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Sp1 and Sp3 Activate the Testis-Specific Histone H1t Promoter Through the H1t/GC-Box

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Abstract The testis-specific linker histone H1t gene is transcribed exclusively in mid to late pachytene primary spermatocytes. Tissue specific expression of the gene is mediated in large part through elements located within the proximal promoter. Previous work in transgenic animals showed that a unique 40 bp promoter element designated H1t/TE is essential for spermatocyte-specific expression. The H1t/TE element contains a GC-box, which is a perfect consensus binding site for members of the Sp family of transcription factors. We have shown that Sp1 and Sp3 are present in testis cells from 9-day-old and adult rats and in pachytene primary spermatocytes and early spermatids and that they can bind to the H1t/GC-box. Mutagenesis of the GC-box reduced H1t promoter activity. Furthermore, a CpG dinucleotide within the GC-box was totally unmethylated in rat testis primary spermatocytes where the gene is transcribed but it was methylated in liver where the gene is silenced. These previous studies supported the importance of the GC-box and suggested that Sp transcription factors contribute to expression of the H1t gene. In this study, we show that co-transfection of Sp1 and Sp3 expression vectors leads to an upregulation of histone H1t promoter activity in several cell lines including testis GC-2spd cells. However, very low H1t promoter activity is seen in GC-2spd cells grown at 39°C, which correlates with lower levels of Sp1 and Sp3 in these cells grown at this elevated temperature. Upregulation of the H1t promoter by Sp1 and Sp3 was also seen in cotransfected NIH3T3 and C127I cell lines. On the other hand, co-transfection of the Sp1 and Sp3 expression vectors does not lead to upregulation of activity of the cell-cycle dependent histone H1d promoter. J. Cell. Biochem. 86: 716–725, 2002.

Key words: Sp1; Sp3; testis; histone H1t; spermatogenesis; gene regulation; transcription

Histones are involved in packaging DNA and eight H1 subtypes have been described [Khadake and Rao, 1995; Drabent and Doenecke, 1997; Tanaka et al., 2001]. Although the functions of individual H1 subtypes are not known, they show differences in expression during development and differentiation [Doenecke et al., 1988] and they have different DNA binding affinities, with H1t having the weakest binding [Khadake and Rao, 1995]. The linker histones also function in regulating gene transcription [Zlatanova, 1990; Meersseman et al., 1991; Juan et al., 1997; Jedrusik and Schulze, 2001].

The testis-specific histone H1t gene is expressed in mid to late pachytene primary spermatocytes, but the protein is found in both primary spermatocytes and early spermatids [Bucci et al., 1982; Grimes, 1986]. The H1t promoter contains four sequence elements conserved among H1 family members including a TATA-box, a CCAAT-box, a GC-rich region, and an AC-box [Coles and Wells, 1985; Dalton and Wells, 1988; van Wijnen et al., 1988; Osley, 1991; Doenecke et al., 1994; Duncliffe et al., 1995]. A unique element within the H1t promoter likely contributes to cell-specific expression [Grimes et al., 1990; Grimes et al., 1992; Wolfe and Grimes, 1993]. This element, a 40 bp enhancer region designated TE [Wolfe et al., 1995], is

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located within the proximal promoter of the H1t gene [Wolfe et al., 1995; Horvath et al., 2001]. Transgenic mice carrying a rat H1t transgene and promoter with either a wild-type or mutant H1t/TE element showed that this element was essential for tissue-specific expression [van Wert et al., 1995; van Wert et al., 1998]. TE is composed of three sub-elements designated TE2, GC-box, and TE1 [Wilkerson et al., 2002]. The GC-box contains a consensus binding site for members of the Sp family of transcription factors.

The Sp family of transcription factors is composed of several members including Sp1, Sp2, Sp3, and Sp4. All have glutamine rich activation domains located near the N-terminus and a highly conserved DNA binding domain composed of three zinc fingers located near the C-terminus [Suske, 1999]. Sp1, Sp3, and Sp4 bind preferentially to a GC-rich sequence [Hagen et al., 1992; Hagen et al., 1994], but Sp2 prefers a GT-rich sequence [Kingsley and Winoto, 1992]. Sp1 and Sp3 are found in most cell types [Saffer et al., 1991; Hagen et al., 1992], but Sp4 is found predominantly in the brain, epithelial tissues, testis, and developing teeth [Hagen et al., 1992; Supp et al., 1996]. Although Sp1 is a potent activator of many genes [Courey and Tjian, 1988; Courey et al., 1989; Pascal and Tjian, 1991], Sp3 can serve as an activator [Udvadia et al., 1995; Dennig et al., 1996] or repressor [Hagen et al., 1994; Birnbaum et al., 1995]. The molecular weight of Sp1 is 95 kDa [Briggs et al., 1986; Kennett et al., 1997], but electrophoretic bands of higher molecular weight are caused by glycosylation [Jackson and Tjian, 1988] and phosphorylation [Jackson et al., 1990]. In addition, 49 and 80 kDa molecular weight species have been reported [Persengiev et al., 1995]. The 49 kDa species results from alternative splicing and is not present in mouse spermatogenic cells. The origin of the 80 kDa species is unknown. Sp3 consists of three predominant species; the largest is approximately 115 kDa [Kingsley and Winoto, 1992; Kennett et al., 1997] and the two smaller ones are 60–70 kDa. The smaller species are formed from two internal AUG codons [Kennett et al., 1997; Suske, 1999].

