BIOLOGY OF REPRODUCTION **68,** 2267–2273 (2003)
Published online before print 22 January 2003.
DOI 10.1095/biolreprod.102.014084

Specific Binding of Nuclear Proteins to a Bifunctional Promoter Element Upstream of the H1/AC Box of the Testis-Specific Histone H1t Gene[1]

Steven A. Wolfe and Sidney R. Grimes[2]

Research Service (151), Overton Brooks Veterans Administration Medical Center, Shreveport, Louisiana 71101-4295
Department of Biochemistry and Molecular Biology, Louisiana State University Health Science Center, Shreveport, Louisiana 71130-3932

ABSTRACT

The testis-specific histone H1t gene is transcribed exclusively in primary spermatocytes during spermatogenesis. Studies with transgenic mice show that 141 base pairs (bp) of the H1t proximal promoter accompanied with 800 bp of downstream sequence are sufficient for tissue-specific transcription. Nuclear proteins from testis and pachytene spermatocytes produce footprints spanning the region covering the repressor element (RE) from 100 to 125 nucleotides upstream of the H1t transcriptional initiation site. Only testis nuclear proteins bind to the 5′-end of the element and produce a unique, low-mobility complex in electrophoretic mobility shift assays. This testis complex is distinct from the complex formed by a repressor protein derived from several cell lines that binds to the 3′-end of the element. The testis complex band is formed when using nuclear proteins from primary spermatocytes, where the H1t gene is transcribed, and band intensity drops 70%–80% when using nuclear proteins from early spermatids, where H1t gene transcription ceases. Protein-DNA cross-linking experiments using testis nuclear proteins produce electrophoretic bands of 59, 52, and 50 kDa on SDS/PAGE gels.

developmental biology, gene regulation, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is a complex process in which spermatogonial stem cells progress through several developmental steps to become spermatozoa. The changes in development are dictated by specific changes in gene expression. During progression from spermatogonial stem cells through the pachytene primary spermatocyte stage of spermatogenesis in mammals, linker and core histone subtypes change dramatically [1]. Somatic linker histone H1 variants (H1a, H1b, H1c, H1d, and H1e) are expressed in early, mitotically dividing spermatogonial stem cells [1]. During the pachytene primary spermatocyte stage of spermatogenesis, the complement of histone H1 subtypes changes with the onset of synthesis of the testis-specific histone H1t, accompanied by reduction in several somatic H1 subtypes. H1t is the major constituent of the H1 pool (~60%) in primary spermatocytes, with H1a and H1c contributing approximately 30% and 10%, respectively [1]. Histone H1 synthesis is undetectable following the primary spermatocyte stage, but H1 and core histones are maintained through two meiotic divisions. The histones are replaced by transition proteins during the midspermatid stage of spermatogenesis. [2].

Histone H1t expression is regulated by stringent transcriptional control. In nongerminal cells, H1t transcription is repressed with contributions from silencing elements found upstream [3] and within the H1t proximal promoter [4] as well as by methylation at specific sites within its proximal promoter [5]. Although the proximal promoter contains all of the consensus elements common to the somatic promoters, including an H1/AC box, a GC box, an H1/CCAAT box, and a TATA box, transcription of the H1t gene is not detected in cells other than testis pachytene primary spermatocytes in animal studies [6–8]. Reporter gene expression, promoted by the testis-specific histone H1t promoter, occurs at a low level in somatic cell lines following transient transfections [8–10].

H1t synthesis is dependent on transcriptional upregulation of the gene in pachytene primary spermatocytes [7, 8]. Regulatory sequence elements that are necessary and sufficient for spermatocyte-specific expression of histone H1t are present within 2.5 kilobases (kb) of upstream DNA and 3.8 kb of downstream DNA flanking the H1t transcriptional start site [11]. The promoter, truncated to within 141 base pairs (bp) of the transcriptional start site in constructs containing the native gene and 800 bp of downstream sequence, maintains testis-specific activity in transgenic mice [12]. Previous studies revealed the importance of the proximal promoter and of testis element (TE) located between the H1/AC box and the H1/CCAAT box [13, 14]. When TE is replaced by a heterologous DNA sequence, expression of the mutant rat H1t gene is disrupted in transgenic mice [11, 15].

In the present study, we examine a repressor element (RE), which is located upstream from the H1/AC box. The DNase I footprinting and electrophoretic mobility shift assays (EMSAs) show that nuclear proteins from primary spermatocytes bind to the 5′-end of the element to generate a unique protein-DNA complex. This testis complex is formed by proteins from the cells in which the H1t gene is transcribed. The level of the testis complex drops in nuclear extracts from early spermatids, which correlates with downregulation of the gene. The EMSA competitions were used to delineate regions within the 5′-end of this bifunctional RE that is responsible for specific nuclear protein binding. Ultraviolet (UV) cross-linking of nuclear proteins bound to the RE revealed three protein/DNA electrophoretic bands with relative molecular weights of 59, 52, and 50 kDa.

[1]Supported by a merit review grant from the Department of Veterans Affairs and by NIH Grant HD29381 (S.R.G.).
[2]Correspondence: Sidney R. Grimes, Medical Research Service (151), Overton Brooks Veterans Administration Medical Center, 510 E. Stoner Ave., Shreveport, Louisiana 71101-4295. FAX: 318 429 5733;
e-mail: srgrimes@prysm.net

Received: 9 December 2002.
First decision: 22 December 2002.
Accepted: 21 January 2003.

ISSN: 0006-3363. http://www.biolreprod.org

These binding proteins are candidates for histone H1t transcriptional activators.

