50% in the 50 bp region upstream and adjacent to the H1/AC box. The experimentally determined transcription start site of the rat gene is indicated by an arrow, and those of the rat, mouse, and human are indicated by a bold character.

assays contained 0.2−0.5 ng of a [^{32}P]-labeled probe and 500 ng of nonspecific competitor DNA poly(dG-dC)•(dG-dC). Assays were performed in a final volume of 20 μL containing 12 mM Hepes/NaOH, pH 7.9, 12% glycerol, 60 mM KCl, 4 mM Tris-HCl, 0.6 mM EDTA,[1] and 0.6 mM DTT. Binding reactions were incubated 30 min at 4 °C and loaded onto a low ionic strength 4% polyacrylamide gel [80:1 acrylamide/bis(acrylamide)]. Samples were electrophoresed at 100 V for 3−3.5 h. Supershift assays were performed by adding Sp1-specific polyclonal antibody to samples following probe binding. Samples were incubated for an additional hour at 4 °C prior to loading on a low ionic strength polyacrylamide gel (see above).

UV Cross-Linking. DNA binding assays were upscaled to a total volume of 100 μL (5-fold increase of standard binding reaction). After binding, samples were pipetted onto parafilm and subjected to two pulses of UV light (120 mJ/cm^2 per pulse) in a Stratalinker 1800 UV cross-linker (Stratagene). Following UV irradiation, 10 μL of SDS sample loading solution (0.625 M Tris-HCl, pH 6.8, 20% (w/v) SDS, and 50% (w/v) 2-mercaptoethanol) was added to each sample prior to boiling for 3 min. Samples were loaded adjacent to prestained SDS−PAGE protein standards (Bio-Rad) on a 7.5% SDS−polyacrylamide gel (2.67% cross-linked gel) having a 5% stacking gel. Samples were electrophoresed at 100 V until bromophenol blue dye reached the bottom of the gel. The gels were dried and exposed to film (Kodak XAR-5) using an intensifying screen (Dupont Cronex Lightning Plus) to detect protein−DNA complexes.

Methylation Interference. Methylation interference experiments were performed essentially as described (Baldwin, 1989) using the strand-specific labeled 19 bp human TE1 element excised from the cloned source (labeled by filling *Eco*R1 digested pSW16 and pSW17).

Blot Analysis of Electrophoretically Separated Proteins. Proteins in nuclear extracts prepared as described above were quantitated, and 150 μg samples were mixed with SDS loading buffer 1:1 (v/v), boiled, loaded on a 7% polyacrylamide gel (0.1% SDS), electrophoresed, and electroblotted to a nitrocellulose membrane. The membrane was blocked by incubation in BLOTTO containing salmon sperm DNA (Johnson et al., 1984; Singh et al., 1988) and probed with a labeled six-copy tandem repeat of the human TE1 element.

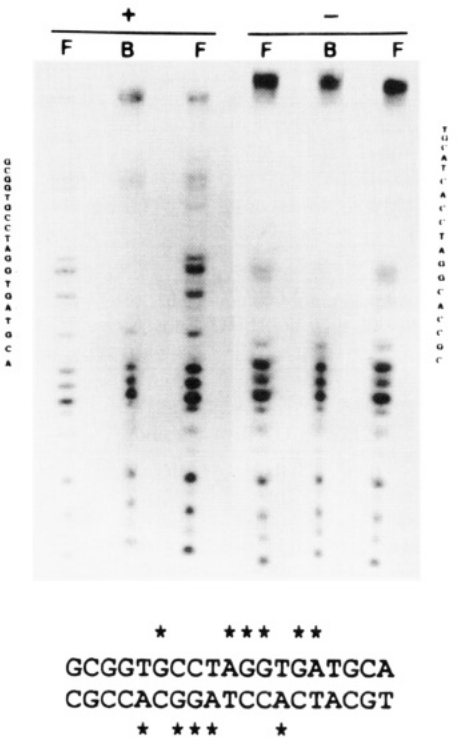

FIGURE 3: Methylation interference assay of testis nuclear proteins bound to the H1t/TE1 element of the H1t promoter (+ and − strands). Methylation interference assays were conducted with the + and− strands of the H1t/TE1 promoter element. F is free probe, and B is bound probe. The G and A residues protected by bound protein are indicated by an asterisk in the double-stranded sequence shown at the bottom of the figure.

