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TE2 and TE1 Sub-Elements of the Testis-Specific Histone H1t Promoter Are Functionally Different

Donald C. Wilkerson,[1,2] Steven A. Wolfe,[1,2] and Sidney R. Grimes[1,2]*

[1]Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130-3932
[2]Research Service (151), Overton Brooks Veterans Affairs Medical Center, Shreveport, Louisiana 71101-4295

Abstract The testis-specific linker histone *H1t* gene is transcribed exclusively in pachytene primary spermatocytes. Tissue specific expression of the gene is mediated in part by transcriptional factors that bind elements located within the proximal and distal promoter. A 40 bp promoter element, designated H1t/TE, that is located within the proximal promoter between the CCAAT-box and AC-box, is known to be essential for *H1t* gene transcription in transgenic animals. In the present study, we show by SDS–PAGE analysis of UV crosslinked protein and DNA and by electrophoretic mobility shift assays (EMSA) of testis nuclear proteins separated on a non-denaturing glycerol gradient that the TE1 sub-element is bound by a protein complex. Mutation of TE1 leads to a drop in H1t promoter activity in germinal GC-2spd cells as well as in nongerminal Leydig, NIH3T3, and C127I cell lines. Although TE1 and TE2 sub-elements have similar sequences, mutation of the TE2 sub-element causes an increase in promoter activity in C127I and Leydig cells. The rat TE1 but not TE2 contains a CpG dinucleotide and this cytosine is methylated in liver but not in primary spermatocytes. Methylation of the cytosine at this site almost eliminates nuclear protein binding. Thus, there are significant functional differences in the TE2 and TE1 sub-elements of the H1t promoter with TE1 serving as a transcriptional activator binding site and TE2 serving as a repressor binding site in some cell lines. J. Cell. Biochem. 88: 1177–1187, 2003.

Key words: testis-specific; histone H1t; spermatogenesis; transcription; gene regulation; TE1; TE2

Linker and core histones are involved in packaging DNA in chromatin [Thoma et al., 1979; Hayes and Wolffe, 1993]. Linker histones are also involved in regulating gene expression [Crane-Robinson, 1999; Thomas, 1999]. The eight known mammalian linker H1 histone genes fall into one of three broad groups [Khadake and Rao, 1995; Drabent and Doenecke, 1997; Tanaka et al., 2001]. The first major group includes the cell cycle regulated linker histone genes that are maximally expressed during S-phase to provide histone for packaging newly synthesized DNA. The second group includes the basally expressed *H1°* gene that is constitutively transcribed at a low rate throughout the cell cycle in terminally differentiated cells and tissues [Doenecke and Alonso, 1996; Peretti and Khochbin, 1997]. The third group include the tissue-specific *H1* genes such as the ovary-specific H1oo [Tanaka et al., 2001] and the testis-specific H1t [Cole et al., 1986; Grimes et al., 1987].

H1t mRNA accumulates only in pachytene primary spermatocytes, and this appears to be the only cell type where the gene is transcribed and where the protein is synthesized [Grimes et al., 1987]. Histone H1t eventually comprises about 60% of the linker histone complement in spermatocytes and it is found at a similar level in early spermatids because it is retained through the two meiotic cell divisions. Of the linker histones, H1t binds weakest to DNA [Khadake and Rao, 1995; De et al., 2002]. There is also an entire complement of testis-specific and testis-enriched core histone genes that are expressed in spermatocytes along with H1t, so

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*Correspondence to: Sidney R. Grimes, Medical Research Service (151), Overton Brooks Veterans Affairs Medical Center, 510 E. Stoner Avenue, Shreveport, LA 71101-4295. E-mail: srgrimes@prysm.net

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that a major portion of the histones in spermatocytes and spermatids are testis histones [Shires et al., 1976; Trostle-Weige et al., 1982, 1984; Bramlage et al., 1997; Celeste et al., 2002].

Clues concerning the function of a gene can frequently be learned by observation of the phenotype produced after mutations to inactivate or "knock out" the gene. For example, mutation of the testis core histone H2A.X leads to DNA instability and a blockage of spermatogenesis [Celeste et al., 2002]. Therefore, this testis core histone and perhaps all of the testis histones may be critical for DNA repair during spermatogenesis. However, no phenotype was observed with mice containing a histone *H1t* gene knockout; spermatogenesis appeared to be normal, and the mice were fertile [Drabent et al., 2000; Lin et al., 2000; Fantz et al., 2001]. Since, the weight ratio of total linker histone to DNA did not drop in germinal cells of the knockout mice, other linker histones that are normally expressed in germinal cells along with H1t compensated for the missing histone. Furthermore, inactivation of the $H1^{o}$ variant alone or in addition to a second H1 in dual knockouts had no effect on the mice [Fan et al., 2001]. Thus, each of the linker histone variants is likely critical and compensatory mechanisms exist to replace the histone when its level is low. However, this does not rule out a possible role for H1t in DNA repair.

Mammalian histone H1 promoters contain several conserved elements. Shared elements required for maximal promoter activation during S-phase of the cell cycle include a TATA-box, a CCAAT-box, a GC-rich region, and an H1/AC-box [Stein et al., 1989; Osley, 1991]. The testis-specific H1t promoter contains additional elements that are required for binding transcriptional activators in primary spermatocytes and elements that are needed for binding transcriptional repressors in other cell types [Grimes et al., 2002]. H1t-specific elements required for activation include the TE element located between the CCAAT-box and AC-box [vanWert et al., 1995, 1998], a portion of the RE element located between −130 and −106 bp just upstream from the AC-box [Wolfe and Grimes, 2002a,b], and a TG-box located at about 500 bp upstream from the mRNA start site [Drabent and Doenecke, 1997]. Elements required for repression in nongerminal cells include a GC-rich region downstream from the H1t TATA-box, designated GC-box 2 [Clare et al., 1997], the 5′ end of the RE element just upstream from the AC-box [Wolfe and Grimes, 2002b], and an element located between −948 and −780 bp upstream from the mRNA start site [Wolfe et al., 1999].

The focus of the present study is the TE element, which is composed of three sub-elements. Two of the sub-elements are designated TE1 and TE2. A GC-rich element, designated GC-box 1, is located between TE1 and TE2 (Fig. 1). Although the levels of Sp1 and Sp3 are low in primary spermatocytes, they can bind to the GC-box 1 in electrophoretic mobility shift assays (EMSA), and mutation of GC-box 1 inactivates the H1t promoter in transient expression assays [Wilkerson et al., 2002a,b]. Coexpression of Sp1 or Sp3 in transient expression assays along with an H1t promoter driven luciferase expression vector activates the H1t promoter [Wilkerson et al., 2002b].