MATERIALS AND METHODS

Animal Studies

Animal studies were conducted in an AAALAC-accredited facility in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Reagents and Supplies

Radiochemicals were purchased from New England Nuclear (Boston, MA). X-OMAT XAR-5 x-ray film (Eastman Kodak, Rochester, NY) was purchased from Sigma (St. Louis, MO). Deoxynucleotides and Ampli-Taq for polymerase chain reaction (PCR) were purchased from Perkin-Elmer (Foster City, CA). The DNA sequence used for site determination in DNase I footprinting was performed using ^{32}P-labeled sequencing primers with the fmol DNA Cycle Sequencing System from Promega (Madison, WI). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). Oligonucleotides were purchased from Genosys (Woodlands, TX).

Oligonucleotides

Primers for PCR amplification included a primer (5′-CAGAGAGCACACATGCAA-3′) located from 126 to 143 bp upstream from the H1t transcriptional start site and a primer (5′-AAGTACAGGAGAGCGAAC-3′) located from 28 to 8 bp downstream from the H1t transcriptional initiation point. Before amplification, the 5′-terminus of the upstream primer was labeled using [γ^{32}P]ATP with T4 polynucleotide kinase [16]. Following PCR amplification (30 cycles of 94°C for 2 min, 54°C for 1 min, and 72°C for 1 min) with plasmid pPS3 as template [8], the 174-bp product was isolated from a 0.7% low-melting agarose gel [17] before use in footprinting. Double-stranded fragments for EMSA probes and for competitions were prepared from oligonucleotides based on sequences shown in Figure 1.

In Vitro DNase I Footprinting

The DNase I footprinting reactions were performed using nuclear extracts from unfractionated testis cells and enriched populations of pachytene primary spermatocytes. The DNase I digestions were conducted in a total volume of 40 μl as previously described [3]. Following DNase I digestion, the samples were extracted twice with phenol:chloroform (1:1) and DNA precipitated from the aqueous phase with 2.5 volumes of ethanol at −20°C for 1 h. The DNA was recovered by centrifugation for 20 min at 16 000 × *g* at 4°C. Ethanol was removed and the DNA pellet was dried partially and dissolved in 3 μl of loading solution before electrophoresis [3]. Samples were heated at 90°C for 3 min before loading onto a denaturing polyacrylamide sequencing gel (6% polyacrylamide [19:1, acrylamide: bisacrylamide] containing 7 M urea and 1× TBE [0.09 M Tris-Borate, 0.002 M EDTA, pH 8.3]). After electrophoresis at 1200 mV for 120–165 min, the gels were soaked for 20 min in a solution containing 10% methanol and 10% acetic acid before transfer to Whatman 3MM paper (Whatman Laboratory Products, Clifton, NJ) and drying. Dried gels were exposed to Kodak X-OMATIC XAR 5 film using a Sigma Lite Plus intensifying screen (Sigma Chemical Company, St. Louis, MO).

Preparation of Nuclear Extracts and EMSAs

Nuclear protein extracts were prepared from crude nuclei derived from several rat organs (testis, liver, brain, spleen, and kidney) as previously described [13, 14, 18]. Nuclear extracts were also prepared from enriched populations of rat pachytene spermatocytes and early spermatids obtained by centrifugal elutriation as previously described [8]. Nuclear extracts enriched for the testis-binding proteins were obtained by ion-exchange chromatography. Briefly, 1 ml of testis nuclear extract was fractionated by binding to 0.5 ml of phosphocellulose P11 in a batch procedure in a 1.5-ml microfuge tube at 4°C. After addition of testis extract, the phosphocellulose was washed five times (0.5-ml washes) with Dignam Buffer D containing 0 M KCl [18]. The phosphocellulose was then washed with Buffer D containing 0.3 M KCl to elute proteins. The 0.3 M KCl fraction was dialyzed against Buffer D containing 0 M KCl to obtain an extract that is enriched for proteins that binds to the RE. Binding of nuclear proteins to labeled DNA probes for EMSA was performed on ice as described previously [13] in a total volume of 20 μl with 500 ng of the nonspecific competitor poly dG-dC added to each assay. When unlabeled specific competitor DNA was used, it was added 15 min before binding of nuclear proteins to labeled probe. Following binding to radiolabeled probe, samples were electrophoresed through 4% polyacrylamide gels (60:1, acrylamide:bisacrylamide) using low-ionic-strength buffer containing 6 mM Tris-HCl (pH 7.9), 3.3 mM sodium acetate, and 1 mM EDTA with buffer recirculation. After electrophoresis, gels were dried, exposed to a SR phosphor screen, and processed using a Cyclone PhosphorImager (both from Packard Instrument Company, Meriden, CT).

Cross-Linking of Protein and DNA

Proteins bound to radiolabeled DNA in a 40-μl EMSA reaction (twice the standard EMSA reaction) were cross-linked to the DNA by three pulses of 254-nm UV light (120 mJ/cm^2) in a Stratalinker 1800 (Stratagene, La Jolla, CA). The SDS-PAGE sample loading mix was added to give a final concentration of 0.06 M Tris (pH 6.8), 2% (w/v) SDS, 0.72 M 2-mercaptoethanol, and 0.0005% bromophenol blue [19]. Samples were boiled for 5 min and loaded on an SDS polyacrylamide gel (width, 8 cm; length, 7 cm; 12.0% separating gel with a 5% stacking gel) and electrophoresed at 200 V for 1.25 h in buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The gel was dried at 60°C, exposed to a SR phosphor screen, and processed using a Cyclone PhosphorImager. The relative molecular masses of the protein/DNA adducts were determined using Quantity One software (Bio-Rad, Hercules, CA) and Precision Plus Protein standards (Bio-Rad).

RESULTS

A Protein-Binding Site Is Located Upstream from the AC-Box Within the H1t Promoter

The testis-specific H1t promoter sequence has been described previously [7, 8, 20], and a schematic diagram of the promoter elements essential for transcription are presented in Figure 1. TE, located between the CCAAT box and the AC box, is essential for spermatocyte-specific transcription of the H1t gene in transgenic mice. A new element, discovered in part by DNase I footprinting, is located adjacent to and upstream from the H1/AC box between nucleotides −129 and −107. It was designated the RE, because the 3′-end serves as a binding site for a transcriptional repressor in some nongerminal cell lines [20, 39].