RESULTS

Conservation of the Mammalian Histone H1t Gene Promoter. The testis-specific histone H1t promoter is similar in several respects to replication-dependent histone H1 proximal promoters. Figure 1 is an alignment depicting promoters of the H1t gene and replication-dependent H1 genes showing elements important for replication-dependent regulation including the H1/AC box, the GC-rich sequence, the H1/CCAAT box, and the TATA box. All four of these elements are also present within the H1t promoter. The H1/CCAAT box is located approximately the same distance from the TATA box in replication-dependent H1 and testis-specific H1t promoters, whereas the H1/AC box in H1t is closer to the TATA box by approximately 10 bp (except the human H1.1 H1/AC box, which is 3 bp closer to the TATA box than the H1t element). The GC-rich region is not drawn in

[1] Abbreviations: EDTA, ethylenediaminetetraacetic 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).

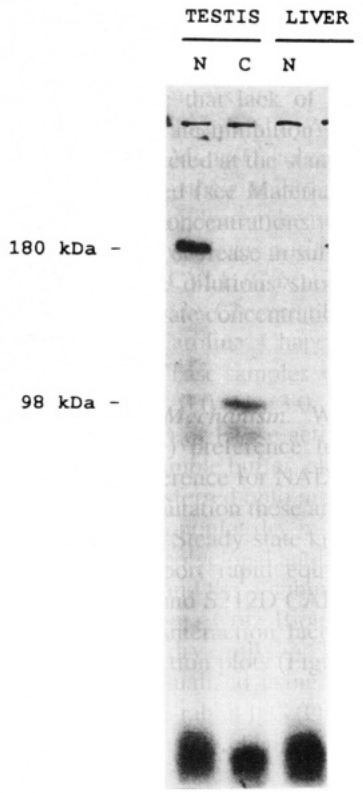

FIGURE 4: UV Cross-linking analysis of protein binding to H1t/TE1. Binding of proteins to the [^{32}P]-labeled H1t/TE1 promoter element was conducted. Bound proteins were cross-linked by UV irradiation using a Stratalinker, and the cross-linked products were analyzed on a 7.5% polyacrylamide−SDS gels. Lanes marked testis N and C show cross-linking with rat testis nuclear and cytoplasmic proteins. Liver proteins (liver N) show very little nonspecific cross-linking. One major cross-linked band was formed with testis nuclear proteins. The apparent molecular mass of the complex forming this band is 180 kDa. The major bound cytoplasmic complex is smaller with an apparent molecular mass of 98 kDa.

the H1t promoter because it is overlapped on either side by TE1 and TE2.

Four mammalian H1t promoters were examined to identify potential elements unique to this variant that may contribute to its testis-specific expression pattern. Figure 2 shows alignment of H1t proximal promoters from human, monkey, mouse, and rat extending from five nucleotides 5′ upstream of the H1/AC box (positioned at −107 bp in relation to the transcriptional initiation site) to the ATG start codon. In the region from the AC box at −107 to −24 located one bp 3′ of the TATA element, the H1t promoter is highly conserved, having 83% nucleotide identity between the rat and human. Between position −24 and the ATG start codon the nucleotide identity dramatically decreases. A significant decrease in nucleotide identity results from deletions yielding variations in lengths of the mRNA untranslated leaders. Alignment of human, mouse, and rat nucleotide sequences from −218 to −107 revealed a reduction in nucleotide identity to 53% (a 30% reduction when compared to the −107 to −24 region) (data not shown).

