Journal of Cellular Biochemistry 75:555–565 (1999)

Binding of Nuclear Proteins to an Upstream Element Involved in Transcriptional Regulation of the Testis-Specific Histone H1t Gene

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Abstract The testis-specific histone H1t is synthesized during spermatogenesis exclusively in late pachytene primary spermatocytes. Transcription of the H1t gene is repressed in every tissue except testis. Within the testis, transcription is repressed during development before the spermatocyte stage and in later stages of germinal cell maturation. Mechanisms involved in transcriptional repression of the H1t gene are unknown. To assess the contribution of upstream H1t promoter sequence to transcriptional silencing in nonexpressing cells, H1t-promoted reporter vectors were constructed using pGL3 Basic. Transient expression assays with these reporter vectors driven by H1t promoter deletions allowed us to identify a region from 948 to 780 bp upstream from the H1t transcriptional initiation site that functions as a silencer. Examination of nuclear protein binding to this DNA regulatory region by electrophoretic mobility shift assays using extracts from C127I cells, rat testis, and pachytene spermatocytes revealed a low mobility band produced only by nuclear proteins derived from nonexpressing cells that may contain proteins that repress H1t gene transcription. J. Cell. Biochem. 75:555–565, 1999. Published 1999 Wiley-Liss, Inc.†

Key words: histone H1t gene; tissue-specific transcription; gene regulation; repression; transient transfection; luciferase assay

H1 histones are composed of seven subtypes in mammals and represent the most divergent histone class [Khadake and Rao, 1995; Drabent and Doenecke, 1997]. There are differences in expression patterns during development and differentiation [Brown et al., 1996] and in binding affinity to chromatin among the H1 histone subtypes with H1t having the weakest binding [De Lucia et al., 1994; Khadake and Rao, 1995]. H1 histones may facilitate the transition from a 10 nm chromatin filament to a 30 nm fiber and in this way they are involved in the formation of higher order chromatin structure [Thoma et al., 1979]. Thus, histone H1 acts as a global gene regulator through chromatin binding and remodeling [Guo et al., 1983]. H1 histones also interact with components of the transcription initiation complex to block transcription [Croston et al., 1991; Juan et al., 1994].

The proximal promoter of the testis-specific histone H1t gene contains the consensus elements found in somatic H1 promoters. These include an H1/AC box, a GC box, an H1/CCAAT box, and a TATA box [Coles and Wells, 1985; Dalton and Wells, 1988; van Wijnen et al., 1988; Osley, 1991; Doenecke et al., 1994; Duncliffe et al., 1995]. The H1/AC box and the H1/CCAAT box have been shown to enhance histone H1 gene transcription during S-phase of the mitotic cell cycle [Dalton and Wells, 1988; Gallinari et al., 1989; La Bella et al., 1989]. The H1t gene proximal promoter contains an additional enhancer element designated TE that binds factors responsible for activation of transcription in the testis [Grimes et al., 1992a,b; Wolfe et al., 1995; vanWert et al., 1996; Grimes et al., 1997]. The H1t promoter also contains a poly C

Grant sponsor: National Institutes of Health; Grant number: HD29381; Grant sponsor: Department of Veterans Affairs.

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Received 11 May 1999; Accepted 12 May 1999

element termed the GC box 2 [Clare et al., 1997a,b] that contributes to silencing of H1t gene transcription in somatic cells. Since there are common elements within the proximal promoters of the seven H1 histone genes, it has been assumed that factors that bind to elements farther upstream modulate histone H1t gene transcription. Transcriptional factors that bind to the upstream region may interact with transcriptional factors that bind to the proximal promoter repressing transcription.

Regions of DNA needed for proper testis-specific transcription of the H1t gene were confirmed using transgenic animals. Sequences required for proper developmental and spermatocyte-specific transcription of the H1t gene were shown to be present within a 6.85 kb genomic fragment containing the rat H1t gene [vanWert et al., 1995].

Upstream elements that contribute to testis-specific histone H1t gene transcription were demonstrated in studies with proliferating cell lines [Kremer and Kistler, 1992; Drabent and Doenecke, 1997]. H1t promoted transcription of a reporter gene was low compared to transcription from somatic H1 constructs in cells undergoing mitosis [Kremer and Kistler, 1992]. The low transcriptional activity of the H1t promoter indicated the presence of an upstream silencer element or significant differences within the proximal promoter that render H1t less efficient in interacting with transcriptional factors utilized by the somatic variants. Regulation of H1t gene transcription during the mitotic cell cycle is influenced by sequences upstream from the H1t proximal promoter [Kremer and Kistler, 1992; Drabent and Doenecke, 1997]. A key question addressed in this study concerns mechanisms that silence H1t gene transcription in somatic cells.