A footprint covered most of the RE and part of the AC box, spanning nucleotides −125 through −100, when nuclear protein binding to the proximal promoter was examined using in vitro DNase I footprinting. Even the lowest concentration of nuclear proteins, derived from an enriched population of pachytene primary spermatocytes, generated a footprint, as shown on the right in Figure 2. The nuclease digestion pattern of free DNA is shown in the left lane of each image. The remaining lanes show the digestion patterns produced by protein binding using increasing amounts of nuclear proteins. Spermatocyte nuclear proteins also produced footprints over the TE that is located between the CCAAT box and the AC box.

Footprints over the RE and TE were weaker or absent when using proteins from nuclear extracts from unfractionated testis cells, as shown on the left in Figure 2. This probably results from a lower concentration of the binding proteins from primary spermatocytes in the unfractionated testis cell population. An enrichment in primary spermatocytes of at least 10-fold was found when comparing the concentration of spermatocytes in a population of unfractionated testis cell to a population of cells enriched in primary spermatocytes by centrifugal elutriation. Therefore, the binding proteins likely were derived from primary spermatocytes.

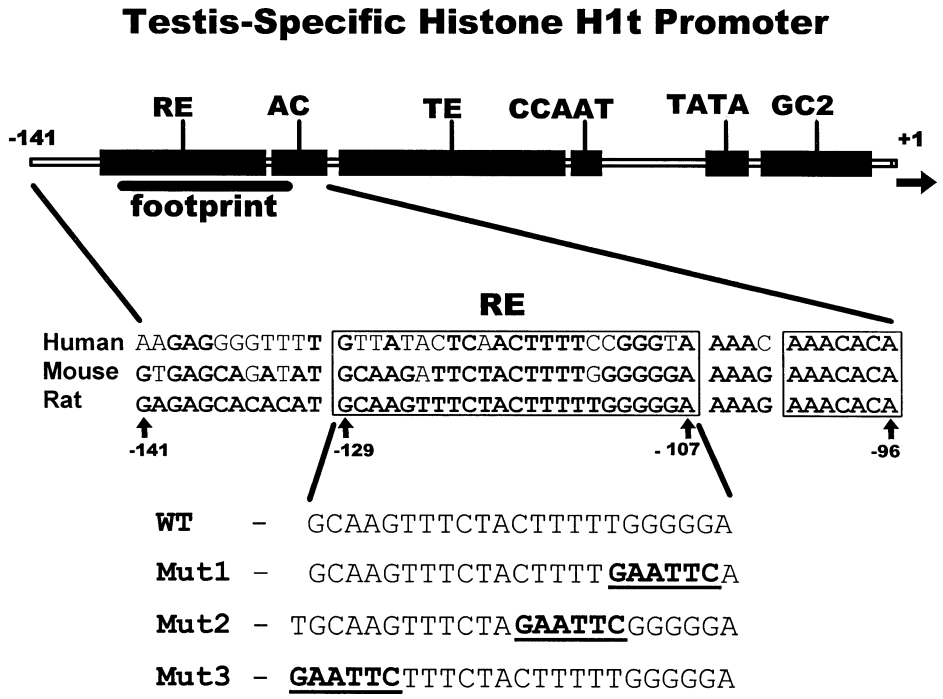

FIG. 1. Diagram of the testis-specific histone H1t promoter. This figure presents a diagram of the proximal promoter elements of the testis-specific Histone H1t gene, including the TE (testis element) and RE (repressor element) promoter elements described in the present study. The region of RE and the AC box bound by spermatocyte nuclear proteins in footprint analysis are indicated. Alignment of mouse, rat, and human H1t promoter sequences is shown below the promoter model, with identical bases shown in bold. Wild-type and mutant sequences used in EMSA and in EMSA competitions are shown in the lower portion, and the mutant sequence is in bold. Numbering represents the number of bases upstream from the mRNA start site.

RE Is Conserved in Mouse, Rat, and Human Histone H1t Promoters

We used the rat testis-specific histone H1t promoter in the DNase I footprint experiments shown in Figure 2. Previous studies identified several sequence elements within the proximal promoter of this gene, starting upstream at the H1/AC box and ending at the GC box 2 located within the mRNA leader region downstream from the TATA box [20]. Several promoter elements important for regulating transcription of the rat H1t gene include the H1/AC box, the H1t/TE element, the H1/CCAAT box, the TATA box, and the GC box 2. All of these elements, except the GC box 2, are conserved in mice and humans.

To examine the sequence conservation of the RE in mammals, we aligned the known mouse, rat, and human H1t promoter sequences as shown in Figure 1. A significant degree of conservation of the element is found. The human and mouse sequences are 65% identical within the 23-bp boxed region; the mouse and rat sequences are 91% identical. Bases that are identical to the rat sequence are indicated by bold characters. A 6-bp core region (ACTTTT) is completely conserved among the species examined.

Primary Spermatocyte Nuclear Proteins Bind Specifically to the RE

We examined the binding affinity of nuclear proteins to the RE promoter element using EMSA. In an initial experiment, we compared binding of nuclear proteins derived from rat testis to binding of nuclear proteins derived from several other organs. The left lane of Figure 3 shows the mobility of free RE probe. The remaining lanes, from left to right, show binding of nuclear proteins from testis, liver, brain, spleen, and kidney. Testis proteins produce two low-mobility bands that are marked in the figure. The upper band, designated testis complex, is not seen in other tissues. A higher-mobility testis band, marked with an asterisk, represent nonspecific binding based on competition assays [39]. Other tissues produce only higher-mobility, nonspecific bands.