TE1 and TE2 sub-element are imperfect inverted repeats that bind nuclear proteins found only in primary spermatocytes and early spermatids, when comparing binding of proteins derived from different tissues. Replacement of the entire TE sequence with heterologous DNA completely inactivates the H1t promoter in transgenic mice [vanWert et al., 1998]. Mutation of the TE1 sub-element alone leads to inactivation of the H1t promoter in transient expression assays [Wilkerson et al., 2002a]. Therefore, TE1 like the GC-box 1 sub-element is required for maximal promoter activity.

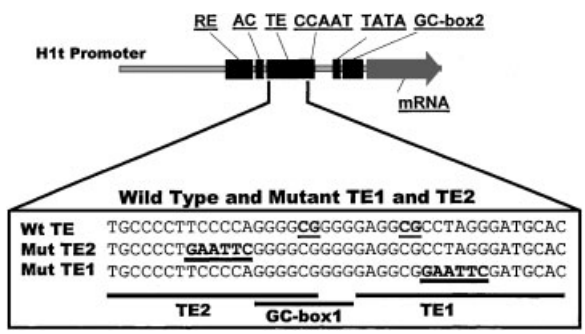

Fig. 1. The testis-specific histone H1t TE promoter element is composed of three sub-elements. The drawing shows the H1t promoter used in expression assays in this study. Starting at the mRNA start site, the elements include the GC-rich repressor region (GC-box 2), the TATA-box, the CCAAT-box, the TE element, the AC-box, and the RE element. The leader region of the mRNA is also shown. The sequence shows the location of the TE2, H1t/GC-box, and TE1 sub-elements within the TE element. The TE2 and TE1 mutants are indicated and the two CpG dinucleotides that are methylated in liver are underlined in the wild type (Wt TE) sequence.

In this study we have examined in the roles of the TE1 and TE2 sub-elements in H1t promoter function. We show that the TE1 sub-element is bound by a complex of proteins. Mutation of the TE1 sub-element leads to a drop in H1t promoter activity and this drop was consistent in every cell line assayed. However, mutation of the TE2 sub-element actually increased promoter activity in two of the four cell lines assayed and did not decrease activity in any cell line tested. In addition, the core of TE1 but not TE2 contains a CpG dinucleotide that can be methylated in liver. Methylation of this site blocks binding of nuclear proteins in EMSA.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Genosys (The Woodlands, TX). Sprague-Dawley rats used for preparing testis extracts were obtained from Harlan Sprague-Dawley (Madison, WI). Cell lines used were the mouse GC-2spd cell line derived from germinal cells that was provided by José Luis Millán [Hofmann et al., 1994], an NIH3T3 mouse cell line (ATCC No. CRL1658), a rat Leydig cell line obtained from American Type Culture Collection (ATCC), and a C127I mouse mammary cell line (ATCC No. CRL1616). GC-2spd cells were grown at 32°C [Hofmann et al., 1994], other cell lines were grown at 37°C and all cells were maintained in a 5% CO_2 atmosphere. Radioisotopes were purchased from New England Nuclear (Boston, MA).

Electrophoretic Mobility Shift Assays (EMSA)

Testis nuclear proteins were prepared by the Dignam procedure [Dignam et al., 1983]. Some samples for the UV crosslinking experiment were further purified by binding to Whatman phosphocellulose P11 in Dignam buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl) without KCl, washing four times with the same buffer without KCl, and eluting with the same buffer with 300 mM KCl. The samples were dialyzed against buffer D. EMSA were performed as describe previously [Grimes et al., 1990; Wolfe and Grimes, 1993; Wilkerson et al., 2002a]. The EMSA TE1 probe used for Figures 4 and 5 was prepared by annealing the following upper (U) and lower (L) oligonucleotide pairs: (U) 5′-GAGGCGCCTAGGG-3′; (L) 5′-GTGCATCCCTAGGCGCC-3′. The annealed DNA fragments were then filled with dATP, dGTP, dTTP, and [α-^{32}P] dCTP using the Klenow fragment of DNA polymerase as described previously [Wolfe et al., 1995]. Wild type (Wt) and methylated (Methyl) TE1 probes and competitors for the methylation study were prepared by annealing the following upper (U) and lower (L) oligonucleotide pairs: Wt: (U) 5′-GAGGCGCCTAGGG-ATGCAC-3′; (L) 5′-GTGCATCCCTAGGCGCC-TC-3′; Methyl: (U) 5′-GAGGC^{M}GCCTAGGGA-TGCAC-3′; (L) 5′-GTGCATCCCTAGGC^{M}GCC-TC-3′. C^{M} in the Methyl TE1 oligonucleotides denotes methylated cytosine. These annealed DNA fragments to be used for probes were end labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase as described previously [Wolfe et al., 1995]. EMSA gels were dried and radioactivity was detected using a Cyclone Phosphorimager (Packard Instrument Company, Meriden, CT).

Transient Transfection Assays

Transfection assays were performed as previously described [Wolfe et al., 1999; Wilkerson et al., 2002a]. Wild type H1t (Wt H1t) and mutant TE1 (mutTE1) and TE2 (mutTE2) expression vectors were constructed as described previously [Wolfe et al., 1999; Wilkerson et al., 2002a] and sequences of the relevant mutants are shown in Figure 1.

UV Crosslinking

Nuclear proteins were bound to a radioactive TE1 DNA probe as for EMSA with all components doubled to give a final volume of 40 μl rather that the standard 20 μl binding reaction. Following binding, the samples in microfuge tubes were crosslinked with 3 pulses of UV light (0.12 Joules/pulse) in a Stratagene Stratalinker. After adding 10 μl of 5X Laemmli SDS sample loading buffer [Laemmli, 1970] without glycerol to each, samples were boiled for 5 min, and 30 μl of each was loaded onto an SDS–PAGE gel (7 cm long and 8 cm wide) composed of a 5% stacking and 12% separating gel. Electrophoresis was conducted at 200 V for about 1.5 h until the 20 kDa BioRad prestained Precision Plus Standard reached the end of the gel. Gels were dried for 1 h at 60°C under vacuum onto Whatmann 3 MM paper and exposed to a phosphorimager screen for 17 h. Prestained bands on the dried gel were photographed for alignment and comparison with bands from the phosphorimager image. Molecular weights of electrophoretic bands in these

images were estimated using Bio-Rad Quantity One software.