Sp1 and Sp3 are present in primary spermatocytes and early spermatids and they bind to the testis-specific histone H1t/GC-box [Wilkerson et al., 2002]. Mutagenesis of the H1t/GC-box eliminates binding of Sp1 and Sp3 and this loss of binding is correlated with a drop in H1t promoter activity in transient expression assays [Wilkerson et al., 2002]. However, it was not clear that Sp1 and Sp3 were directly responsible for this upregulation. In this study, we have extended those studies and have shown that co-transfection of Sp1 or Sp3 expression vectors upregulates H1t promoter activity and that these factors act through the H1t/GC-box. We have also found that Sp1 and Sp3 do not affect the activity of all linker histone genes; the rat H1d promoter is not upregulated by co-transfection of Sp1 or Sp3 expression vectors.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Genosys (The Woodlands, TX). Sprague–Dawley rats used for preparing testis extracts (including centrifugal elutriation to isolate enriched populations of testis cells) were obtained from Harlan Sprague–Dawley (Madison, WI). The 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), and a C127I mouse mammary cell line (ATCC No. CRL1616). The GC-2spd cells were grown at 32 or 39°C as indicated [Hofmann et al., 1994]. Other cell lines were grown at 37°C and all cells were maintained in a 5% CO_2 atmosphere. Sp1 and Sp3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody, α-IgG-HRP, was in an ECL kit (Amersham Life Science, Inc., Arlington Heights, IL). Radioisotopes were purchased from New England Nuclear (Boston, MA).

Electrophoretic Mobility Shift Assays (EMSA)

EMSA were performed as previously described [Grimes et al., 1990; Wolfe and Grimes, 1993]. Synthetic upper (U) and lower (L) oligonucleotide pairs (shown 5′ to 3′) were annealed and used as probes. The "C^M" in the Methyl GC oligonucleotides denotes a methylated cytosine.

- Sp Cons: (U) ATTCGATCGGGGCGGGGC-GAGC; (L) GCTCGCCCCGCCCCGATC-GAAT
- WtGC: (U) TCCCCAGGGGCGGGGGAGG-C; (L) GCCTCCCCCGCCCCTGGGGA
- MutGC: (U) TCCCCAGGAATTCGAAA-GGC; (L) GCCTTTCGAATTCCTGGGGA

- Methyl GC: (U) CCCTTCCCCAGGGGC^{M}G; (L) CCCC^{M}GCCCCTGGG

Oligonucleotides were labeled and EMSA competitions and supershift assays were performed as previously described [Wilkerson et al., 2002]. Unlabeled wild type or mutant GC-box sequences were used as competitors at a 20-fold molar excess. Gels were dried and radioactivity was detected using a Cyclone Phosphorimager (Packard Instrument Company, Meriden, CT).

Western Blots

Nuclear proteins were prepared from adult rat testis, GC-2spd cells, and other cells lines [Dignam et al., 1983] and western blots were performed as described previously [Wilkerson et al., 2002]. Chemiluminescence was visualized by exposure to Kodak XAR film.

Transient Transfection Assays

Co-transfection assays were performed with an H1t promoter driven luciferase expression vector (pGL3-1866) with either a wild type or mutant GC-box. These vectors were constructed as described previously [Wolfe et al., 1999; Wilkerson et al., 2002] and the wild type and mutant GC-box sequences are shown in Figure 1. The H1d promoter construct was described previously [Wolfe et al., 1999]. Cytomegalovirus driven vectors expressing mammalian Sp1 or Sp3 were provided by Guntram Suske [Hagen et al., 1994]. Cells were grown in 60 mm dishes and transfected using 5 μg of lipofectamine (Invitrogen, Carlsbad, CA) and 3 μg of total DNA and included 1 μg of expression vector and 1 μg of CMV-Sp1 or CMV-Sp3 as indicated. The plasmid pUC19 was used to bring the total DNA content to 3 μg per dish. Co-transfections were performed in triplicate to correct for slight variations in transfection efficiency and cell density. Protein concentrations were determined spectrophotometrically as described previously [Wolfe et al., 1999].

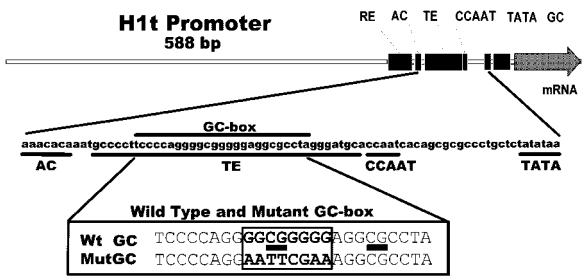

Fig. 1. A map of the testis-specific histone H1t promoter indicating the location of the GC-box within the TE element. The top drawing shows the wild type H1t promoter (designated 1866) used in expression assays in this study. The proximal promoter includes consensus elements RE (repressor element), AC (H1 AC-box), TE (H1t/TE element), CCAAT (the H1 CCAAT-box), TATA (TATA-box), and GC (the H1t GC-rich region downstream from the TATA-box). The mRNA start site is shown and the map extends to the ATG start codon. The sequence includes nucleotides present in the region from the AC-box to the TATA-box and shows the location of the H1t/GC-box within the TE element. The lower box is an enlargement of the wild type GC-box located within the H1t/TE element. The mutant GC-box sequence is shown for comparison.