To determine the contribution of the upstream 5′ flanking region of the proximal promoter to transcriptional silencing of the H1t gene, we prepared reporter vectors whose transcription was driven by truncated H1t promoter constructs and we examined transcription in transfected mouse somatic cells. A region of the H1t promoter located between 948 to 875 nucleotides upstream from the H1t transcriptional start site appeared to act as a silencer of H1t gene transcription in mouse C127I cells. This element significantly reduced transcription when placed upstream from the rat somatic H1d proximal promoter in a luciferase reporter plasmid construct [Wolfe et al., 1999]. Electrophoretic mobility shift assays (EMSA) were performed using this upstream element and nuclear extracts from mouse C127I cells, testis, and pachytene spermatocytes. One low mobility band produced by C127I and testis nuclear proteins was not produced by pachytene extracts. This band may contain proteins that contribute to repression of H1t gene transcription in somatic tissues.

MATERIALS AND METHODS

Plasmids

Recombinant DNA construction followed standard procedures [Sambrook et al., 1989]. Plasmid DNA was harvested from cultures of *Escherichia coli* strain HB101 using alkaline lysis [Birnboim and Doly, 1979] followed by passage through a Wizard DNA clean up minicolumn (Promega, Madison, WI). DNA concentration was estimated by reading absorbance at 260 nm and confirmed by ethidium bromide staining of restriction fragments after gel electrophoresis.

Histone H1t-Promoted Luciferase Reporter Plasmids

Construction of plasmids pGL3B *Eco*R I, pGL3B *Pvu* II, and pGL3B *Acc* I from pGL3Basic (Promega) was described previously [Wolfe et al., 1999]. The plasmid pPS5 [Grimes et al., 1990] served as the source for the histone H1t promoter fragments used in the construction of the luciferase based reporter vectors. All of the H1t promoted reporter vectors had identical fusion points to the luciferase reporter.

Animal Cells and Mammalian Cell Lines

Male Sprague-Dawley rats were the source of all tissues used in these experiments. Enriched populations of pachytene spermatocytes were obtained by centrifugal elutriation [Meistrich et al., 1981]. Mouse C127I mammary cells and HeLa cells obtained from American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Transient Transfection Assays

Cells were transfected using LipofectAMINE (GibcoBRL, Gaithersburg, MD) as described by the supplier's protocol. C127I cells from a single

cell suspension were plated on 60-mm dishes and grown to a density of 40–60% before transfection. Transfections were performed in triplicate to help control for variation in cell number and culture conditions. Cells in serum-free medium were cotransfected using 2 μg of a specific plasmid construct plus 200 ng of pRL TK complexed with 20 μl of LipofectAMINE per dish. LipofectAMINE/DNA complexes were incubated with cells 5 h before feeding with an equal volume of DMEM containing 20% fetal bovine serum. Cell lysates used for luciferase assays were made from transfected cells 48 h after feeding. Assays were conducted using slight modifications of the dual-luciferase reporter assay system protocols supplied by Promega.

Luciferase Assays

To prepare cell lysates following transfection, the growth medium was removed and 4 ml of phosphate buffered saline was added to each 60 mm dish of cells. The dishes were gently swirled to wash the cell surfaces, followed by removal of the rinse solution and addition of 400 μl of 1 × passive lysis buffer (Promega) to each dish. The dishes were incubated for 15 min at room temperature before harvesting the cell lysates by scraping the bottom of the dishes with disposable plastic scrapers. Each lysate was pipetted several times to obtain a homogenous solution and transferred to a microfuge tube. Lysates were then cleared by centrifugation in the microfuge at 4°C for 1–2 min. Lysates were transferred to fresh tubes and stored at −70°C.

Dual-luciferase assays were performed in a Beckman LS6000SC scintillation counter with the coincidence counter disabled. Firefly luciferase and the renilla luciferase assays were performed manually in one reaction tube. Firefly luciferase activity and renilla luciferase activity were sequentially measured for 2 min each as directed in the protocol for manual luminometers in the dual-luciferase reporter assay system technical manual. Protein determinations were made spectrophotometrically using a program based upon the Warburg and Christian [1942] coefficients.