Glycerol Gradient Analysis

Rat testis nuclear proteins were prepared as for EMSA [Dignam et al., 1983]. Nuclear protein extract (approximately 2 mg of protein in a volume of 100 μl of Dignam buffer D in 1% glycerol) was layered over a 5–20% glycerol gradient in buffer D and centrifuged 18 h at 132,000*g* in a Beckman SW55Ti rotor. A mixture of proteins (lysozyme: 14,400 kDa; BSA: 67,000 kDa; aldolase: 158,000 kDa; and calalase: 232,000 kDa) was layered on a similar gradient in a separate tube. Fractions of 0.25 ml were collected for analysis. Proteins in each gradient fraction were assays for binding activity by EMSA using a labeled TE1 element as DNA probe. Fractions containing protein standards were analyzed by SDS–PAGE to determine the distance of migration of the different proteins during centrifugation.

RESULTS

TE1 and TE2 Promoter Elements of the *H1t* Gene Have Similar Sequences

To gain insight into the mechanisms of tissue-specific transcription of the *H1t* gene, we analyzed the H1t promoter. A number of important regulatory elements within the proximal promoter are indicated in Figure 1. We have shown previously that the H1t/TE element is necessary for tissue-specific expression of the histone *H1t* gene [Wolfe et al., 1995]. Transgenic mice that carried a rat H1t transgene with a mutant TE element did not express the rat *H1t* gene, while transgenic mice with a wild type TE element expressed the rat H1t normally in primary spermatocytes [vanWert et al., 1998]. This transgenic mouse experiment shows that sequences within the TE element are essential for H1t gene expression.

The tripartite TE element contains a GC-rich element designated GC-box 1 that is located between the TE1 and TE2 sub-elements (Fig. 1). TE1 and TE2 bind nuclear proteins, whose appearance coincides with initiation of transcription of the *H1t* gene [Grimes et al., 1992]. The GC-box 1 is important for activating the H1t promoter [Wilkerson et al., 2002a] and binding of Sp1 or Sp3 to GC-box 1 enhances activity of the H1t promoter in transient expression assays [Wilkerson et al., 2002b].

Figure 1 also shows H1t promoter elements shared with other H1 promoters including the TATA-box, CCAAT-box, and AC-box as recently reviewed [Grimes et al., 2002]. The 3′ end of the RE element, located just upstream from the AC-box, serves as a binding site for a transcriptional repressor in some cell lines [Wolfe and Grimes, 2002b]. On the other hand, the 5′ end of this element may serve as a binding site for a transcriptional activator in primary spermatocytes [Wolfe and Grimes, 2002a]. The GC-rich region, designated GC-box 2, downstream from the TATA-box serves as a potent repressor in some cell lines [Clare et al., 1997].

In the sequence alignment in the top panel of Figure 2, the TE1 and TE2 sub-elements are shown to be imperfect inverted repeats. The forward (f) strand and the reverse (r) strand of the entire TE element are aligned for comparison. Both strands are shown in the 5′ to 3′ direction with identical bases highlighted. The underlined regions of TE1 and TE2 are identical in 10 out of 13 positions. In the bottom panel of Figure 2, it is possible to see that the TE1 and TE2 sub-elements are conserved among mammals including rat, mouse, and human. The human sequence has an additional nucleotide in both the TE1 and TE2 sub-elements.

The promoter element alignment in Figure 3 shows the conserved TE1 and TE2 sub-elements along with similar promoter elements from other genes. *Testisin* [Hooper et al., 2000], *c-mos* [van der Hoorn et al., 1991; Zhao et al., 1991], *RT7* [van der Hoorn and Tarnasky, 1992], and a

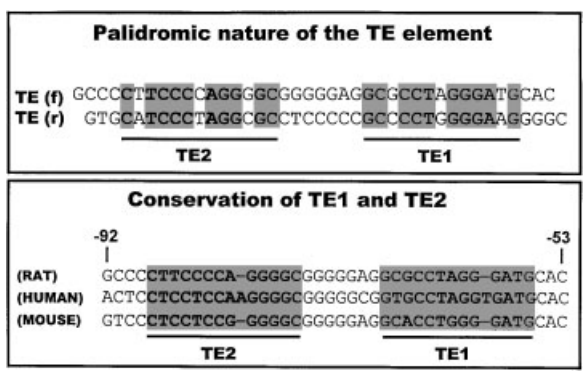

Fig. 2. The TE1 and TE2 sub-elements are imperfect inverted repeats. The rat TE sequence and its reverse complement are aligned in the **upper panel** to show the similarity of the two sub-elements. Only three bases differ between TE1 and TE2 within the 13 bp regions that are underlined (identical bases are highlighted). In the **lower panel** the rat, mouse, and human TE elements are aligned to show the conservation among these species.

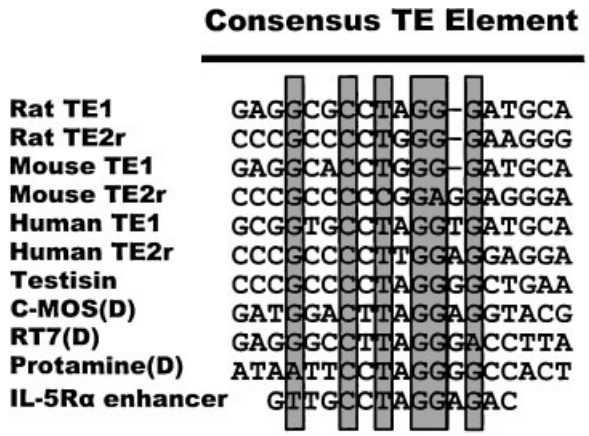

Fig. 3. Several testis-specific genes contain an element similar to the TE1 and TE2 sub-elements. The TE1 and TE2 elements shown in Figure 2 are aligned along with similar regulatory elements found in the promoters of the *testisin, c-mos, RT7, protamine,* and *IL-5Rα* genes. All of these except the IL-5Rα enhancer are known to be testis-specific. However, RFX4 is a testis-specific RFX family member that binds to this IL-5Rα enhancer sequence.

protamine gene [Johnson et al., 1988] are testis-specific genes like the histone *H1t* gene. It is interesting that the testisin element, located about −75 bp from the mRNA start site, is composed of a paired GC-box and CCTAGG element [Hooper et al., 2000], which is very similar to the arrangement of the GC-box and CCTAGG element in the testis histone H1t promoter as shown in Figure 1. *MHC II* gene X-box elements bind RFX family members [Morotomi-Yano et al., 2002]. The *IL-5Rα* gene enhancer (GTTGCCTAGGAGAC) is shown as a representative of the group. RFX4 is a testis-specific member of the RFX family that lacks a transcriptional activation domain and that might function through selective interactions with other RFX members in transcriptional regulation [Morotomi-Yano et al., 2002]. Therefore, these data support the hypothesis that nuclear proteins that bind the TE1 and TE2 subelements may be involved in regulating transcription of the testis-specific histone *H1t* gene.