RESULTS

Sp1 and Sp3 Can Bind to the Wild Type But Not to the Mutant Histone H1t GC-Box

Previous work in our laboratory demonstrated the importance of the H1t/TE element for tissue-specific expression of the histone H1t gene [Wolfe et al., 1995]. Recently, we showed that the GC-box found within the H1t/TE element was important for H1t gene expression [Wilkerson et al., 2002], but we were unable to demonstrate which transcription factors were functioning through this sequence element. Figure 1 shows the location of the GC-box within the H1t/TE element. This figure also shows elements shared with other H1 promoters including the TATA-box, CCAAT-box, and AC-box [Koppel et al., 1994]. The RE element just upstream from the AC-box is a repressor element recently discovered in our laboratory (Wolfe and Grimes, manuscript in preparation) and the GC-rich region downstream from the TATA-box serves as a repressor [Clare et al., 1997a]. Studies presented in this paper were designed to examine factors that bind to the GC-box and to determine whether these factors are involved in regulating H1t gene expression. The GC-box within the H1t/TE element is a consensus binding site for members of the Sp family of transcription factors.

To assay the GC-box for its ability to control H1t promoter activity, we wanted to conduct transient transfection assays in GC-2spd cells, a germinally derived testis cell line, as well as in other cell lines. When GC-2spd cells are grown at 39°C, they display low or undetectable levels of lactate dehydrogenase C_4 isozyme and the cytochrome c_t isoform, two specific markers for meiotic germ cells [Hofmann et al., 1994]. When

grown at 32°C, a population of these cells express these markers. Therefore, we wanted to compare levels and binding activities of Sp1 and Sp3 in GC-2spd cells grown at 32 and 39°C. EMSA supershifts showed that Sp1 and Sp3 in GC-2spd cells could bind to an Sp consensus oligonucleotide using antibodies specific to these factors (Fig. 2). In this binding reaction with nuclear extracts from GC-2spd cells grown at 32°C, lane 1 shows bands corresponding to Sp1 and Sp3 as well as a third higher mobility non-specific band. The addition of polyclonal antibodies specific to Sp1 (lane 2) generates an Sp1 supershifted band (Sp1ss) while the addition of Sp3 antibodies (lane 3) generate an even lower mobility supershifted band (Sp3ss). Interestingly, when extracts from GC-2spd cells grown at 39°C were assayed, very low levels of binding activity were seen other than the non-specific band. When antibodies specific for Sp1 (lane 5) or Sp3 (lane 6) were added, supershifted bands were formed, but they are difficult to see due either to the low level of Sp1 and Sp3 protein or to low binding activity of these factors. Non-specific bands have been reported by others [Baek et al., 2001] and it is possible that the non-specific band observed in these experiments are caused by single stranded DNA binding activity [Harrington et al., 1988].

Before we compared activities of the H1t promoter with a wild type or mutant GC-box in transient transfection assays, it was important to demonstrate that the GC-box mutation eliminated Sp factor binding. EMSA competitions were performed using an oligonucleotide with either a wild type or mutant GC-box and nuclear extracts from GC-2spd cells at 32°C (Fig. 3A) and from adult rat testis (Fig. 3B). GC-2spd extracts grown at 39°C were not assayed due to poor Sp factor binding. Lanes 1–3 in Figure 3A correspond to a wild type GC-box probe incubated with nuclear extracts from GC-2spd cells. Lane 2 includes an excess of unlabeled wild type GC-box (S, Self) added as a specific competitor. Lane 3 includes an excess of unlabeled mutant GC-box competitor (NS, Non-self). Lane 4 corresponds to GC-2spd extracts incubated with the mutant GC-box probe. A comparison of lanes 2 and 3 shows that the mutant GC-box sequence competes poorly, while lane 4 shows that Sp1 and Sp3 binding to the mutant GC-box is eliminated. The same pattern was seen in Figure 3B when testis nuclear extracts were used. The experiment shows that Sp1 and Sp3 binding to the mutant GC-box was eliminated.

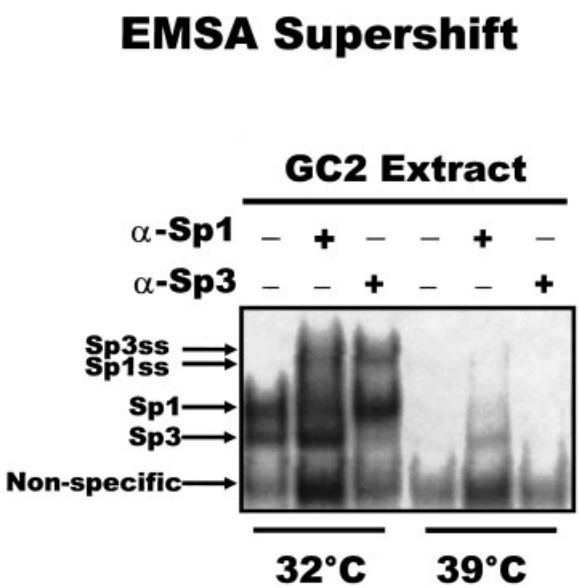

Fig. 2. Sp1 and Sp3 from GC-2spd cells grown at 39°C bind weakly compared to those from cells grown at 32°C. This EMSA supershift assay compares binding of nuclear proteins from GC-2spd cells grown at 32 and 39°C to an Sp consensus probe (Santa Cruz Biotechnology). **Lane 1** shows strong binding of proteins from cells grown at 32°C to the probe and the positions of bands representing binding of Sp1 and Sp3 are shown. Supershifted bands (Sp1ss and Sp3ss) form when antibodies against Sp1 (**lane 2**) and Sp3 (**lane 3**) are added to the binding reaction. Proteins from GC-2spd cells grown at 39°C bind weakly as shown in **lanes 4–6**. Only faint bands are produced using the same quantity of proteins as used from cells grown at the lower temperature. This may be due either to low levels of Sp1 and Sp3 or to low binding activities.