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from crude nuclei derived from mouse C127I cells and from several rat tissues (testis, liver, and brain) as previously described [Dignam et al., 1983; Wolfe et al., 1995]. Nuclear extracts were also prepared from enriched germinal cell populations from rat testis [Grimes et al., 1990]. Binding of nuclear extracts to labeled DNA probes was performed on ice as described previously [Grimes et al., 1992a,b] in a total volume of 20 μl with 2 μg of the nonspecific competitor poly dI-dC added to each assay. When used, unlabeled specific competitor DNA was added 15 min prior to binding of nuclear proteins to labeled probe. Following binding, samples were electrophoresed through 4% polyacrylamide gels (60:1, acrylamide:bisacrylamide) using buffer containing 6 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, and 1 mM EDTA with buffer recirculation. Following electrophoresis, gels were dried and exposed to Kodak XOMAT XAR-5 film at −70°C.

Probes

The plasmid pM3R was digested with *Xba* I followed by isolation and recovery of the 150 bp *Xba* I fragment by electrophoresis through a 1.0% LMT agarose gel followed by hot phenol extraction. The purified 150 bp *Xba* I fragment was labeled by filling the 3′ recessed ends with [α-^{32}P] dCTP using the Klenow fragment of DNA polymerase [Sambrook et al., 1989]. The resulting radioactive DNA fragment was isolated on an 8% polyacrylamide gel, excised, and recovered by diffusion in annealing buffer (10 mM Tris, pH 8.0, 5 mM $MgCl_2$) for use in EMSA.

RESULTS

H1t-Promoted Transcription Is Silenced in Nongerminal Cells

The histone H1t gene is transcribed only during spermatogenesis within pachytene primary spermatocytes as depicted in Figure 1. Transient transfection of nongerminal cell lines with histone H1t promoted reporters reveal that distal elements contribute to transcription control [Kremer and Kistler, 1992; Drabent and Doenecke, 1997]. Transcription of reporter genes driven by the H1t proximal promoter was low in transiently transfected cells compared to somatic histone H1 variant transcription [Kremer and Kistler, 1992]. These transient transfection studies revealed a cyclic pattern of H1t promoted transcription similar to the pattern of somatic histone H1 promoted transcription [Dailey et al., 1988]. Various sequences upstream

from the proximal promoter have been reported to enhance or repress transcription [Kremer and Kistler, 1992; Drabent and Doenecke, 1997]. For example, a TG box located between 521 and 305 bp upstream from the mouse H1t transcriptional initiation site is involved in enhanced H1t gene transcription in transiently transfected mouse testicular cells [Drabent and Doenecke, 1997].

In the current study luciferase based transcription expression vectors were used. Four reporter vectors, containing 2,385, 1,472, 948, and 875 bp of sequence upstream from the rat histone H1t transcriptional initiation site, were constructed (Fig. 2, inset). These constructs were used in transient transfections of mouse C127I cells to identify sequences that contribute to silencing of H1t-promoted transcription in nongerminal cells.

Expression resulting from transient transfection of C127I cells is shown in Figure 2. The longest construct designated pGL3 *Eco* RI contained 2385 nucleotides of sequence upstream from the H1t transcriptional start site and exhibited activity slightly greater than pGL3 Basic, a promoterless control vector. The first two deletions of the histone H1t promoter led to minimal increases in the level of transcription (Fig. 2). These two constructs designated pGL3B *Kpn* I and pGL3 *Pvu* II contained 1,472 and 948 nucleotides, respectively, of sequence upstream from the H1t transcriptional start site and exhibited luciferase activities that were only slightly higher than pGL3 *Eco* RI. Removal of DNA to 875 in the vector pGL3 *Acc* I led to increased transcription to a level approximately 40-fold higher than that provide by pGL3 *Eco* RI (Fig. 2). Apparently, a short region of DNA (73 bp) located between the *Pvu* II and *Acc* I restriction sites contributed significantly to transcriptional silencing.