Previous work indicated that H1t/TE1 binds to proteins in testis nuclear extracts but not to proteins in nuclear extracts from other tissues (Grimes et al., 1992a,b). Using the alignment shown in Figure 2, a second copy of the TE1 element was identified. This element, designated H1t/TE2, is an imperfect inverted copy of TE1 centered approximately 19 bp upstream from TE1. The two TE elements flank and

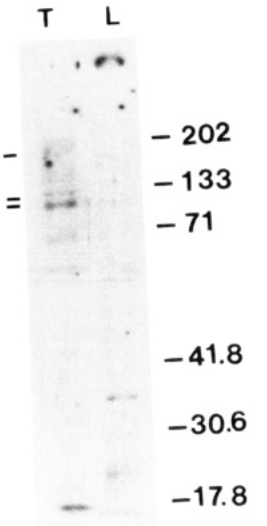

FIGURE 5: Analysis of proteins that bind to the H1t/TE1 sequence element. Binding of a 6-copy TE1 element (Table 1) to nuclear proteins electrophoresed on SDS−polyacrylamide gels and blotted to nitrocellulose. Nuclear extracts used in mobility shift assays were mixed with SDS gel loading buffer, boiled, and electrophoresed on a 7% polyacrylamide gel containing SDS. Electrophoretically separated proteins were electroblotted to nitrocellulose membrane and probed with a [^{32} P]-labeled double-stranded 6-copy TE element. Lanes 1 and 2 show binding to testis (T) and liver (L) nuclear proteins. Two major bands migrating at 100 and 130 kDa, bound by this probe, are marked in the figure. A fainter band migrating at 180 kDa is also marked in the figure.

partially overlap the GC-rich sequence that contains a consensus Sp1 binding site.

Testis Nuclear Proteins Bind to H1t/TE1. To study binding of nuclear proteins to the H1t proximal promoter, four double stranded oligodeoxynucleotide fragments representing specific regions of the promoter were prepared (Table 1). One probe represents the 17 bp H1t/TE2 element, one encompasses the GC-rich element, one, an 18 bp H1t/TE1 element, binds exclusively to proteins in testis nuclear extracts, and one, a 39 bp double-stranded oligonucleotide, spans the complete region being examined and contains H1t/TE1, H1t/TE2, and the GC-rich sequence.

Previous experiments demonstrated that only proteins in nuclear extracts from testis of sexually mature rats would bind to H1t/TE1 (Table 1). Binding activity to this probe can be specifically eliminated by competition with 5 ng (10-fold excess) of homologous oligonucleotide (Grimes et al., 1992a). A logical extension of this work was to identify specific nucleotides within the element necessary for binding. Methylation interference assays were conducted using a probe containing the H1t/TE1 element that had been methylated on an average of one nucleotide per molecule. Analysis of both strands indicates that 11 methylated guanine and adenine residues interfere with binding, six residues on the plus (+, coding) strand and five residues on the minus (−, noncoding) strand. Interfering sites are centrally located and symmetrically distributed about the axis of dyad symmetry of the 6 bp palindrome, CCTAGG (Figure 3).

UV cross-linking experiments were undertaken to determine the relative molecular mass of nuclear protein(s) binding to the H1t/TE1 element. Testis nuclear and cytoplasmic extracts and liver nuclear extracts were compared to identify any common or nonspecific binding. A major cross-linked product from testis nuclear protein has an