A Large Testis Nuclear Protein Complex Binds to the TE1 Sub-Element

Testis nuclear proteins generate a low mobility band in EMSA when using the TE1 and TE2 DNA probes [Wolfe et al., 1995; Wilkerson et al., 2002a]. Nuclear proteins from germinal GC-2spd cells also bind but produce a slightly higher mobility band with TE1 and TE2 probes [Wilkerson et al., 2002b]. The TE binding complex formed with testis nuclear proteins is shown in lane 2 of the left panel of Figure 4. Lane 3 shows the banding pattern produced

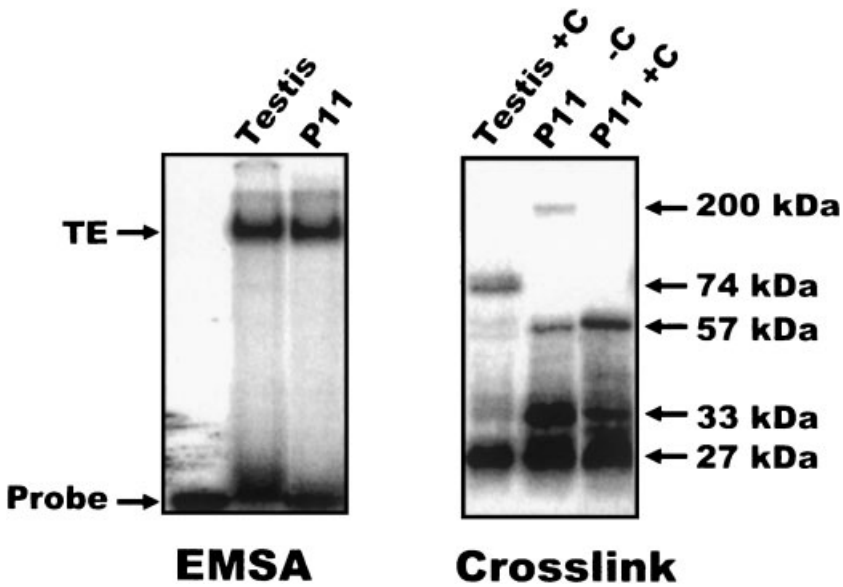

Fig. 4. A testis nuclear protein complex binds to the TE1 sub-element. The **left panel** shown an EMSA to demonstrate testis nuclear proteins binding to the TE1 sub-element. Free probe is in the **lane 1**, bound testis nuclear proteins are in **lane 2**, and bound proteins from phosphocellulose P11 purified testis nuclear proteins are in **lane 3**. The purified proteins were prepared from the same testis nuclear protein stock. They were bound to phosphocellulose P11 and eluted with 0.3 M KCl as described in the Materials and Methods. The **right panel** shows a UV crosslink experiment using nuclear proteins from testis and P11 purified protein with (+C) and without (−C) added competitor DNA.

when using the same testis nuclear protein preparation that was partially purified before analysis. Purification was accomplished by binding to phosphocellulose P11 and elution with 0.3 M KCl as described in the Materials and Methods. The banding profiles of the partially purified material and the original extract appear to be identical.

To characterize the bound nuclear proteins, we conducted protein–DNA crosslinking using testis nuclear proteins with nonspecific competitor (500 ng dG–dC) (lane 1), phosphocellulose P11 purified proteins bound without nonspecific competitor (lane 2), and P11 purified proteins bound with nonspecific competitor (lane 3) (right panel of Fig. 4). Nuclear proteins were bound to a radioactive TE1 probe, crosslinked with UV light, and analyzed by SDS–PAGE on a 12% gel. A phosphorimage of the dried gel is shown in the right panel of Figure 4. Compared to prestained protein molecular weight standards run on the same gel, the apparent masses of the major bands were approximately 27, 33, and 57 kDa when using the P11 purified proteins with nonspecific competitor (lane 3). The 33 kDa band decreased with addition of competitor and it may represent binding of nonspecific protein (compare lanes 2 and 3). The proteins that form the 57 kDa band may represent a dimer of the protein that forms the 27 kDa band.

An additional low mobility band (200 kDa) formed when nonspecific competitor was absent (lane 2), but accurate molecular weight determinations for such large proteins by SDS–PAGE composed of a 12% polyacrylamide separating gel is difficult. The 74 kDa band produced by the original testis nuclear protein extract (lane 1) was not present in the P11 purified proteins and therefore appears to be a nonspecific binding protein removed during purification.

To further examine the TE binding protein complex, testis nuclear proteins were layered over a 5–20% glycerol gradient and centrifuged for 18 h at 132,000 *g* in a Beckman SW55Ti rotor. Protein molecular weight markers were layered over the same type of gradient in a separate centrifuge tube. After centrifugation, testis protein gradient fractions were collected, dialyzed against Dignam nuclear protein buffer D, and the dialyzed proteins examined for binding activity using EMSA. Fractions containing protein standards were analyzed by SDS–PAGE to determine the distance of migration during centrifugation of protein markers within the gradient.

TE1 binding activity of each fraction is shown in the EMSA and locations of protein molecular weight markers are shown below the EMSA in Figure 5. The first lane is a control (C) banding pattern with testis nuclear proteins to show the low mobility TE complex. Native TE1 binding activity migrates in the glycerol gradient with a mobility similar to the mobility of the 158 kDa protein marker. Binding proteins that formed higher mobility bands traveled further through the glycerol gradient and appear to be larger than the proteins that form TE complex. It is interesting that the mass of the testis protein complex that binds to the TE1 probe appears to be at least 158 kDa. Such a large mass for the complex is consistent with the large mass (about 200 kDa) seen in the UV crosslinking experiment (lane 2 of the right panel of Fig. 4). On the other hand, the SDS–PAGE gel shows major