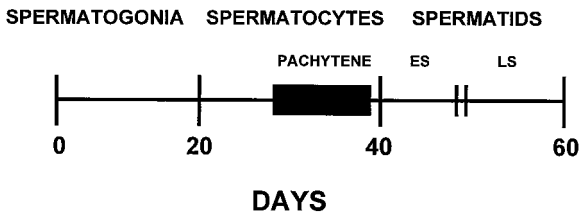

Fig. 1. A schematic representation of the stages of spermatogenesis. A time line for spermatogenesis in the rat showing cell types in the three major divisions of spermatogenesis: (1) spermatogonia, (2) spermatocytes, and (3) spermatids. Pachytene primary spermatocytes (indicated by the filled rectangle) are shown within the spermatocyte stage, and early spermatids and late spermatids are shown within the spermatid stage. Enriched populations of pachytene primary spermatocytes, early spermatids (ES), and late spermatids (LS) were prepared by centrifugal elutriation.

Localization of Protein-DNA Binding Sites Within the Silencer Region

Previous protein binding studies indicated a weak footprint in the upstream region located between the *Pvu* II site (−948) and the *Xba* I site (−930) [Wolfe et al., 1999]. Because this region falls within the silencer region, nuclear protein binding was examined by EMSAs. A 29 bp competitor 1 probe (Fig. 3) was used to detect nuclear protein binding to the 18 bp region between the *Pvu* II site and the upstream *Xba* I site at −930 within the silencer region.

In EMSAa using the 29 bp competitor 1 probe (Fig. 4), a single major shifted band designated R1 formed (Fig. 4, lane 2) which was specifically eliminated with cold competitor (Fig. 4, lane 3). Band R1 was also competed by cold competitor 7 (Fig. 4, lane 4). This was likely due to sequence similarity between one region from each fragment (TCTTATTTC in competitor 1 and TCAGATTTC in the complement strand of competitor 7). Competitions with competitor 2 or 5 did not eliminate specific bands (Fig. 4). The band labeled NS in Figure 4 represents a nonspecific band.

EMSAs also were performed with the 29 bp competitor 1 probe to compare binding patterns formed by nuclear proteins from C127I cells, rat testis, and rat pachytene spermatocytes. As stated before, extracts from C127I cells produced a major band designated R1 (Fig. 5). A broader and slightly faster migrating band was formed when nuclear extracts were derived from rat testis (Fig. 5). Band R1 was significantly reduced when extracts were derived from pachytene primary spermatocytes (Fig. 5).

A larger 150 bp *Xba* I restriction fragment overlapping the 3′ end of competitor 1 was used to examine nuclear protein binding to the region 930 to 780 bp upstream from the H1t transcriptional initiation site (Fig. 3). When EMSAs were performed with the 150 bp *Xba* I restriction fragment, nuclear extracts from C127I cells formed two major shifted bands numbered 1 and 6 and at least 4 less distinct minor bands numbered 2 through 5 (Fig. 6, lane 2 "Control"). Incubation with an excess of the unlabeled 150 bp *Xba* I fragment in mobility