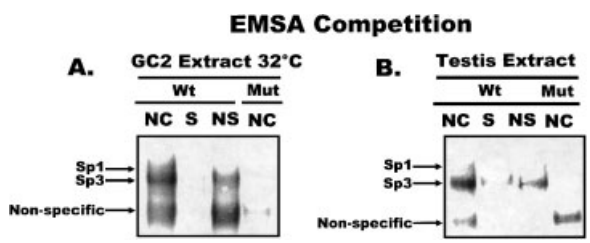

Fig. 3. Mutation of the GC-box disrupts binding of Sp1 and Sp3. Electrophoretic mobility shift assays (EMSA) were conducted using the mutant (Mut GC) and wild type (Wt GC) GC-box probes shown in Figure 1 and nuclear extracts derived from GC-2spd cells grown at 32°C (**panel A**) and from a testis cell preparation (**panel B**). Lanes are labeled NC (no competitor), S (self competitor), and NS (non-self competitor). Competitions were performed with a 20-fold molar excess of unlabeled double-stranded wild type or mutant DNA fragments. Note that the mutant GC-box (Mut GC) fails to bind either Sp1 or Sp3.

Western Blots Show That Sp1 and Sp3 Levels Vary in Testis GC-2spd Cells at Different Temperatures

After observing low Sp binding activity in GC-2spd cells grown at 39°C versus 32°C, we wanted to compare the levels of Sp1 and Sp3 in these cells grown at these two temperatures. Consistent with the results from the EMSA supershifts, our Western blots show that GC-2spd cells grown at 39°C have lower levels of both Sp1 and Sp3 (Fig. 4). The 105, 95, and 60 kDa bands of Sp1 are lower at 39°C than at 32°C and the 115, 70, and 80 kDa bands of Sp3 are lower at 39°C. This figure also shows that Sp1 and Sp3 are present in testis and C127I nuclear extracts.

Over-Expression of Sp1 and Sp3 Enhances H1t But Not H1d Promoter Activity

We next wanted to determine the effect of co-transfection of Sp1 or Sp3 expression vectors on the activity of the H1t promoter. We compared the germinal GC-2spd cell line grown at either 32 or 39°C. We also assayed NIH3T3 cells, a mouse fibroblast cell line, and C127I cells, a mouse mammary cell line. To examine transcription, we performed transient co-transfection assays using H1t promoter driven luciferase expression vectors containing either a wild type (WtGC) or mutant GC-box (MutGC) and cytomegalovirus driven vectors which express mammalian Sp1 or Sp3 (designated CMV-Sp1 and CMV-Sp3). The results of these assays are shown in Figure 5A–D. GC-2spd cells grown at 32°C and transfected with the H1t promoter vector alone have higher expression levels than the other cell lines assayed. When these cells are co-transfected with both the wild type H1t promoter driven luciferase vector (WtGC) and the CMV-Sp1 there is a 6.4-fold increase in expression of the H1t promoter (Fig. 5A). If the H1t promoted luciferase vector containing the GC-box mutation (MutGC) is co-transfected with CMV-Sp1 the activity increases only 2.7-fold. Co-transfecting the wild type H1t promoted luciferase vector with CMV-Sp3 results in a 2.4-fold increase in activity compared to the 6.4-fold increase with co-transfection with Sp1. When these vectors were co-transfected into GC-2spd cells grown at 39°C, H1t promoter activity was very low compared to GC-2spd cells at 32°C (Fig. 5B). In addition, the effects of over-expression of Sp1 or Sp3 were less pronounced.

H1t promoter activity was relatively low in C127I and NIH3T3 cells compared to activity in GC-2spd cells grown and 32°C, but not as low as

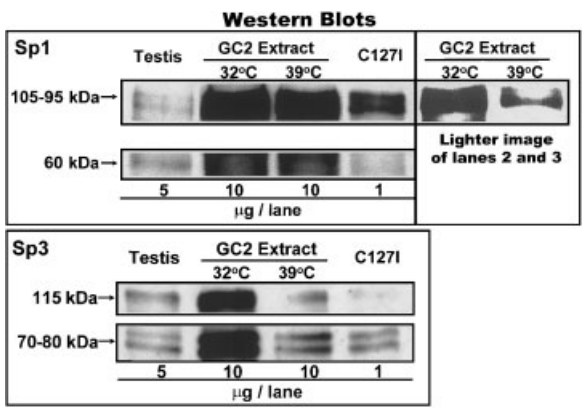

Fig. 4. Sp1 and Sp3 levels are lower in GC-2spd cells grown at 39°C than in GC-2spd cells grown at 32°C. Proteins from the different sources were loaded in amounts indicated and separated by SDS–PAGE. Lanes from left to right contained nuclear proteins from the unfractionated rat testis cells, the testis GC-2spd cell line cultured at 32 and at 39°C, and a C127I cell line. After electrophoresis and electroblotting of the proteins, membranes were probed with either anti-Sp1 antibodies or anti-Sp3 to detect proteins. The 60, 95, and 105 kDa bands of Sp1 and the 60, 70, and 115 kDa bands of Sp3 are indicated. Note that the levels of Sp1 and Sp3 are lower in GC-2spd cells grown at 39°C than in GC-2spd cells grown at 32°C.