In another experiment, we examined binding of nuclear proteins derived from enriched populations of pachytene primary spermatocytes and early spermatids as shown on the right in Figure 3. The two major low-mobility bands produced with testis nuclear proteins were produced by these two protein samples, but the intensity of the testis complex was maximal when proteins were derived from primary spermatocytes. The intensity of the testis complex band dropped approximately 70%–80% when proteins were derived from early spermatids as determined by densitometric scanning of the digital images. Therefore, proteins that form the testis complex are germinal cell-type specific and are produced largely in pachytene spermatocytes. A strong correlation is found between the cell type where the testis-specific histone H1t gene is transcribed and where we see strong binding of nuclear proteins to the RE element of the H1t promoter to form the testis complex.

Spermatocyte Nuclear Protein Binding Occurs Within the 5′-End of the Bifunctional RE

The EMSA competition experiments were conducted to identify regions within the RE element responsible for binding nuclear proteins to produce the testis complex. In these experiments, a wild-type, double-stranded RE element and three mutant RE elements were prepared as shown on the left in Figure 4. The mutations were made by replacing regions of wild-type sequence that were 6 bp in length with heterologous sequences within the 3′ end of the element (Mut1), within the central region of the element (Mut2), or within the 5′ end of the element (Mut3) as shown in Figures 1 and 4.

The first lane (P) in Figure 4A shows free wild-type probe, and the second lane (C) shows binding of testis nuclear extract to the wild-type probe. The next four lanes show competitions with 20 μg of wild-type (Wt), Mut1 (M1), Mut2 (M2), and Mut3 (M3) unlabeled DNA competitors, respectively. An excess of wild-type or Mut1 DNA competes binding to the testis complex and the nonspecific low-mobility band. Higher-mobility bands are not compet-

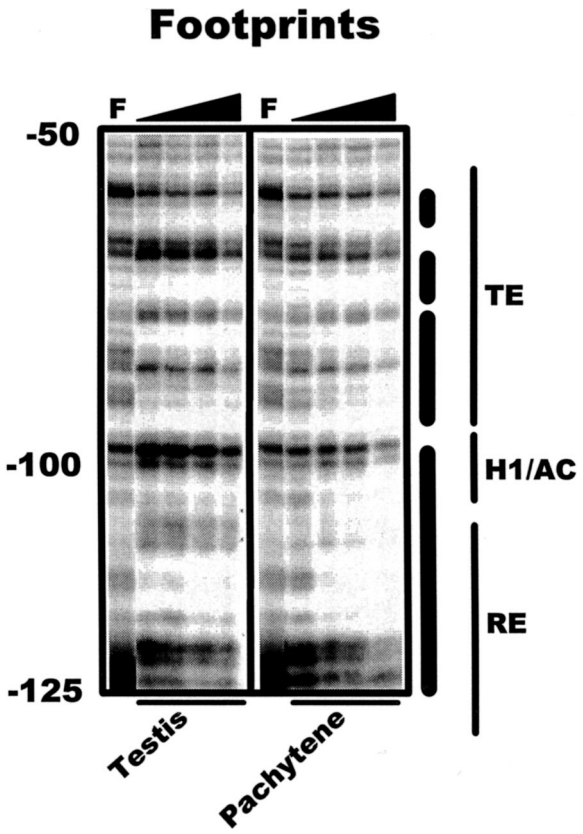

FIG. 2. Detection of nuclear protein binding to the histone H1t promoter by in vitro DNase I footprinting. The DNase I digestion patterns of free DNA and of DNA bound by testis nuclear proteins are shown. The patterns of free DNA and of DNA bound by proteins from unfractionated testis cells are presented on the left. The left lane shows the pattern produced using protein-free DNA. The remaining lanes show patterns produced using increasing amounts (0.7, 0.8, 0.9, and 1.1 μg/μl) of nuclear proteins isolated from unfractionated testis cells. The DNase I digestion patterns of free DNA and of DNA bound by proteins from primary spermatocytes are shown on the right. The first lane on the left is the digestion pattern produced using free DNA. Remaining lanes from left to right show the patterns produced by protein binding using increasing amounts (0.6, 0.8, 1.0, and 1.2 μg/μl) of nuclear proteins isolated from an enriched population of pachytene primary spermatocytes.

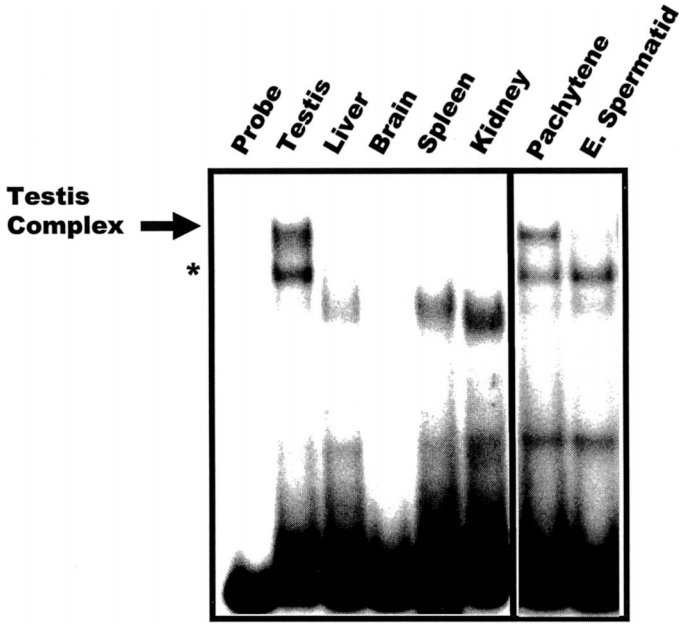

FIG. 3. Specific binding of nuclear proteins to the RE promoter. We used EMSA to examine binding of nuclear proteins to the RE promoter. Equal amounts of nuclear proteins were used in each reaction. The sequence of the wild-type RE probe used in this assay was based on the rat H1t promoter sequence shown in Figure 1. The first lane shows free probe. The next five lanes show binding patterns of nuclear proteins from testis, liver, brain, spleen, and kidney, respectively. The lowest mobility band formed by testis, designated testis complex, is marked by an arrow. A nonspecific band formed with testis nuclear proteins is marked with an asterisk. Proteins from other tissues form higher-mobility bands. We also examined binding of nuclear proteins from enriched populations of rat germinal cells. The last two lanes show binding patterns of nuclear proteins derived from a cell population enriched in pachytene primary spermatocytes and from a cell population enriched in early spermatids. The intensity of the testis complex decreases when using nuclear proteins from early spermatids.

ed with these probes. The Mut3 probe competes binding only to the major low-mobility, nonspecific band. On the other hand, Mut2 fails to compete the testis complex and the low-mobility, nonspecific band.