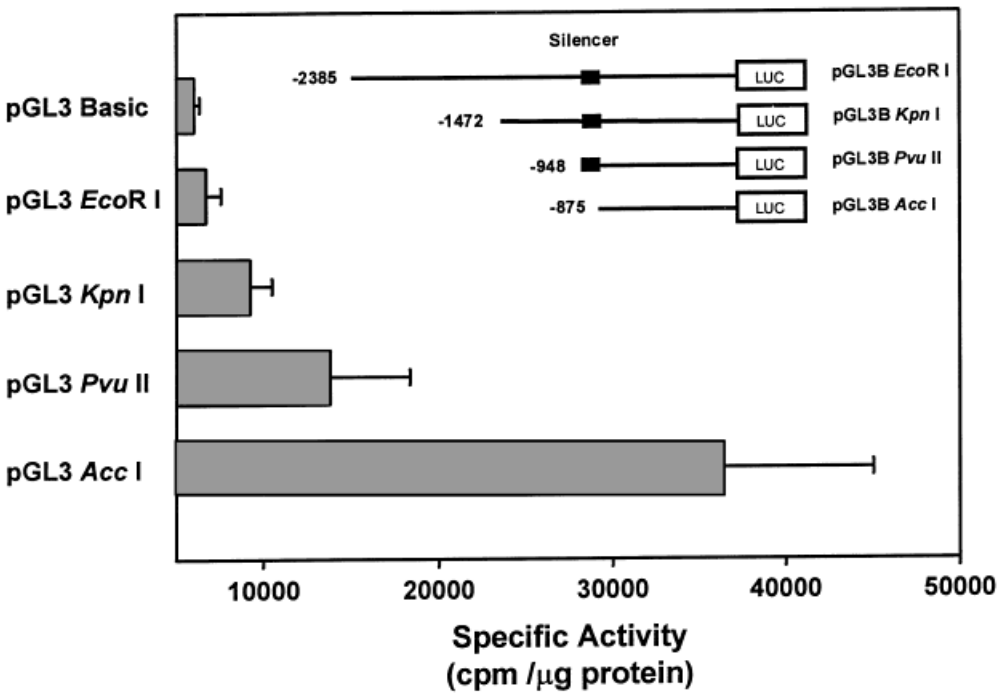

Fig. 2. Transient expression of a luciferase reporter under the control of the rat H1t promoter in mouse C127I cells. **Inset:** H1t promoter fragments fused to the firefly luciferase reporter gene used for expression studies. The negative number to the left of each construct indicates the distance in nucleotides relative to the respective H1t transcriptional initiation site. All of the H1t promoter constructs have identical fusion points to the firefly luciferase gene. The silencer region from the *Pvu* II restriction site at −948 to the *Acc* I restriction site at −875 is shown by a closed rectangle. The bar graph shows normalized luciferase-specific activities from the H1t-promoted constructs. The three H1t-promoted constructs are arranged along the y-axis from the longest promoter fragment (pGL3B *Eco*R I) to the shortest (pGL3B *Acc* I). The level of luciferase-specific activity is shown on the x-axis. The means and standard errors of the means for three independent transfections are plotted.

shift assays effectively competed all of the shifted complexes (Fig. 6, lane 3 "*Xba* I").

Competition of the 150 bp *Xba* I probe with a restriction fragment that contained the region from the upstream *Mae* III site at −1,019 to the *Acc* I site at −875 specific bands reduced the intensities of bands 1, 4, and 5 (Fig. 6, lane 5 "Upstream"). Competition with a restriction fragment covering the downstream region from the *Acc* I site at −875 to the *Mae* III site at −735 eliminated all bands except band 6 (Fig. 6, lane 4 "Downstream").

To further examine protein binding to the labeled 150 bp *Xba* I fragment and to identify specific binding elements we used several double-stranded competitors to perform EMSAs. Positions and sequences of the specific competitors used are shown in Figure 3. Incubation of C127I nuclear extracts with Competitors 1 and 7 reduced the relative level of minor band 4 by approximately 50% compared to lane 2 with no competitor (Fig. 7, lanes 3 "Comp1" and 9 "Comp7" compared to lane 2 "C127I"). Comparisons of relative band intensities were made by scanning the autoradiograms and measuring peak areas of the bands (data not shown). These data are consistent with those obtained previously using the 150 bp *Xba* I probe revealing competition of band R1 by competitors 1 and 7 (Fig. 4). No competition was detected with competitors 2, 3, and 5 (Fig. 7, lanes 4, 5, and 7). However, competitor 4 reduced bands 5 and 6 (Fig. 7, lane 6). Competitor 6 reduced bands 2 and 3 (Fig. 7, lane 8). Competitor 8, a fragment overlaping competitors 4, 5, 6, and 7, eliminated all bands except bands 4 and 6 (Fig. 7, lane 10). This 51 bp DNA competitor spans the *Acc* I site and covers the distal rat H1t promoter from 887 bp to 837 bp upstream from the transcriptional initiation site.

In an experiment complementary to the one shown in Figure 6, an upstream 55 bp *Xba* I-*Acc* I labeled restriction fragment formed two bands in EMSAs (Fig. 8, left panel, lane 2 "C127I"). Band 6 was competed by competitor 4 (Fig. 8, left panel, lane 3 "Comp4"). EMSAs with C127I