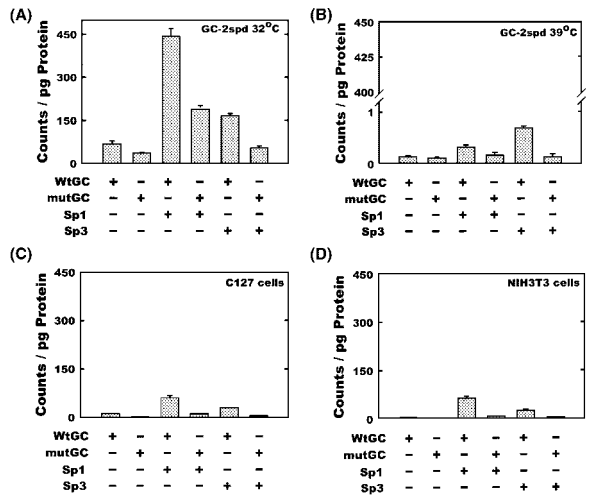

Fig. 5. Overexpression of Sp1 and Sp3 upregulates activity of the H1t promoter. Cells were transfected with the expression vectors shown below the graph. These vectors include the wild type testis-specific histone H1t promoter vector (Wt GC), the mutant GC-box (MutGC), the Sp1 expression vector (Sp1), and the Sp3 expression vectors (Sp3). The WtGC and MutGC vectors were transfected either alone or in combination with Sp1 or Sp3. The wild type and mutant H1t vectors have the same upstream and downstream fusion points in the pGL3B expression vector. The columns represent the means of triplicate samples and the error bars indicate the standard error of the mean.

in GC-2spd cells at 39°C. Co-transfection of Sp1 or Sp3 with the wild type H1t promoted luciferase vector within these cell lines also activates the H1t promoter as seen in Figure 5C,D. There were no synergistic effects when both CMV-Sp1 and CMV-Sp3 were co-transfected with the H1t-promoted luciferase vectors in any of the cell lines (data not shown).

Although Sp1 and Sp3 activate the H1t promoter, this is not a general phenomenon that occurs with all H1 promoters. When we tested the rat cell-cycle dependent H1d promoter in transient expression assays, neither Sp1 nor Sp3 expression upregulated H1d promoter activity (Fig. 6). This is interesting because the region between the AC-box and CCAAT-box of the H1d promoter, like the H1t promoter, is GC-rich [Koppel et al., 1994].

Methylation of the H1t/GC-Box Blocks Binding of Sp1 and Sp3

Previous work in our lab revealed that the GC-box within the H1t/TE element is unmethylated in pachytene primary spermatocytes where the gene is actively transcribed, but it is methylated in liver where the gene is silenced [Singal et al., 2000]. Methylation of the H1t promoter decreases expression of a luciferase reporter vector [Singal et al., 2000]. However, it was not clear that methylation altered binding of Sp to the H1t/GC-box. In the light of our findings in this study that Sp1 and Sp3 alter H1t promoter activity, we wanted to determine whether methylation of this CpG dinucleotide alters Sp1 and Sp3 binding. EMSA competitions were performed using methylated and unmethylated H1t/GC-box oligonucleotides with nuclear extracts prepared from GC-2spd cells (Fig. 7). Lane 1 (left panel) shows binding to an unmethylated GC-box probe using an extract from GC-2spd cells grown at 32°C with no competitor. Lanes 2 and 3 include an excess of unlabeled unmethylated GC-box DNA (S, Self) or methylated H1t/GC-box DNA (NS, Non-self), respectively. The right panel shows GC-2spd cell nuclear extracts incubated with the methylated H1t/GC-box probe in the absence of specific competitor (lane 4) or presence of methylated (lanes 5) or unmethylated (lane 6) H1t/GC-box competitor. Sp1 and Sp3 binding is eliminated when the GC-box is methylated (lanes 3–6). The methylated probe does not compete binding of Sp to the wild-type GC-box probe.

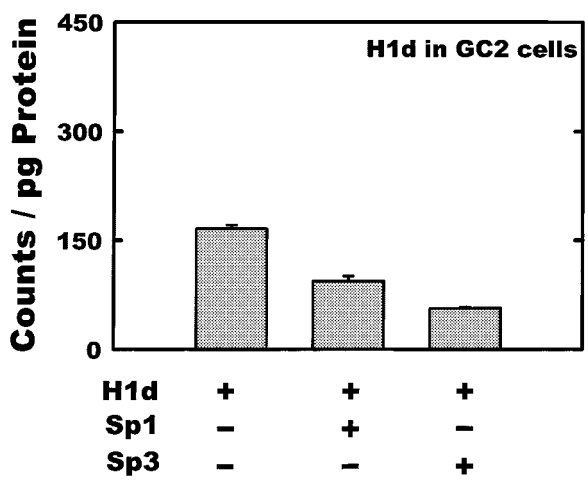

Fig. 6. The H1d promoter is not upregulated by co-transfection of Sp1 and Sp3 expression vectors. The same type of transient expression assay shown in Figure 5 was conducted, but using a rat H1d rather than H1t promoted expression vector. Overexpression of either Sp1 or Sp3 did not enhance the activity of the H1d promoter.