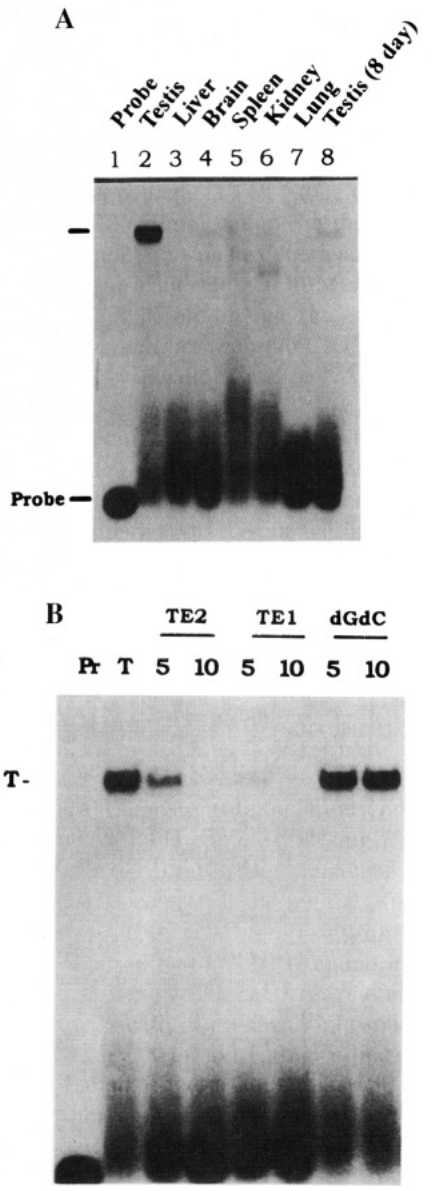

FIGURE 6: Binding of testis nuclear proteins to the H1t/TE2 element. (A) Testis-specific binding. A probe of the H1t/TE2 region of the H1t promoter −92 to −76 (Table 1) was used in these mobility shift assays. Probe alone is in lane 1. Mobility shift assays conducted using nuclear extracts from testis, liver, brain, spleen, kidney, lung, and testes of 8 day old rats demonstrate testis-specific binding to this element. Note that only a faint band is formed with testis extracts from testis from 8 day old animals. (B) Competitions with homologous and heterologous DNA. TE2 and TE1 at levels of 5 and 10 ng (10- and 20-fold molar excess) successfully compete binding to labeled TE2, but 5 and 10 ng additional nonspecific competitor dGdC does not compete. Testis nuclear extracts were used for these assays.

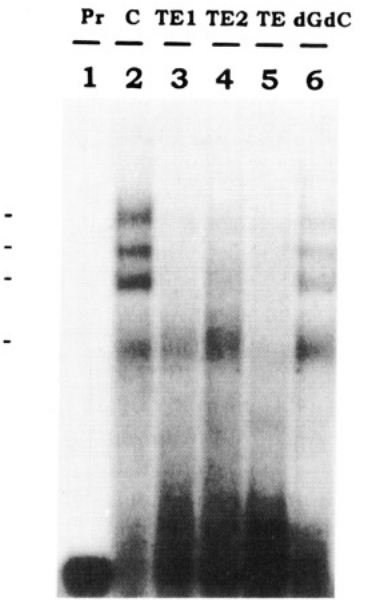

FIGURE 7: Binding of protein to the H1t/TE probe spanning H1t/TE1 and H1t/TE2 subelements. The 39 bp H1t/TE DNA covering the promoter from the TE2 element through the TE1 element (Table 1) was used as a probe in these mobility shift assays. Probe alone is shown in lane 1. Note that four major bands are formed with testis proteins with the H1t/TE probe (lane 2 designated C for control) compared to the one major band formed with H1t/TE2 (Figure 6). Competitions were conducted with competitor DNA as follows: H1t/TE1 element, 200-fold excess (lane 3); H1t/TE2 element, 200-fold excess (lane 4); the full-length H1t/TE element, 100-fold excess (lane 5); and 500 ng of nonspecific competitor poly(dG-dC)•poly(dG-dC) (lane 6). Note that the three specific competitors competed the three major bands but not the higher mobility fourth band.

apparent molecular mass of 180 kDa (Figure 4). Cross-linking of testis cytoplasmic extracts yields a cross-linked product with an apparent molecular mass of 98 kDa (Figure 4). Liver nuclear extracts did not yield significant cross-linked products (Figure 4).

Further characterization of nuclear proteins was conducted by transferring electrophoretically separated proteins to nitrocellulose and probing with a radiolabeled tandem repeat of the H1t/TE1 element. As seen in Figure 5, three major bands and several minor bands appear unique to testis compared to liver. The major bands have relative molecular masses of 180, 130, and 100 kDa, the largest having a mass similar to the mass of the testis proteins that cross-linked to TE1 (Figure 4).