The testis complex appears to be generated by the binding of nuclear proteins to the region covering Mut2 and Mut3. Therefore, we expected to see normal binding to the Mut1 probe that contains an altered protein-binding site in the 3′-end of the RE probe. To confirm that nuclear proteins bind to the Mut1 RE probe, EMSAs were conducted comparing the binding patterns of the wild-type and Mut1 probes. In Figure 4B, the binding pattern produced by the wild-type probe is shown in lane 2 (Wt), and the binding pattern produced by the Mut1 probe is shown in lane 3. The banding patterns using these two probes are identical, suggesting that testis proteins can bind to RE that has a mutation in the 3′-end of the element, as indicated in the model shown in Figure 5B.

Characterization of the Testis Proteins that Bind to the RE Promoter Element

Nuclear protein extracts from unfractionated testis cells were used in the same type of DNA-binding reaction that was used for EMSA. Bound proteins were cross-linked to the radioactive DNA probe by UV irradiation and were separated by SDS-PAGE. The major band seen in a phosphorimage of the dry gel had an apparent molecular weight of 59 kDa, as shown in the left lane on the left in Figure 5A. Two lower-intensity bands with apparent molecular weights of 52 and 50 kDa were also formed. The testis nuclear protein sample used in this experiment, designated P11 +C, was partially purified by binding to phosphocellulose P11, washed, and eluted with 0.3 M KCl before the binding reaction. The +C indicates that nonspecific competitor was added to the binding reaction. The binding patterns of these partially purified proteins are indistinguishable from the original proteins, but the cleanup step lowers the background significantly in the cross-linking experiment. A faint band of approximately 250 kDa was formed when competitor was omitted, as shown in the second lane (P11 −C).

DISCUSSION

The histone H1t gene is the only linker histone variant gene to exhibit testis-specific transcription [20]. Transcription of the gene is limited to primary spermatocytes within the testis [1]. Some sequence elements found in the testis-specific histone H1t promoter are also found in other linker histone promoters [6, 21–25]. These include the H1/AC box, the H1/CCAAT box, and the TATA box [8, 26]. Both

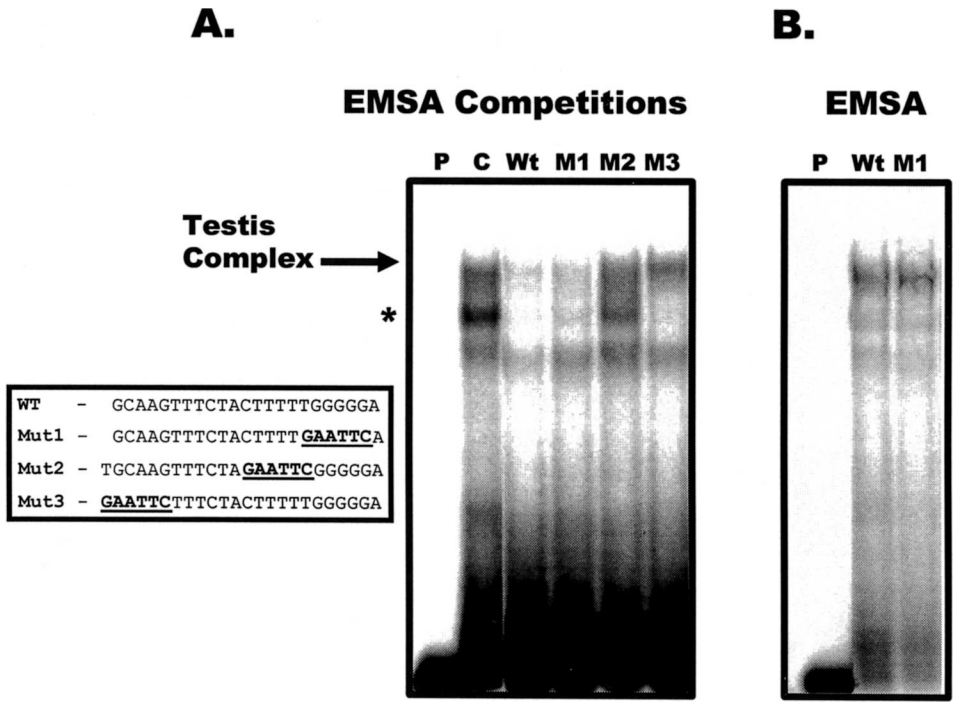

FIG. 4. Identification of the region of the RE responsible for testis-specific binding. **A**) The probe used was the wild-type RE. Unlabeled competitors included the wild-type element, and the three mutant elements are shown in the box to the left of the EMSA. The left lane shows free probe, and the second lane shows binding of testis nuclear proteins to the wild-type probe. The next four lanes show competitions with 20 ng of wild-type, Mut1 (M1), Mut2 (M2), or Mut3 (M3) elements. Only M1 competes binding to the wild-type RE. **B**) An EMSA was conducted to show that testis nuclear proteins could bind to the M1 probe to form the testis complex. The first lane shows free probe. The next two lanes show binding to the wild-type (WT) RE probe and to the M1 probe. The M1 forms the testis complex, and the patterns produced using the WT and M1 probes are indistinguishable.