DISCUSSION

The H1t promoter has a GC-box located within the H1t/TE element, a region important for tissue-specific expression of the gene [van Wert et al., 1995; Wolfe et al., 1995; Clare et al., 1997a,b; van Wert et al., 1998]. Previously, we detected Sp1 in nuclear extracts from testis, from enriched populations of primary spermatocytes and early spermatids, and from several cells lines [Wilkerson et al., 2002]. We were able to show that Sp1 and Sp3 from these sources could bind to an Sp consensus as well as to the H1t/GC-box probe. Protein binding to a mutant

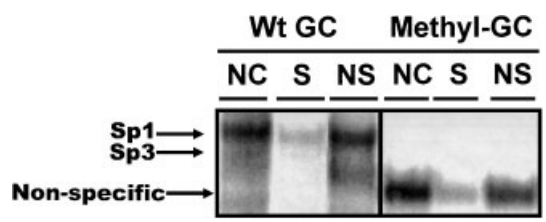

Fig. 7. Methylation of the CpG dinucleotide within the GC-box disrupts binding of Sp1 and Sp3. The wild type GC-box (Wt GC) and methylated GC-box (Methyl-GC) probes (described in the Methods) were used in an EMSA competition assay with nuclear proteins from GC-2spd cells grown at 32°C. The Lanes are labeled NC (no competitor), S (self competitor), and NS (non-self competitor). Competitions were performed with a 20-fold molar excess of unlabeled double-stranded wild type or mutant DNA fragments. Note that the methylated GC-box fails to bind either Sp1 or Sp3. Only the higher mobility non-specific band is formed.

GC-box probe was eliminated and loss of binding correlated with the loss of activity in transient expression assays [Wilkerson et al., 2002].

Thus, Sp1 and Sp3 are present in the testis and in the cell lines being examined in our studies and mutation of the H1t/GC-box eliminates protein binding and reduces H1t promoter activity. However, it was not shown that Sp1 and/or Sp3 were the transcription factors that actually caused activation of the H1t promoter. Thus, we extended our studies to examine the effect of Sp1 and Sp3 on the H1t promoter. We confirm that Sp1 and Sp3 are present in the testis derived GC-2spd cell line and in the other cell lines used in this study and that they bind to the Sp-consensus sequence from Santa Cruz as well as to the H1t/GC-box (Figs. 2 and 3). We also show by EMSA competition assays that the H1t/GC-box mutant eliminates binding of Sp proteins to the GC-box (Fig. 3). The mutant shown in Figure 1 was used in the EMSA assays as well as in the transcription assays presented in this study.

We found a low abundance of Sp1 and Sp3 binding activity in GC-2spd cells at 39°C. This cell line was established by cotransfecting with the simian virus 40 large tumor antigen (LTAg) and with a temperature-sensitive mutant of the mouse p53 gene [Hofmann et al., 1994]. When GC-2spd cells are grown at 39°C, p53 is inactivated, allowing the LTAg to retain its immortalizing ability. At the permissive temperature of 32°C, excess p53 neutralizes the proliferative effect of the viral protein. In this study, we found abundant Sp1 and Sp3 binding activity in the cells grown at 32°C, but at 39°C the binding activity was low (Fig. 2). It is possible that the proteins are present, but that their binding activity drops at the higher temperature or it is possible that the protein level drops. To distinguish between these two possibilities, we conducted Western blots to detect Sp1 and Sp3 in this cell line. The reduced activity appears to be due in part to a drop in the levels of both Sp1 and Sp3 in the GC-2spd cells at the higher temperature (Fig. 4). We can not rule out the possibility that posttranslational modifications of Sp1 or Sp3 contribute to the reduced activities.

Another finding in this study is that Sp1 and Sp3 activate the H1t promoter. To show this activation, several different cells lines were transiently transfected with an H1t promoter-luciferase expression vector containing either a wild type or mutant GC-box. Some of the transfected cells were cotransfected with either the Sp1 or Sp3 expression vector. There was activation of the H1t promoter in GC-2spd cells grown at 32°C by co-transfection of the Sp1 expression vector. Sp3 also activated the promoter in these cells but to a lower degree (Fig. 5A). Activation by Sp1 and Sp3 was reduced or eliminated when the H1t/GC-box mutant expression vector was used. Sp1 and Sp3 may also act indirectly on other factors important for histone H1 transcription. This can be seen with activation of the H1t promoter by Sp1 and Sp3, even though it has a mutation in the GC-box that weakens or prevents binding of Sp1 and Sp3. However, when we tested the H1d promoter, there was no apparent activation by either Sp1 or Sp3 (Fig. 6) and the H1d promoter, like the H1t promoter, has a TATA-box, a CCAAT-box, a GC-rich region, and an AC-box.

When GC-2spd cells were grown at the non-permissive temperature of 39°C, H1t promoter activity was low compared to cells grown at the permissive temperature of 32°C (Fig. 5A,B). Neither Sp1 nor Sp3 activated the promoter to a significant degree in GC-2spd cells grown at 39°C. This lower H1t promoter activity in cells grown at the non-permissive temperature correlates with the lower level and lower binding activity of Sp1 and Sp3 in these cells. Furthermore, we found that co-transfection of Sp1 and Sp3 into these cells fails to activate H1t promoter activity to a significant degree. It is possible that Sp1 and Sp3 are expressed from these vectors at the non-permissive temperature, but that they become modified so that they lose binding activity or they become compartmentalized so that they are not available for activation of the H1t promoter. Alternatively, essential transcriptional activators may be present at low levels in cells grown at 39°C or they may have reduced activities. It is also possible that a repressor is present in cells grown at the higher temperature. It is interesting that the activity of the H1t promoter is low in the C127I and NIH3T3 cell lines (Fig. 5C,D). Activities are not as low as in GC-2spd cells grown at the non-permissive temperature, but they are significantly lower than in GC-2spd cells grown at 32°C.