Testis Nuclear Proteins Bind to H1t/TE2. Analysis of the nucleotide sequence located between the H1/CCAAT motif and the H1/AC box revealed a second copy of the H1t/TE1 element from nucleotide −92 to −76 (Figure 2, Table 1). This element, designated H1t/TE2, is an imperfect inverted repeat of H1t/TE1 (Table 1) and is centered 19 nucleotides upstream from H1t/TE1. Mobility shifts using testis nuclear extracts and the −92 to −76 H1t/TE2 probe (Table 1) demonstrate testis-specific binding (Figure 6A). Note that only a faint band is formed with testis extracts from 8 day old animals, which are sexually immature and deficient in primary spermatocytes. It should be mentioned that there are also faint bands formed with DNA-binding proteins from other tissues, most notably from brain (Figure 6). Since the sequences of TE2 and TE1 are similar, it is not surprising that both TE2 and TE1 successfully compete binding to labeled TE2, but excess nonspecific competitor dGdC does not compete (Figure 6B).

Since the two TE elements are imperfect copies of each other, additional competition experiments were performed to determine whether the individual TE elements would compete for binding with the larger 39 bp TE element in mobility shift experiments. Competitions were performed using the −92 to −54 H1t/TE labeled probe that contains both TE1 and TE2 subelements (Table 1). Figure 7 shows that competition with the full-length TE or with either TE1 or TE2 subelements alone can eliminate all three of the lowest mobility shifted bands specific to testis nuclear

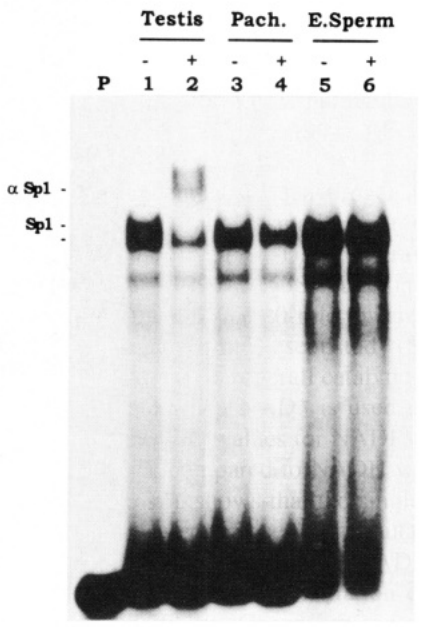

FIGURE 8: Sp1 is present in testis nuclear extracts. Commercially prepared Sp1 probe (Santa Cruz Biotechnology, Inc., Table 1) was used in this mobility shift assay. Free probe is in the lane designated P. Lane 1 shows binding of proteins in rat testis nuclear extracts, and lane 2 shows a supershift of the lowest mobility band when anti-Sp1 antibodies are added to the assay. Lanes 3 and 4 represent binding to proteins from enriched primary spermatocytes, and lanes 5 and 6 show binding to proteins from early spermatid. No supershift was detected with the anti-Sp1 antibodies with either of these extracts, lanes 4 and 6. Therefore, Sp1 is abundant in unfractionated testis nuclear extracts but not in enriched primary spermatocytes and early spermatids. The plus sign (+) indicates antibody addition, and the minus sign (−) indicates no antibody addition.

extracts. The present data are consistent with the possibility that similar testis nuclear proteins interact with each of the subelements. However, direct proof of the relationship between the proteins that bind to these two sites awaits further experimentation. Higher levels of the nonspecific competitor dGdC did not compete binding. A fourth high mobility band marked in the figure is not eliminated by these competitors.

Low Abundance of Sp1 in Primary Spermatocytes and Early Spermatids. A GC-rich Sp1 consensus sequence common to replication-dependent H1 promoters is located between and overlaps TE1 and TE2. Mobility shift assays using a GC-rich probe (−81 to −70 probe, Table 1) representing the Sp1 consensus sequence and testis nuclear extracts exhibit very weak binding (data not shown). However, this may be due to the fact that this probe may not have enough flanking sequence for efficient binding. On the other hand, proteins in the same testis extracts bind to a commercial 22 bp Sp1 consensus oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) (Table 1), as seen in Figure 8, lane 2. Anti-Sp1 polyclonal antibodies were used in a supershift assay with the testis nuclear extracts. Antibodies bind and supershift the lowest mobility band formed by proteins in total testis nuclear extracts as seen in Figure 8, lane 2. The antibodies also shift a specific low mobility band produced by liver extracts (data not shown). Two major low mobility bands and one or more higher mobility bands form with this commercial probe, and it is possible but not known whether all of the bands are formed by Sp1 family members (Hagen et al., 1994; Sogawa et al., 1993). However, only the lowest mobility band formed with testis extracts is shifted by the Santa Cruz anti-Sp1 polyclonal antibody (Figure 8, lane 2). On the other hand, this testis Sp1 binding activity was not detected in primary spermatocyte or early spermatid nuclear extracts as determined by the failure to supershift (Figure 8, lanes 4 and 6).