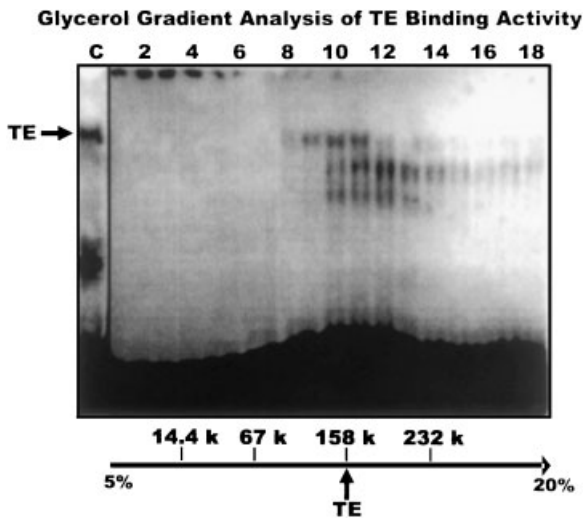

Fig. 5. Glycerol gradient analysis of the testis-nuclear protein complex that binds to the TE1 sub-element. Rat testis nuclear proteins in Dignam buffer D but with 1% glycerol were layered over a 5–20% glycerol gradient and centrifuged 18 h at 132,000 *g* in a Beckman SW55Ti rotor. Protein standards were layered on a similar gradient in a separate tube as described in the Materials and Methods. Gradient fractions of 0.25 ml were collected for analysis. Proteins in each gradient fraction were assayed for binding activity by EMSA using a labeled TE1 element as DNA probe. The **first lane** (C) shows the TE band that is formed using unfractionated testis nuclear proteins. Fractions containing protein standards were analyzed by SDS–PAGE to determine their mobilities through the gradient and the mobilities of these proteins are indicated by their molecular weight. Proteins that bind to form the TE complex migrate at peak fraction 11 at the same rate as the 158 kDa protein standard.

lower molecular weight UV crosslinked bands (27, 33, and 57 kDa). Therefore, the smaller DNA binding components may associate with other testis nuclear proteins to form the large TE1 binding complex (158–200 kDa).

Transcriptional Activators Binds TE1 but Transcriptional Repressors may Bind TE2

Since testis nuclear proteins appear to be able to bind to both TE1 and TE2, we wanted to examine the relative contribution of each sub-element to H1t promoter activity. To assay each for H1t promoter activity, each element was individually mutated in the context of a pGL3 luciferase reporter vector containing the H1t proximal promoter. We chose mutations that do not mutate the GC-box that is located between TE1 and TE2 (Fig. 1). We also showed previously that these mutant sequences do not form artifactual enhancer or repressor binding sites [Wilkerson et al., 2002a]. The specific nucleotides mutated within the Mut TE2 and Mut TE1 are underlined in Figure 1. Wild type and mutant expression vectors were transiently transfected into four different cell lines and assayed for luciferase activity (Fig. 6A–D).

In this experiment we confirmed that mutation of TE1 reduced promoter activity compared to the wild type promoter in GC-2spd cells grow at 32°C. We extended these studies showing that the TE1 mutant also reduced H1t promoter activity in NIH3T3, Leydig, and C127I cells. Thus, the TE1 sub-element serves as a binding site for a transcriptional activator. This site appear to contribute almost as much to transcriptional activation of the H1t promoter as the GC-box 1 [Wilkerson et al., 2002a].

In the same experiment we showed that mutation of the TE2 sub-element caused a slight but reproducible increase in promoter

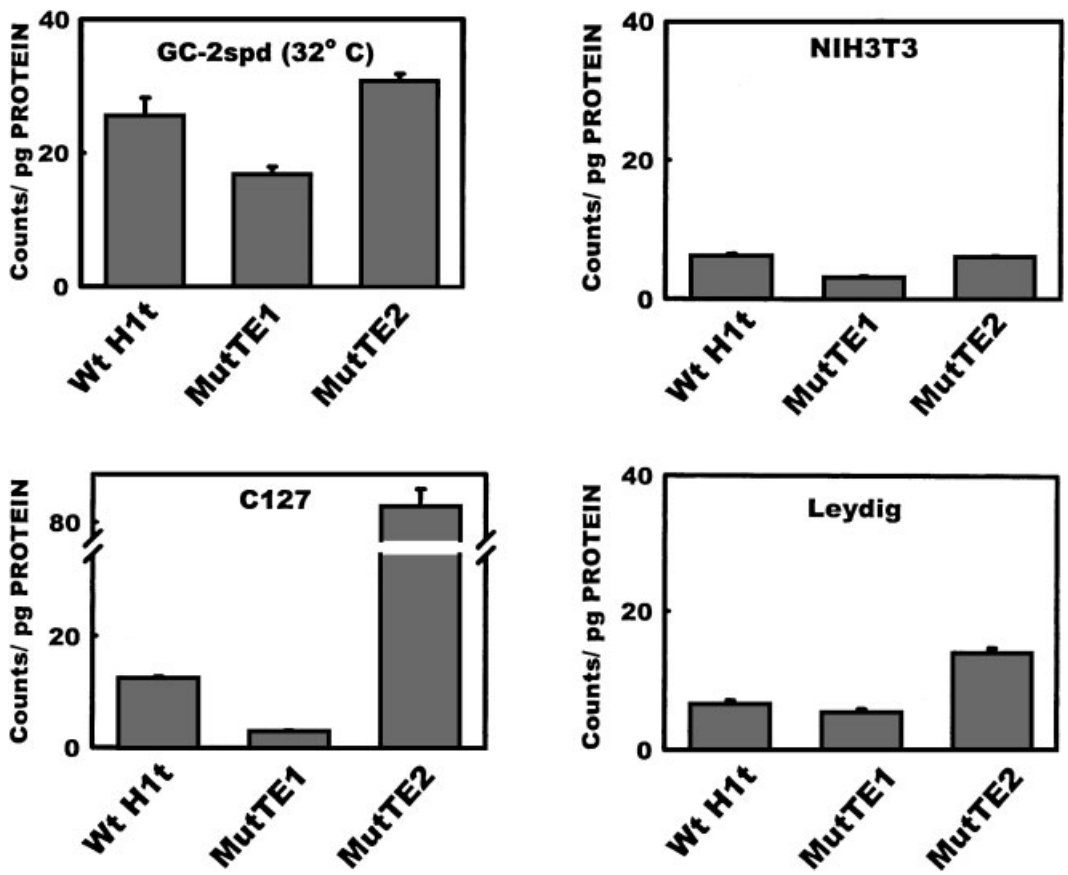

Fig. 6. Transient expression assay of H1t promoter mutants. Four different cell lines (testis GC-2spd, NIH3T3, C127, and testis Leydig cells) were transfected with the expression vectors as indicated. From left to right in each panel these vectors are the wild type testis-specific histone H1t promoter vector (Wt H1t), the H1t promoter vector with a TE1 mutation (MutTE1), and the H1t promoter vector with a TE2 mutation (MutTE2). All vectors have the same upstream and downstream fusion points in pGL3B and they have the same sequence as wild type (Wt H1t) except for the nucleotides indicated in Figure 1. The columns represent the means of triplicate samples and the error bars represent the standard error of the mean.

activity in GC-2spd and Leydig cells. However, there was a surprising increase of almost seven fold in activity in C127I cells. There was little or no change in activity in NIH3T3 cells. Thus, mutation of the TE2 element did not cause repression of activity in any cell line tested. We conclude that the function of the TE2 sub-element differs substantially from that of the TE1 sub-element. While TE1 serves as a binding site for transcriptional activators, TE2 apparently serves as a binding site for transcriptional repressors. This is most apparent in C127I cells.