As mentioned above, we also examined the activity of the cell cycle dependent H1d promoter in GC-2spd cells grown at 32°C. Because the wild type H1d promoter is active in this cell line,

we expected to see enhanced activity from the H1d promoter as with the H1t promoter during coexpression of either Sp1 or Sp3. However, it was not upregulated in the co-transfection assay (Fig. 6). The rat H1d promoter, like the H1t promoter, contains a GC-rich sequence located between the AC-box and CCAAT-box, but may not contain a classical GC-box [Koppel et al., 1994]. Thus, Sp1 and Sp3 do not appear to activate all histone H1 promoters even though most have a GC-rich sequence located between the AC-box and CCAAT-box. The differences in response of the testis-specific H1t and the somatic H1d promoters to Sp transcription factors is interesting and may reflect differences that are important for tissue-specificity.

Another factor that may be related to tissue-specific expression is the methylation status of the H1t promoter. We have demonstrated that there is hypomethylation of CpG dinucleotides located within the H1t proximal promoter in rat primary spermatocytes where the gene is expressed and hypermethylation of the same CpG dinucleotides in rat liver where the gene is silenced [Singal et al., 2000]. Two of these CpG dinucleotides are located within the 40 bp H1t/TE element as indicated by the underlined CpG dinucleotides in Figure 1. Our previous results suggested that methylation of this promoter region may contribute to silencing of the gene in non-expressing cells. Because we saw a positive effect of Sp1 and Sp3 on H1t promoter activity, we decided to see if methylation of the CpG dinucleotide within the GC-box altered binding of Sp1 and Sp3 to the GC-box. We found that methylation of the GC-box eliminated binding of Sp1 and Sp3 under our test conditions. Binding of Sp1 and Sp3 is weakened if not eliminated by methylation (Fig. 7). It is possible that methylation of this CpG dinucleotide blocks Sp binding in vivo and is responsible in part for silencing of the gene in non-expressing cells.

We have shown that Sp1 and Sp3 from adult testis, primary spermatocytes, and early spermatids bind to the H1t/GC-box [Wilkerson et al., 2002]. There are also other GC-box and GT-box binding proteins such as BTEB1 (basic transcription element binding protein) [Imataka et al., 1992] and TIEG1 and TIEG2 (TGFβ-inducible early protein genes I and 2) [Subramaniam et al., 1995; Cook et al., 1998] that share characteristics with Sp transcription factors and have nearly identical binding properties [Sogawa et al., 1993; Cook et al., 1998]. Germinal cell-specific Sp isoforms or similar Sp-like proteins may function during spermatogenesis. To determine whether such proteins regulate H1t or other gene transcription during spermatogenesis requires further experimentation.

Although a cell line derived from germinal cells was used for these transient expression assays, there are important differences in this cell line and normal germinal cells. For example, we have not been able to see H1t gene expression in the GC-2spd line, although basal expression below the level of detection by northern blots is possible. Nevertheless, this GC-2spd cell line and several non-germinal cell lines have proven to be useful in transient expression assays to examine H1t promoter elements that enhance or silence transcription.

In summary, Sp1 and Sp3 are present in primary spermatocytes as well as other germinal cell types and non-germinal cells. The levels and binding activities of Sp1 and Sp3 are high in GC-2spd cells grown at 32°C where activity of the H1t promoter-luciferase expression vector is high, but the levels and binding activities are low in GC-2spd cells grown at 39°C where activity of the H1t promoter is low. Co-transfection of Sp1 and Sp3 expression vectors in several cell lines leads to activation of the H1t promoter and it appears that this is due, in part, to a direct effect of Sp1 and Sp3 on the H1t/GC-box.

It is possible that ubiquitous transcription factors such as Sp1 and Sp3 contribute to transcriptional regulation of the H1t gene, and it seems very likely that other factors such as the TE binding proteins are involved in directing tissue-specific transcription of this gene. Proteins that bind to other proximal and distal elements also contribute to tissue-specific expression. Proximal elements include the GC-rich region downstream from the TATA-box [Clare et al., 1997a] and the RE element upstream from the AC-box (Wolfe and Grimes, in preparation) that contribute to silencing the H1t gene in non-germinal cells (Fig. 1). Distal elements also have been described that contribute to regulating transcription of the H1t gene [Drabent and Doenecke, 1997; Wolfe et al., 1999].

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REFERENCES

Baek SJ, Horowitz JM, Eling TE. 2001. Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3. J Biol Chem 276:33384–33392.

Birnbaum MJ, van Wijnen AJ, Odgren PR, Last TJ, Suske G, Stein GS, Stein JL. 1995. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. Biochemistry 34:16503–16508.

Briggs MR, Kadonaga JT, Bell SP, Tjian R. 1986. Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. Science 234:47–52.

Bucci LR, Brock WA, Meistrich ML. 1982. Distribution and synthesis of histone 1 subfractions during spermatogenesis in the rat. Exp Cell Res 140:111–118.

Clare SE, Fantz DA, Kistler WS, Kistler MK. 1997a. 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.

Clare SE, Hatfield WR, Fantz DA, Kistler WS, Kistler MK. 1997b. Characterization of the promoter region of the rat testis-specific histone H1t gene. Biol Reprod 56:73–82.

Coles LS, Wells JR. 1985. An H1 histone gene-specific 5′ element and evolution of H1 and H5 genes. Nucleic Acids Res 13:585–594.