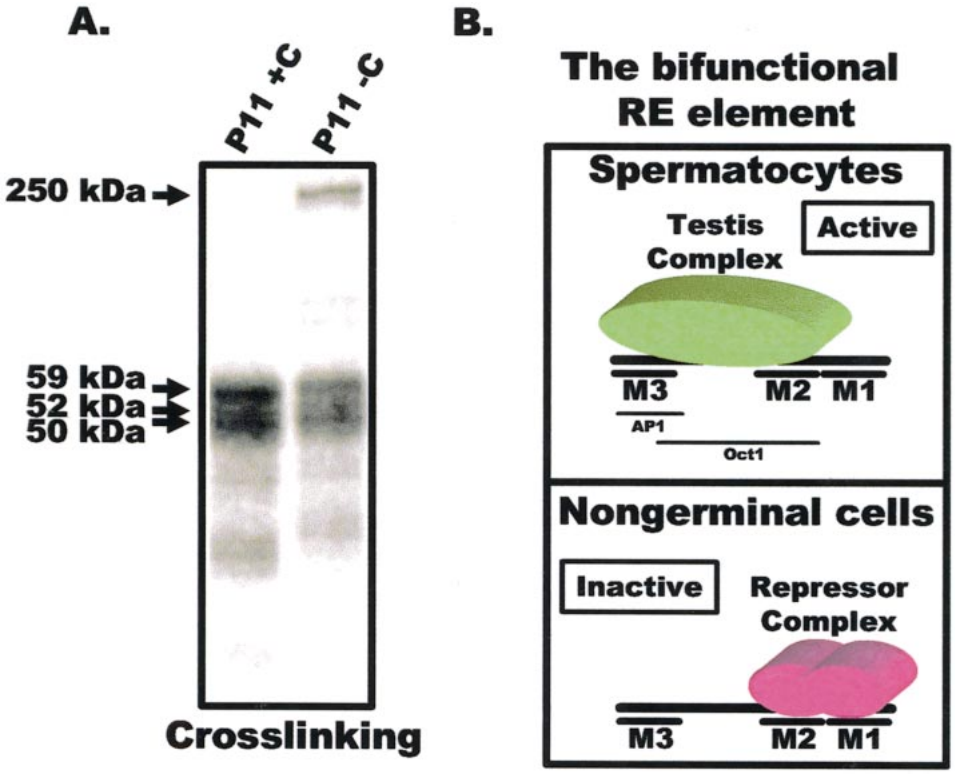

FIG. 5. Cross-linking of testis nuclear proteins to the RE. **A**) To initiate characterization of proteins that bind to the RE, testis nuclear proteins were partially purified by binding to phosphocellulose P11, washed, and eluted with 0.3 M KCl. The proteins were bound to a labeled RE probe as in an EMSA and then cross-linked by exposure to UV light. Cross-linked proteins and DNA were separated by SDS-PAGE, and the gel was dried and then exposed to reveal labeled bands. One binding reaction had a normal level of nonspecific DNA competitor added (P11 +C), and a second reaction lacked nonspecific DNA competitor (P11 −C). Bio-Rad prestained protein electrophoretic standards were analyzed in adjacent lanes for estimation of molecular weights. **B**) A model of the binding of nuclear proteins to the bifunctional RE. The top portion illustrates binding of nuclear protein derived from primary spermatocytes to the 5′-end of the RE to form the testis complex. The binding proteins cover both the Mut3 and Mut2 regions as indicated. Consensus AP1- and Oct-1-binding sites, located in this region, are shown. The Mut1 region is not required for formation of the testis complex in EMSA, as shown in Figure 4. At least one of the DNA-binding components appears to be 50–60 kDa in molecular weight, as shown in **A**. The bottom portion illustrates the binding of a repressor protein complex to the 3′-end of the RE. When this end of the RE is mutated, activity of the H1t promoter increases in nongerminal cell lines in transient expression assays [39].

the H1/CCAAT box and the H1/AC box are required for mitotic cell-cycle regulation of histone H1 gene expression [6]. All of these elements except the TATA box are also found in the basally expressed H1° gene [27].

Within the H1t promoter, additional elements are important for testis-specific expression of the histone H1t gene [4, 6, 10, 11, 15, 20]. These include those that are involved in transcriptional activation of the gene in primary spermatocytes and those that are involved in transcriptional repression in other cell types. An H1t promoter element, designated TE, which is essential for activation of testis-specific transcription of the H1t gene, is located between the H1/AC box and the H1/CCAAT box [11, 13–15, 28] (Fig. 1). This element is composed of three subelements: TE1, TE2, and GC box 1.

Experiments in somatic cells indicate that both the TE1 subelement and the GC box 1 are binding sites for transcriptional activators [28, 29]. Mutation of either site reduces transcription significantly in transient expression assays [28]. However, mutation of the TE2 subelement leads to transcriptional activation in some cell lines, indicating that this site binds a transcriptional repressor [30]. A GC-rich sequence located within the leader region of the rat promoter downstream from the TATA box has been reported to function as a transcriptional repressor-binding site in some nongerminal cell lines [4]. Most of the H1t promoter regulatory elements are located within the proximal promoter, but some upstream elements also contribute to transcriptional regulation. For example, we identified a silencer region located between 875 and 948 bp upstream from the transcription initiation site [3]. In addition, an upstream TG-rich element has been identified as a binding site for a transcriptional activator [31].

To identify additional H1t promoter elements involved in either transcriptional activation or repression, we examined a small region of the H1t promoter located immediately upstream from the AC box. We identified an element, designated RE, that serves as a binding site for factors that lead to transcriptional repression in some nongerminal cell lines [39], and we found sequence conservation among the rat, mouse, and human REs. When the 3′-end of this RE is mutated, promoter activity increases in transiently transfected cell lines. Therefore, the 3′-end of the RE is conserved and appears to be involved in transcriptional repression in nongerminal cells [20, 39].