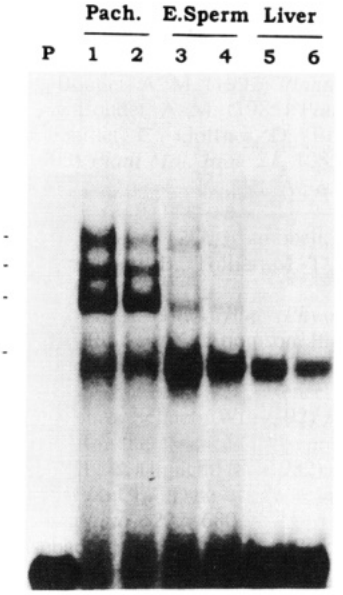

FIGURE 9: Testis Sp1 does not appear to bind to the H1t/TE element. The 36 bp H1t/TE probe used in Figure 7 that spans TE1 and TE2 and the GC-rich region (Table 1) was used in this experiment. The lane designated P shows free probe. Lanes 1−4 show the same banding pattern (four major bands) seen in Figure 7 with protein extracts from enriched primary spermatocytes (lanes 1 and 2) and early spermatids (lanes 3 and 4). Liver extracts form only one high mobility band (lanes 5 and 6). Anti-Sp1 antibodies failed to supershift any bands in primary spermatocytes (lane 2) or early spermatids (lane 4). The major shifted band produced with liver extract does not supershift with antibodies against Sp1 (lane 6).

To determine whether testis Sp1 could bind to the H1t promoter, a supershift experiment was conducted using the large 39 bp H1t/TE probe (−92 to −54 probe, Table 1) that contains the GC-rich Sp1 consensus sequence. Supershift assays using the same testis nuclear extract and conditions used with the commercial 22 bp Sp1 consensus oligonucleotide did not alter the mobility of any bands. For example, Sp1 polyclonal antibodies did not alter the binding profile of the −92 to −54 H1t/TE probe using nuclear extracts from primary spermatocytes, early spermatids, or liver (Figure 9, lane 2: spermatocytes; lane 4: spermatids; lane 6: liver). The results presented in Figures 8 and 9 indicate that Sp1 is present in both testis and liver nuclear extracts, but it is absent or present in low abundance in primary spermatocytes and early spermatids. Even though Sp1 is present in liver and testis, it does not appear to bind to the GC-rich element within the H1t promoter. The failure to see a supershift in primary spermatocytes and early spermatids may be due to the low abundance of Sp1 and cannot be taken to mean that Sp1 will not bind to the GC-rich region of the H1t promoter. Note that liver extracts which have Sp1 (data not shown) only produce one high mobility band (lanes 5 and 6) which appears to comigrate with a band formed by spermatocytes (lanes 1 and 2) and early spermatids (lanes 3 and 4). There is no supershift when anti-Sp1 antibodies are used with liver (lane 6).