Cook T, Gebelein B, Mesa K, Mladek A, Urrutia R. 1998. Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-beta-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. J Biol Chem 273:25929–25936.

Courey AJ, Tjian R. 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55:887–898.

Courey AJ, Holtzman DA, Jackson SP, Tjian R. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 59:827–836.

Dalton S, Wells JR. 1988. A gene-specific promoter element is required for optimal expression of the histone H1 gene in S-phase. EMBO J 7:49–56.

Dennig J, Beato M, Suske G. 1996. An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. EMBO J 15:5659–5667.

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, Tonjes R, Kress H. 1988. The H1 and core histone subtypes: Differential gene expression and varied primary structures. Adv Enzyme Regul 27:107–120.

Doenecke D, Albig W, Bouterfa H, Drabent B. 1994. Organization and expression of H1 histone and H1 replacement histone genes. J Cell Biochem 54:423–431.

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.

Duncliffe KN, Rondahl ME, Wells JR. 1995. A H1 histone gene-specific AC-box-related element influences transcription from a major chicken H1 promoter. Gene 163:227–232.

Grimes SR, Jr. 1986. Nuclear proteins in spermatogenesis. Comp Biochem Physiol B 83:495–500.

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. Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone H1t gene. Arch Biochem Biophys 296:402–409.

Hagen G, Muller S, Beato M, Suske G. 1992. Cloning by recognition site screening of two novel GT box binding proteins: A family of Sp1 related genes. Nucleic Acids Res 20:5519–5525.

Hagen G, Muller S, Beato M, Suske G. 1994. Sp1-mediated transcriptional activation is repressed by Sp3. EMBO J 13:3843–3851.

Harrington MA, Jones PA, Imagawa M, Karin M. 1988. Cytosine methylation does not affect binding of transcription factor Sp1. Proc Natl Acad Sci USA 85:2066–2070.

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

Horvath GC, Clare SE, Kistler MK, Kistler WS. 2001. Characterization of the H1t promoter: Role of conserved histone 1 AC and TG elements and dominance of the cap-proximal silencer. Biol Reprod 65:1074–1081.

Imataka H, Sogawa K, Yasumoto K, Kikuchi Y, Sasano K, Kobayashi A, Hayami M. 1992. Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene. EMBO J 11:3663–3671.

Jackson SP, Tjian R. 1988. *O*-glycosylation of eukaryotic transcription factors: Implications for mechanisms of transcriptional regulation. Cell 55:125–133.

Jackson SP, MacDonald JJ, Lees-Miller S, Tjian R. 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell 63:155–165.

Jedrusik MA, Schulze E. 2001. A single histone H1 isoform (H1.1) is essential for chromatin silencing and germline development in Caenorhabditis elegans. Development 128:1069–1080.

Juan LJ, Utley RT, Vignali M, Bohm L, Workman JL. 1997. H1-mediated repression of transcription factor binding to a stably positioned nucleosome. J Biol Chem 272:3635–3640.

Kennett SB, Udvadia AJ, Horowitz JM. 1997. Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. Nucleic Acids Res 25:3110–3117.

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.

Kingsley C, Winoto A. 1992. Cloning of GT box-binding proteins: A novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol 12:4251–4261.

Koppel DA, Wolfe SA, Fogelfeld LA, Merchant PS, Prouty L, Grimes SR. 1994. Primate testicular histone H1t genes are highly conserved and the human H1t gene is located on chromosome 6. J Cell Biochem 54:219–230.

Meersseman G, Pennings S, Bradbury EM. 1991. Chromatosome positioning on assembled long chromatin. Linker histones affect nucleosome placement on 5 S rDNA. J Mol Biol 220:89–100.

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

Pascal E, Tjian R. 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev 5:1646–1656.

Persengiev SP, Saffer JD, Kilpatrick DL. 1995. An alternatively spliced form of the transcription factor Sp1 containing only a single glutamine-rich transactivation domain. Proc Natl Acad Sci USA 92:9107–9111.

Saffer JD, Jackson SP, Annarella MB. 1991. Developmental expression of Sp1 in the mouse. Mol Cell Biol 11: 2189–2199.

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.

Sogawa K, Kikuchi Y, Imataka H, Fujii-Kuriyama Y. 1993. Comparison of DNA-binding properties between BTEB and Sp1. J Biochem (Tokyo) 114:605–609.

Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC. 1995. Identification of a novel TGF-beta-regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucleic Acids Res 23: 4907–4912.

Supp DM, Witte DP, Branford WW, Smith EP, Potter SS. 1996. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. Dev Biol 176:284–299.

Suske G. 1999. The Sp-family of transcription factors. Gene 238:291–300.

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.

Udvadia AJ, Templeton DJ, Horowitz JM. 1995. Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: Superactivation by Rb requires amino acids necessary for growth suppression. Proc Natl Acad Sci USA 92:3953–3957.

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

Van Wert 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.

van Wijnen AJ, Wright KL, Massung RF, Gerretsen M, Stein JL, Stein GS. 1988. Two target sites for protein binding in the promoter region of a cell cycle regulated human H1 histone gene. Nucleic Acids Res 16:571–592.

Wilkerson DC, Wolfe SA, Grimes SR. 2002. The H1t/GC-box and the H1t/TE1 element are essential for promoter activity of the testis-specific histone H1t gene. Biol Reprod (in press).

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, 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.

Zlatanova J. 1990. Histone H1 and the regulation of transcription of eukaryotic genes. Trends Biochem Sci 15:273–276.