Methylation of the H1t/TE1 Sub-Element Alters Nuclear Protein Binding

The core of the rat TE1 and the GC-box 1, sub-elements located within TE element, contain a CpG dinucleotide (underlined in the WT TE sequence shown in Fig. 1) and cytosines in these CpG dinucleotides are known to be methylated in liver where transcription of the gene is repressed but unmethylated in pachytene primary spermatocytes where the gene is transcribed [Singal et al., 2000]. Methylation of the H1t promoter decreases expression of a luciferase reporter vector [Singal et al., 2000] and methylation of GC-box 1 blocks binding of Sp1 and Sp3 [Wilkerson et al., 2002b]. Sp family members do not bind to the TE1 site, but we wanted to test the possibility that methylation also altered nuclear protein binding to the TE1 element.

Therefore, EMSA competitions were performed using either methylated or unmethylated rat H1t/TE1 oligonucleotides with nuclear extracts prepared from the GC-2spd cell line (Fig. 7). Lane 1 (left panel) shows protein binding to the unmethylated TE1 probe using an extract from GC-2spd cells grown at 32°C with no competitor (NC). Lanes 2 and 3 include an excess of unlabeled unmethylated TE1 DNA (UM) competitor or unlabeled methylated TE1 DNA (M) competitor, respectively. The unmethylated DNA fragment competed binding but the methylated TE1 fragment failed to compete. The right panel shows GC-2spd cell nuclear extracts incubated with the methylated TE1 probe in the absence of specific competitor (lane 1) or presence of methylated (lane 2) or unmethylated (lane 3) H1t/TE1 competitor. Protein binding is almost eliminated when the probe is methylated (right panel). Only a faint band with a slightly higher mobility is formed in lane 1 when the methylated probe is used in the assay.

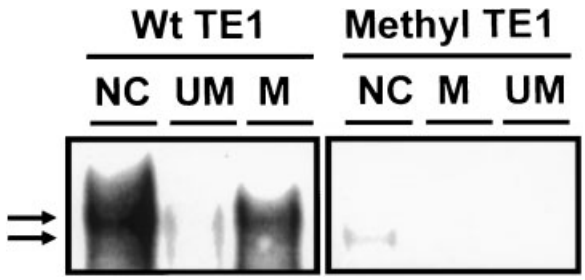

Fig. 7. Methylation of the CpG dinucleotide within the TE1 sub-element block binding of nuclear proteins. The wild type TE1 (Wt TE1) and methylated TE1 (Methyl-TE1) probes (described in the Materials and Methods) were used in an EMSA competition assay with nuclear proteins from GC-2spd cells grown at 32°C. The lanes are labeled NC (no competitor), UM (the identical unlabeled and unmethylated sequence as competitor), and M (the identical unlabeled competitor but with a methylated CpG dinucleotide as competitor). Competitions were performed with a 20-fold molar excess of unlabeled double-stranded DNA. The unmethylated TE1 sequence was used as a probe in the **left panel** and the methylated TE1 sequence was used as a probe in the **right panel**. Note that nuclear proteins almost fail to bind to the methylated probe.

DISCUSSION

The testis-specific linker histone *H1t* gene is expressed only in primary spermatocytes. Transcriptional regulation contributes significantly to this tissue-specific expression. A major goal of our research has been to identify promoter elements that confer tissue-specific transcription to the *H1t* gene. Several laboratories have identified conserved elements within the proximal promoters of the linker histone genes that are required for active transcription. For example, the TATA-box, CCAAT-box, and AC-box, are found in promoters of almost all *H1* genes including the *H1t* gene. The TATA-box is important for formation of a transcription initiation complex and the CCAAT-box and AC-box contribute to maximal S-phase transcription to provide histones for packaging newly synthesized DNA. Although these elements are required for maximal activity of the H1t promoter, they may not make a significant contribution to testis-specific transcription.

Promoter elements that contribute to tissue-specific transcription include those that activate transcription in primary spermatocytes and those that repress transcription in other cell types. Within the proximal promoter, the

TE element [vanWert et al., 1995, 1998] and the 5′ end of the RE element [Wolfe and Grimes, 2002a] function to activate transcription; and within the distal promoter, a TG-rich element [Drabent and Doenecke, 1997] has been reported to be important for activation. Elements known to be important for transcriptional repression include the GC-box 2 [Clare et al., 1997] located downstream from the TATA-box within the leader region, the 3′ portion of the RE element [Wolfe and Grimes, 2002b], which is located just upstream from the AC-box between 130 and 106 bp upstream from the start site, and a distal promoter element [Wolfe et al., 1999] located between 948 and 780 bp upstream from the mRNA start site.

In this study we focused upon the roles of TE1 and TE2 sub-elements that are located within the larger TE element. In transgenic mouse studies, the H1t promoter is totally inactive when TE is replaced by heterologous DNA sequence. In vitro transcription assays with two different deletions within the TE element showed that removal of a portion of TE2 and all sequences upstream of TE2, had little effect on transcription. A second deletion, that removed a portion of TE1 and all sequences upstream of TE1, including the GC-box and TE2, decreased transcription. However, these studies failed to reveal the individual contributions of TE2, GC-box 1, and TE1 to transcription.

More recently, we showed that mutation of either TE1 or the GC-box 1 reduced transcription in transient expression assays [Wilkerson et al., 2002a]. However, these initial studies showed that mutation of TE2 did not reduce transcription in GC-2spd cells. The TE1 element is present in promoters of several other testis-specific genes including *testisin*, *c-mos*, *RT7*, and *protamine* as seen in Figure 3 and a portion of the element is also present in the leader region of the testis-specific LDHc promoter [Zhou et al., 1994]. The sequences of TE1 and TE2 are similar with only 3 bp differences as shown in Figures 2 and 3. In addition, we have shown that testis nuclear proteins bind to both TE1 and TE2 to give similar banding patterns in EMSA and the TE1 and TE2 sequences complete with each other for nuclear protein binding in EMSA [Wolfe et al., 1995; Wilkerson et al., 2002a]. Therefore, based upon the transgenic mouse studies, the in vitro transcription studies, and protein–DNA binding studies, we originally proposed that the same or very similar testis nuclear proteins bind to TE1 and TE2 and possibly form a homodimer or heterodimer.