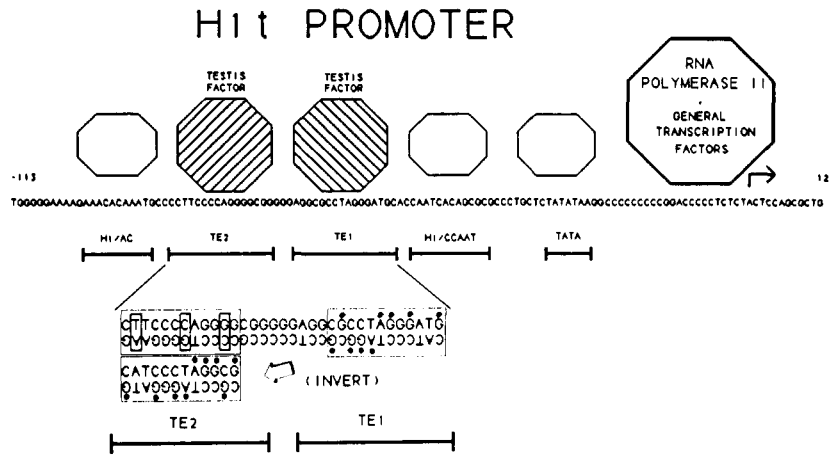

FIGURE 10: Model of the testis-specific histone H1t promoter. This model of the histone H1t proximal promoter shows DNA–protein binding sites based upon published data obtained from studies of other H1 genes (H1/AC box, H1/CCAAT box, and TATA box). It also shows H1t/TE1 and the imperfect inverted repeat designated H1t/TE2 found within mammalian H1t promoters. Specific binding to the H1t/TE1 element has been confirmed by methylation interference (Figure 3), and bases showing interaction are marked with an asterisk. The model omits binding of Sp1 to the GC-rich region based upon experiments which fail to detect Sp1 binding.

Data concerning the H1t proximal promoter are summarized in the model shown in Figure 10. The model shows that individual factors are bound to the TE1 and TE2 subelements because the full-length TE probe generates three testis-specific low mobility bands when bound to testis proteins. Furthermore, it appears that the same testis factor binds to TE1 and TE2, and it is possible that the factor binds as a homodimeric complex. However, if different factors bind to TE1 and TE2, then a heterodimeric complex may bind to this region of the promoter.

DISCUSSION

Histone H1t is expressed exclusively in the testis where it is found only in primary spermatocytes and early spermatids (Grimes et al., 1977; Kistler et al., 1989; Meistrich, 1989). Histone H1t may comprise approximately 60% of the H1 complement in these germinal cells (Lennox, 1984; Meistrich, 1989). Although present in both spermatocytes and early spermatids, synthesis occurs only in primary spermatocytes (Lennox, 1984; Meistrich, 1989; Grimes et al., 1977). H1t expression correlates with accumulation of H1t mRNA in primary spermatocytes (Doenecke et al., 1994; Grimes et al., 1990). Transcriptional activation coupled with increased messenger RNA stability probably accounts for accumulation during prophase of the meiotic cell cycle. This paradigm parallels observations of replication-dependent transcription of histones in somatic cells during S-phase of the cell cycle and may involve common or similar transcriptional regulatory factors such as CDC2, cyclin A, and RB related proteins (vanWijnen et al., 1994).

Since testis-specific expression of the H1t gene appears to be regulated at the transcriptional level, we initiated an examination of the H1t promoter. Appropriate high level tissue-specific expression of the rat H1t gene was found previously in transgenic mice bearing the rat H1t carried on a DNA fragment containing 2.45 kb upstream and 3.78 kb downstream of the H1t gene (vanWert et al., 1995a,b). In parallel studies transcription in nonexpressing cells by a 2384 bp promoter is dramatically repressed or silenced compared to a shorter 141 bp promoter (data not shown). These data extend and are consistent with previous data showing very low level transcription using the longer H1t promoter in nonexpressing cells (Grimes et al., 1990) and data showing repression by upstream H1t promoter sequences in nonexpressing cells (Kremer & Kistler, 1992).

Since transient expression experiments revealed high level transcription contributed by the proximal promoter, we extended our work by comparing four mammalian histone H1t promoters to identify regions important for regulation as revealed by their homology. Comparison reveals homology from the H1/AC box through the TATA box. In addition to the four common motifs in replication-dependent histone H1 promoters, we found an element designated H1t/TE1 that binds specifically to proteins from testis nuclear extracts (Grimes et al., 1992a,b). Closer examination revealed a second imperfect inverted copy of TE1 designated TE2, which was centered 19 bp upstream from the first element. Proteins that bind to the H1t/TE2 element exhibit identical tissue distribution and produce similar electrophoretic mobility shifts as proteins binding to the H1t/TE1 element (i.e., they appear to be restricted in large part to primary spermatocytes and early spermatids). They may be tissue-specific DNA-binding proteins, but a faint band corresponding to the major shifted band produced by testis nuclear proteins is produced by some other tissues such as brain. It is not clear whether these faint bands represent very low levels of the same TE-binding protein that is abundant in testis nuclei or whether they represent different DNA-binding proteins that produce similar mobilities. At this time, there is no direct experimental evidence that TE1 and TE2 binding proteins are identical, but it is tempting to speculate that common proteins are involved.