The initial evidence for binding of testis nuclear proteins to the H1t proximal promoter region adjacent to and upstream from the H1t/AC box was obtained by DNase I footprint analysis. A footprint was formed over the RE region by the binding of nuclear proteins derived from unfractionated testis cells and from an enriched population of pachytene primary spermatocytes. Although some minor differences were observed in the footprint patterns of proteins from testis and spermatocytes, these likely resulted from additional proteins present in the unfractionated testis sample derived from nongerminal cells and from germinal cells that do not express the H1t gene.

Because footprint analyses indicated binding of nuclear proteins to this region of the H1t promoter, we wanted to confirm the testis-specific binding to this site and examine in more detail the interaction of nuclear proteins with this region of the promoter. Specific binding of testis proteins to this region of the promoter was confirmed using EMSA. When assays were conducted using nuclear proteins from testis cell populations enriched in either primary spermatocytes and early spermatids, spermatocytes contained the highest level of binding activity, correlating with the maximal transcriptional activity of the H1t gene. We are not aware of another example in which a transcription factor decreases during the transition from pachytene spermatocytes to early spermatids.

The EMSA competition assays show that the testis complex is formed only by the binding of nuclear proteins to the 5′-end of the RE. Competition with Mut2 and Mut 3 (Fig. 4) fail to compete binding to the testis complex, but competition with Mut1 eliminates the testis complex, as shown in Figure 4A. It is possible that more that one protein may bind to this 5′-region. One protein could bind specifically to the sequence within the Mut3 region, and another could bind within the Mut2 region. If multiple proteins bind to these sites, the proteins may associate with each other so that a mutation in the sequence of either Mut3 or Mut2 alone will not eliminate the complex. It may be necessary to mutate both Mut3 and Mut2 to eliminate formation of the testis complex. Alternatively, the binding protein may bind to a region between the Mut3 and Mut2 sites.

Nuclear proteins bind to the Mut1 probe just as they do to the wild-type RE probe (Fig. 4B), and a model showing how these testis proteins might bind to the RE is shown in Figure 5B. In a preliminary protein-DNA cross-linking study to characterize the binding proteins, masses of the binding proteins appear to range from 50 to 60 kDa. If the binding activity contains multiple components, the entire complex may be larger, and this may explain the large complex of approximately 250 kDa that is seen when nonspecific competitor DNA is removed from the binding assay, as shown in lane 2 of Figure 5A. It is also possible that only one protein component binds to the DNA and that there may not be higher-molecular-weight bands on the gel (UV cross-linking preferentially cross-links protein to DNA rather than protein to protein). Our cross-linking conditions may make it likely that even if more than one protein is involved, only one will be cross-linked to the DNA.

Potential transcription factor consensus-binding sites are within the RE. An Oct-1 site is found within rat, mouse, and human REs, and an AP1 site is found within the rat and human REs (Fig. 5B). Both factors can form multicomponent complexes [32, 33]. Multiple testis isoforms of Oct-1 are somewhat shorter than the full-length, 766-amino acid protein [32, 34], but the exact sizes are unknown. Oct-4, which recognizes the same DNA sequence, is shorter and migrates on SDS-PAGE as a 45- to 50-kDa protein. Fos, a component of AP1, is important for spermatogenesis [35]. Mammalian c-fos is 380 amino acids in length [36], but isoforms migrate on SDS-PAGE as 40- to 60-kDa proteins. Fos B is 338 amino acids in length and migrates as a 35- to 40-kDa protein [37].

In conclusion, transcriptional regulation of the testis-specific histone H1t gene is complex. Transcription is upregulated in primary spermatocytes by binding of activators to specific promoter elements and is downregulated in other cell types by binding of repressors to the same or to other elements. The 3′-end of the bifunctional RE serves as a binding site for a transcriptional repressor in nongerminal cells, and the 5′-end serves as a binding site for a transcriptional activator in primary spermatocytes (Fig. 5B). In the present study, we show that nuclear proteins derived from primary spermatocytes bind to the 5′-end of the RE. Only nuclear proteins from pachytene primary spermatocytes bind tightly to this element to generate a low-mobility testis complex band in EMSAs. It is also possible that transacting factors from primary spermatocytes that bind to the

5′-end of the RE serve as transcriptional coactivators along with factors that bind to the AC box, the GC box 1, the TE1 subelement, and the CCAAT box.

In previous studies, we used transient transfection assays to examine activities of wild-type and mutant H1t promoters in nongerminal cells. With development of new germinal cell lines that can differentiate and progress through spermatogenesis [38], it may be possible to repeat these assays in germinal cells to examine normal and mutant H1t promoter constructs to more clearly determine the importance of the RE to transcription of the H1t gene. If this is not possible, then we can test the importance of the RE for enhanced testis-specific transcription of the H1t gene in transgenic mice [15].