In the present study we extended our studies on the TE1 and TE2 sub-elements. We first examined the size of the nuclear protein or nuclear protein complex that binds to the TE1 element and then we compared the effects of mutations of the two sub-elements on transcription. Concerning the testis nuclear proteins that bind to TE, previous studies showed that proteins with similar properties bind to both TE1 and TE2. To determine the size of the DNA binding proteins, we used centrifugation to separate testis nuclear proteins on a non-denaturing glycerol gradient. The TE binding proteins comigrate with the protein marker aldolase that has a molecular weight of approximately 158 kDa (Fig. 5). The results are consistent with previous binding studies where the protein–DNA complex was cross-linked with UV light. SDS–PAGE analysis of proteins cross-linked to the TE1 probe, showed that the proteins or protein complex was approximately 180 kDa [Wolfe et al., 1995]. Although these experiments do not prove that the binding protein is part of a larger protein complex, the data certainly favor the idea that it is part of a protein complex.

We confirm that mutation of TE1 leads to a drop in promoter activity in transient expression assays (Fig. 6). We show that mutation of TE2 leads to an increase in H1t promoter activity in transient expression assays in the GC-2spd cell line (Fig. 6). Although mutation of TE2 had little effect on promoter activity in NIH3T3 cells, it caused significant increases in promoter activity in C127I and Leydig cells.

Thus, the TE1 and TE2 sub-elements have different functions in regulating transcription of the *H1t* gene. Both TE1 and the adjacent GC-box 1 are required for transcriptional activation in transient expression assays. It may be important that this paired arrangement of the GC-box and TE1 element is also seen in the promoter of *testisin*, another testis-specific gene [Hooper et al., 2000]. We have shown that Sp1 and Sp3 are present in testis primary spermatocytes and that they can bind to the GC-box 1 [Wilkerson et al., 2002a]. In addition, overexpression of Sp1 and Sp3 increase transcription in transient expression studies [Wilkerson et al., 2002b]. Therefore, it is possible that nuclear proteins that bind to the TE1 element

and the Sp nuclear proteins that bind to the GC-box associate with each other in activating transcription in spermatocytes. On the other hand, the TE2 sub-element may serve as site for binding a transcriptional repressor in other cell types where the *H1t* gene is not expressed.

One additional experiment emphasized the differences in TE1 and TE2. The rat TE1 but not the TE2 sub-element contains a CpG dinucleotide. The cytosine within this CpG dinucleotide is unmethylated in primary spermatocytes where the promoter is active but it is methylated in liver where the promoter is inactive [Singal et al., 2000]. Therefore, the methylation status of this element correlates with promoter activity as found in many genes. Since methylation of the CpG dinucleotide in GC-box 1 eliminates binding of Sp1 and Sp3, we wanted to determine if methylation of the CpG dinucleotide in TE1 altered protein binding. Figure 7 shows that methylation of this element almost totally blocks protein binding. Furthermore, the methylated DNA probe does not compete with the unmethylated probe for protein binding. It should be mentioned that the CpG dinucleotide is not present in mouse or human TE1 elements.

In summary, TE1 and TE2 have very similar sequences and under conditions used in our EMSA they both appear to bind similar testis nuclear proteins. The protein or protein complex that binds to TE1 has an apparent molecular weight of approximately 160 kDa. However, individual mutation of these sub-elements within that H1t promoter show that there are significant differences in TE1 and TE2. TE1 and GC-box 1 both appear to serve as binding sites for transcriptional activators in transient expression assays. On the other hand TE2 appears to serve as a binding site for a transcriptional repressor in some cell lines. It is not yet possible to determine how these individual sub-elements perform in vivo and thus it will be important to examine the individual TE1 and TE2 mutations in transgenic animals.

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REFERENCES

Bramlage B, Kosciessa U, Doenecke D. 1997. Differential expression of the murine histone genes *H3.3A* and *H3.3B*. Differentiation 62:13–20.

Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC, Nussenzweig A. 2002. Genomic instability in mice lacking histone H2AX. Science 296:922–927.

Clare SE, Fantz DA, Kistler WS, Kistler MK. 1997. The testis-specific histone *H1t* gene is strongly repressed by a G/C-rich region just downstream of the TATA Box. J Biol Chem 272:33028–33036.

Cole KD, Kandala JC, Kistler WS. 1986. Isolation of the gene for the testis-specific H1 histone variant H1t. J Biol Chem 261:7178–7183.

Crane-Robinson C. 1999. How do linker histones mediate differential gene expression? Bioessays 21:367–371.

De S, Brown DT, Lu ZH, Leno GH, Wellman SE, Sittman DB. 2002. Histone H1 variants differentially inhibit DNA replication through an affinity for chromatin mediated by their carboxyl-terminal domains. Gene 292:173–181.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Doenecke D, Alonso A. 1996. Organization and expression of the developmentally regulated *H1(o)* histone gene in vertebrates. Int J Dev Biol 40:395–401.

Drabent B, Doenecke D. 1997. Testis-specific expression of the mouse histone gene *H1t* is regulated by several promoter elements. Biol Reprod 57:1375–1383.

Drabent B, Saftig P, Bode C, Doenecke D. 2000. Spermatogenesis proceeds normally in mice without linker histone H1t. Histochem Cell Biol 113:433–442.

Fan Y, Sirotkin A, Russell RG, Ayala J, Skoultchi AI. 2001. Individual somatic H1 subtypes are dispensable for mouse development even in mice lacking the H1(0) replacement subtype. Mol Cell Biol 21:7933–7943.

Fantz DA, Hatfield WR, Horvath G, Kistler MK, Kistler WS. 2001. Mice with a targeted disruption of the *H1t* gene are fertile and undergo normal changes in structural chromosomal proteins during spermiogenesis. Biol Reprod 64:425–431.

Grimes S, Weisz-Carrington P, Daum H III, Smith J, Green L, Wright K, Stein G, Stein J. 1987. A rat histone *H4* gene closely associated with the testis-specific *H1t* gene. Exp Cell Res 173:534–545.

Grimes SR, Wolfe SA, Anderson JV, Stein GS, Stein JL. 1990. Structural and functional analysis of the rat testis-specific histone *H1t* gene. J Cell Biochem 44:1–17.