Electrophoretic mobility shift experiments using the larger H1t/TE probe that spans the TE1 and TE2 subelements and contains a central GC-rich region exhibit a multiband profile when binding to testis nuclear proteins. With this probe three tissue-specific low mobility electrophoretic bands are detected using extracts from pachytene primary spermatocytes, early spermatids, and total testis compared to one common high mobility electrophoretic band detected using extracts from somatic tissues (Figures 8 and 9). The three testis-specific shifted bands are likely due to combinations of interactions of one or more proteins with the TE1 and TE2 subelements. These may involve protein–DNA or protein–

protein interactions or both. These two subelements are recognized exclusively by proteins in extracts from specific germinal cells (primary spermatocytes and early spermatids) but not by proteins in extracts from somatic cells. On the other hand, the common higher mobility band detected using liver nuclear extracts is also produced by testis extracts.

Since the 39 bp H1t/TE probe contains an Sp1 consensus element (5′-GGGGCGGGG-3′), supershift experiments were performed to determine whether a band was generated by interaction with Sp1. Our results indicate that Sp1 is present in testis and liver extracts, but liver Sp1 does not bind to the GC element found in the H1t/TE probe (Figures 8 and 9). This may be due to other factors, including other members of the Sp1 family competing with Sp1 for binding. Additionally, Sp1 is detected in nuclear extracts from total testis but not from extracts enriched in primary spermatocytes or early spermatids. These findings are consistent with results of Saffer et al. (1991) indicating that Sp1 levels are normally low in spermatocytes.

Repression of testis-specific genes in nonexpressing tissues may be mediated in part by cis-acting negative regulatory elements (Mizuno et al., 1992; Lim & Chae, 1992; Bunick et al., 1990) as well as DNA methylation (Choi & Chae, 1991; Salehi-Ashtiani et al., 1993) within the proximal promoter. Protein factors which repress transcription of the histone H1t gene may be present in nonexpressing tissues. A potential candidate for this repressor may be the protein responsible for generating the high-mobility shifted band common to liver and testis, as seen in Figure 9. The site of binding of this liver protein has not been determined. This binding activity is low in extracts from primary spermatocytes compared to total testis and liver. Enriched populations of primary spermatocytes contain at least 30% contaminating cell types, and therefore this band would be expected to be seen in this mixed population of cells. However, data from parallel studies (data not shown) revealing high level transcription driven by the proximal promoter up to −141 bp argue against a strong repressor binding to the proximal promoter leading to repression of H1t gene transcription in nongerminal cells. However, this possibility has not been eliminated.

Data presented in this study can be used to design a model of the histone H1t promoter. The central region of the proximal promoter designated H1t/TE binds testis proteins at the primary spermatocyte stage of spermatogenesis. Protein binding to one or both TE1 and TE2 subelements may facilitate activation of transcription in these cells. Furthermore, repression in non-expressing tissues may be mediated in part by binding of a repressor to the H1t/TE element (Figure 10). This model does not reflect other proximal or distal elements or interactions that may be involved in transcriptional control such as a potential transcriptional repressor region located between the −948 bp and −520 bp of the H1t promoter. Various cis-acting elements from promoters of several testis-specific expressed genes have been implicated in transcriptional activation (Zhou et al., 1994; van der Hoorn & Tarnasky, 1992; Queralt & Oliva, 1993). Therefore, testis-specific transcriptional activation most likely requires a combination of protein factors (some of which may be testis specific) and appears to be complex, involving different mechanisms, possibly including alterations in DNA methylation. It will be important to test the participation of the H1t/TE element for transcriptional activation of the histone H1t gene in primary spermatocytes and transcriptional repression in nonexpressing cells and tissues in future experiments. Distal upstream sequences also contribute significantly to transcriptional repression in nonexpressing cells, and factors binding to these sequences need to be identified.

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