REFERENCES

1. Bucci LR, Brock WA, Meistrich ML. Distribution and synthesis of histone 1 subfractions during spermatogenesis in the rat. Exp Cell Res 1982; 140:111–118.
2. Grimes SR Jr, Meistrich ML, Platz RD, Hnilica LS. Nuclear protein transitions in rat testis spermatids. Exp Cell Res 1977; 110:31–39.
3. Wolfe SA, Mottram PJ, van Wert JM, Grimes SR. Localization of upstream elements involved in transcriptional regulation of the rat testis-specific histone H1t gene in somatic cells. Biol Reprod 1999; 61:1005–1011.
4. Clare SE, Fantz DA, Kistler WS, Kistler MK. The testis-specific histone H1t gene is strongly repressed by a G/C-rich region just downstream of the TATA Box. J Biol Chem 1997; 272:33028–33036.
5. Singal R, van Wert J, Bashambu M, Wolfe SA, Wilkerson DC, Grimes SR. Testis-specific histone H1t gene is hypermethylated in nongerminal cells in the mouse. Biol Reprod 2000; 63:1237–1244.
6. La Bella F, Gallinari P, McKinney J, Heintz N. Histone H1 subtype-specific consensus elements mediate cell cycle-regulated transcription in vitro. Genes Dev 1989; 3:1982–1990.
7. Grimes S, Weisz-Carrington P, Daum H III, Smith J, Green L, Wright K, Stein G, Stein J. A rat histone H4 gene closely associated with the testis-specific H1t gene. Exp Cell Res 1987; 173:534–545.
8. Grimes SR, Wolfe SA, Anderson JV, Stein GS, Stein JL. Structural and functional analysis of the rat testis-specific histone H1t gene. J Cell Biochem 1990; 44:1–17.
9. Wolfe SA, Grimes SR. Binding of nuclear proteins to an upstream element involved in transcriptional regulation of the testis-specific histone H1t gene. J Cell Biochem 1999; 75:555–565.
10. Kremer EJ, Kistler WS. Analysis of the promoter for the gene encoding the testis-specific histone H1t in a somatic cell line: evidence for cell-cycle regulation and modulation by distant upstream sequences. Gene 1992; 110:167–173.
11. Van Wert JM, Wolfe SA, Grimes SR. Testis-specific expression of the rat histone Hlt gene in transgenic mice. Biochemistry 1995; 34:8733–8743.
12. Bartell JG, Davis T, Kremer EJ, Dewey MJ, Kistler WS. Expression of the rat testis-specific histone H1t gene in transgenic mice. One kilobase of 5′-flanking sequence mediates correct expression of a lacZ fusion gene. J Biol Chem 1996; 271:4046–4054.
13. Grimes SR, Wolfe SA, Koppel DA. 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 1992; 201:216–224.
14. Grimes SR, Wolfe SA, Koppel DA. Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone H1t gene. Arch Biochem Biophys 1992; 296: 402–409.
15. van Wert JM, Panek HR, Wolfe SA, Grimes SR. The TE promoter element of the histone H1t gene is essential for transcription in transgenic mouse primary spermatocytes. Biol Reprod 1998; 59:704–710.
16. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
17. Guo LH, Yang RC, Wu R. An improved strategy for rapid direct sequencing of both strands of long DNA molecules cloned in a plasmid. Nucleic Acids Res 1983; 11:5521–5540.
18. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983; 11:1475–1489.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
20. Grimes SR, Wilkerson DC, Noss KR, Wolfe SA. Transcriptional control of the testis-specific histone H1t gene. Gene 2003; 304:13–21.
21. Coles LS, Wells JR. An H1 histone gene-specific 5′ element and evolution of H1 and H5 genes. Nucleic Acids Res 1985; 13:585–594.
22. Dalton S, Wells JR. A gene-specific promoter element is required for optimal expression of the histone H1 gene in S-phase. EMBO J 1988; 7:49–56.
23. Van Wijnen AJ, Wright KL, Massung RF, Gerretsen M, Stein JL, Stein GS. Two target sites for protein binding in the promoter region of a cell cycle regulated human H1 histone gene. Nucleic Acids Res 1988; 16:571–592.
24. Osley MA. The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 1991; 60:827–861.
25. 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. Cell Cycle Dependent Human Histone Genes: Their Organization and Regulation. In: Hnilica LS, Stein GS, Stein JL (eds.), Histones and Other Basic Nuclear Proteins. Boca Raton, FL: CRC Press; 1989:269–318.
26. Cole KD, Kandala JC, Kistler WS. Isolation of the gene for the testis-specific H1 histone variant H1t. J Biol Chem 1986; 261:7178–7183.
27. Doenecke D, Alonso A. Organization and expression of the developmentally regulated H1° histone gene in vertebrates. Int J Dev Biol 1996; 40:395–401.
28. Wilkerson DC, Wolfe SA, Grimes SR. The H1t/GC-box and the H1t/TE1 element are essential for promoter activity of the testis-specific histone H1t gene. Biol Reprod 2002; 67:1157–1164.
29. Wilkerson DC, Wolfe SA, Grimes SR. Sp1 and Sp3 activate the testis specific histone H1t promoter through the H1t/GC-box. J Cell Biochem 2002; 86:716–725.
30. Wilkerson DC, Wolfe SA, Grimes SR. Functional differences in the TE1 and TE2 subelements of the testis-specific histone H1t promoter. J Cell Biochem 2003; (in press).
31. Drabent B, Doenecke D. Testis-specific expression of the mouse histone gene H1t is regulated by several promoter elements. Biol Reprod 1997; 57:1375–1383.
32. Riss J, Laskov R. Expression of novel alternatively spliced isoforms of the Oct-1 transcription factor. Biochim Biophys Acta 1999; 1444: 295–298.
33. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 1988; 55:917–924.
34. Sturm RA, Das G, Herr W. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev 1988; 2:1582–1599.
35. Cobellis G, Meccariello R, Fienga G, Pierantoni R, Fasano S. Cytoplasmic and nuclear Fos protein forms regulate resumption of spermatogenesis in the frog, *Rana esculenta*. Endocrinology 2002; 143: 163–170.
36. Curran T, Gordon MB, Rubino KL, Sambucetti LC. Isolation and characterization of the c-fos (rat) cDNA and analysis of post-translational modification in vitro. Oncogene 1987; 2:79–84.
37. Siderovski DP, Blum S, Forsdyke RE, Forsdyke DR. A set of human putative lymphocyte G_0/G_1 switch genes includes genes homologous to rodent cytokine and zinc finger protein-encoding genes. DNA Cell Biol 1990; 9:579–587.
38. Feng LX, Chen Y, Dettin L, Pera RA, Herr JC, Goldberg E, Dym M. Generation and in vitro differentiation of a spermatogonial cell line. Science 2002; 297:392–395.
39. Wolfe SA, Grimes SR. Transcriptional repression of the testis-specific *histone H1t* gene mediated by an element upstream of the H1/AC box. Gene 2003; (in press).