Grimes SR, Wolfe SA, Koppel DA. 1992. Temporal correlation between the appearance of testis-specific DNA-binding proteins and the onset of transcription of the testis-specific histone *H1t* gene. Exp Cell Res 201:216–224.

Grimes SR, Wilkerson DC, Noss KR, Wolfe SA. 2002. Transcriptional control of the testis-specific histone *H1t* gene. Gene (In press).

Hayes JJ, Wolffe AP. 1993. Preferential and asymmetric interaction of linker histones with 5S DNA in the nucleosome. Proc Natl Acad Sci USA 90:6415–6419.

Hofmann MC, Hess RA, Goldberg E, Millan JL. 1994. Immortalized germ cells undergo meiosis in vitro. Proc Natl Acad Sci USA 91:5533–5537.

Hooper JD, Bowen N, Marshall H, Cullen LM, Sood R, Daniels R, Stuttgen MA, Normyle JF, Higgs DR, Kastner DL, Ogbourne SM, Pera MF, Jazwinska EC, Antalis TM. 2000. Localization, expression and genomic structure of the gene encoding the human serine protease testisin. Biochim Biophys Acta 1492:63–71.

Johnson PA, Peschon JJ, Yelick PC, Palmiter RD, Hecht NB. 1988. Sequence homologies in the mouse *protamine 1* and *2* genes. Biochim Biophys Acta 950:45–53.

Khadake JR, Rao MR. 1995. DNA- and chromatin-condensing properties of rat testes H1a and H1t compared to those of rat liver H1bdec; H1t is a poor condenser of chromatin. Biochemistry 34:15792–15801.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lin Q, Sirotkin A, Skoultchi AI. 2000. Normal spermatogenesis in mice lacking the testis-specific linker histone H1t. Mol Cell Biol 20:2122–2128.

Morotomi-Yano K, Yano K, Saito H, Sun Z, Iwama A, Miki Y. 2002. Human regulatory factor X 4 (RFX4) is a testis-specific dimeric DNA-binding protein that cooperates with other human RFX members. J Biol Chem 277: 836–842.

Osley MA. 1991. The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 60:827–861.

Peretti M, Khochbin S. 1997. The evolution of the differentiation-specific histone *H1* gene basal promoter. J Mol Evol 44:128–134.

Shires A, Carpenter MP, Chalkley R. 1976. A cysteine-containing H2B-like histone found in mature mammalian testis. J Biol Chem 251:4155–4158.

Singal R, vanWert J, Bashambu M, Wolfe SA, Wilkerson DC, Grimes SR. 2000. Testis-specific histone *H1t* gene is hypermethylated in nongerminal cells in the mouse. Biol Reprod 63:1237–1244.

Stein GS, Stein JL, Chrysogelos S, Dell'Orco R, Hickey E, Kroeger P, Morris T, Nick H, Pauli U, van Wijnen AJ, Weber L, Zambetti G. 1989. Cell cycle dependent human histone genes: Their organization and regulation. In: Hnilica LS, Stein GS, Stein JL, editors. Histones and Other Basic Nuclear Proteins. Boca Raton, Florida: CRC Press, Inc. pp 269–318.

Tanaka M, Hennebold JD, Macfarlane J, Adashi EY. 2001. A mammalian oocyte-specific linker histone gene *H1oo*: Homology with the genes for the oocyte-specific cleavage stage histone (cs-H1) of sea urchin and the B4/H1M histone of the frog. Development 128:655–664.

Thoma F, Koller T, Klug A. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. J Cell Biol 83: 403–427.

Thomas JO. 1999. Histone H1: Location and role. Curr Opin Cell Biol 11:312–317.

Trostle-Weige PK, Meistrich ML, Brock WA, Nishioka K, Bremer JW. 1982. Isolation and characterization of TH2A, a germ cell-specific variant of histone 2A in rat testis. J Biol Chem 257:5560–5567.

Trostle-Weige PK, Meistrich ML, Brock WA, Nishioka K. 1984. Isolation and characterization of TH3, a germ cell-specific variant of histone 3 in rat testis. J Biol Chem 259:8769–8776.

van der Hoorn FA, Tarnasky HA. 1992. Factors involved in regulation of the RT7 promoter in a male germ cell-derived in vitro transcription system. Proc Natl Acad Sci USA 89:703–707.

van der Hoorn FA, Spiegel JE, Maylie-Pfenninger MF, Nordeen SK. 1991. A 43 kD c-mos protein is only expressed before meiosis during rat spermatogenesis. Oncogene 6:929–932.

vanWert JM, Wolfe SA, Grimes SR. 1995. Testis-specific expression of the rat histone *Hlt* gene in transgenic mice. Biochemistry 34:8733–8743.

vanWert JM, Panek HR, Wolfe SA, Grimes SR. 1998. The TE promoter element of the histone *H1t* gene is essential for transcription in transgenic mouse primary spermatocytes. Biol Reprod 59:704–710.

Wilkerson DC, Wolfe SA, Grimes SR. 2002a. The H1t/GC-box and the H1t/TE1 element are essential for promoter activity of the testis-specific histone *H1t* gene. Biol Reprod 67:1157–1164.

Wilkerson DC, Wolfe SA, Grimes SR. 2002b. Sp1 and Sp3 activate the testis-specific histone H1t promoter through the H1t/GC-box. J Cell Biochem 86:716–725.

Wolfe SA, Grimes SR. 1993. Histone H1t: A tissue-specific model used to study transcriptional control and nuclear function during cellular differentiation. J Cell Biochem 53:156–160.

Wolfe SA, Grimes SR. 2002a. Spermatocyte specific nuclear protein binding to the RE element of the testis-specific histone *H1t* gene. Submitted.

Wolfe SA, Grimes SR. 2002b. Transcriptional repression of the testis-specific histone *H1t* gene mediated by an element upstream of the H1/AC box. Submitted.

Wolfe SA, van Wert JM, Grimes SR. 1995. Expression of the testis-specific histone *H1t* gene: Evidence for involvement of multiple cis-acting promoter elements. Biochemistry 34:12461–12469.

Wolfe SA, Mottram PJ, vanWert JM, Grimes SR. 1999. Localization of upstream elements involved in transcriptional regulation of the rat testis-specific histone *H1t* gene in somatic cells. Biol Reprod 61:1005–1011.

Zhao X, Singh B, Batten BE. 1991. The role of c-mos proto-oncoprotein in mammalian meiotic maturation. Oncogene 6:43–49.

Zhou W, Xu J, Goldberg E. 1994. A 60 bp core promoter sequence of murine lactate dehydrogenase C is sufficient to direct testis-specific transcription in vitro. Biol Reprod 51:425–432.