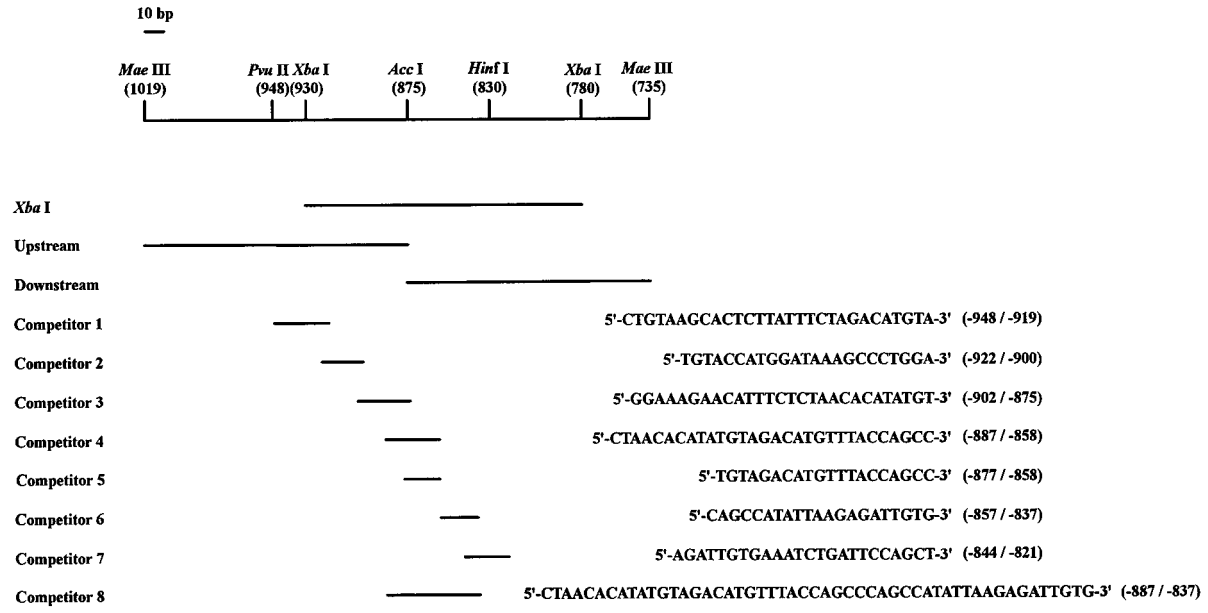

Fig. 3. Diagram of DNA competitors used in protein binding assays (EMSAs). The positions and relative lengths of the restriction fragments and double-stranded oligonucleotides used in EMSA competition experiments are indicated. The nucleotide sequences and the positions of the 5′ and 3′ ends of the double-stranded oligonucleotides relative to the rat H1t transcriptional initiation site are also indicated.

nuclear extracts and a labeled downstream 95 bp *Acc* I—*Xba* I probe formed six bands with mobilities similar to those formed with the larger 150 bp *Xba* I fragment (compare Fig. 8, right panel, to Fig. 6, lane 2 "control"). Band 6 was significantly competed by competitor 4 (Fig. 8, right panel, lane 3 "Comp4").

DISCUSSION

The testis-specific H1t gene is the only member of the mammalian H1 histone family that exhibits tissue specific transcription. Proximal and distal elements have been implicated in the developmental activation and enhanced transcription of the H1t gene in testis primary spermatocytes [Kremer and Kistler, 1992; vanWert et al., 1995, 1996; Wolfe et al., 1995; Drabent and Doenecke, 1997]. Repression of H1t gene transcription in nonexpressing tissues and cells may be mediated through proximal elements such as the GC box 2 [Clare et al., 1997a] and through upstream elements. Previous studies of the rat promoter in somatic cell lines [Kremer and Kistler, 1992] and of the mouse promoter in primary cultures of testicular cells [Drabent and Doenecke, 1997] demonstrated negative regulation of H1t gene transcription by distal upstream promoter regions. In the present study we analyzed several promoter reporter constructs using fragments containing up to 2,385 nucleotides upstream from the rat H1t transcriptional initiation site to extend the examination of the upstream regions of the H1t promoter and to localize distal elements that silence transcription in nonexpressing cells.

Deletion of a short upstream H1t promoter region revealed a potential regulatory region (Fig. 2). The pGL3B *Eco*R I construct, containing 2,385 bp of upstream promoter sequence, exhibited luciferase activity only slightly higher than that of the promoterless plasmid pGL3Basic used as a negative control in these experiments (Fig. 2). Therefore most of the elements necessary for silencing of H1t promoter activity in somatic cells appear to be located within 2.4 kb upstream of the H1t gene transcriptional start site. These results are consistent with the transcription pattern of this testis-specific histone gene and with the results of transgenic mouse studies in our laboratory and in other laboratories [vanWert et al., 1995; Bartell et al., 1996].

Deletion of the promoter to 1,472 nucleotides upstream from the H1t transcriptional start site (pGL3B *Kpn* I) led to luciferase expression levels that were only slightly higher than that obtained from the full-length construct (pGL3B *Eco*R I; Fig. 2). However, removal of the DNA

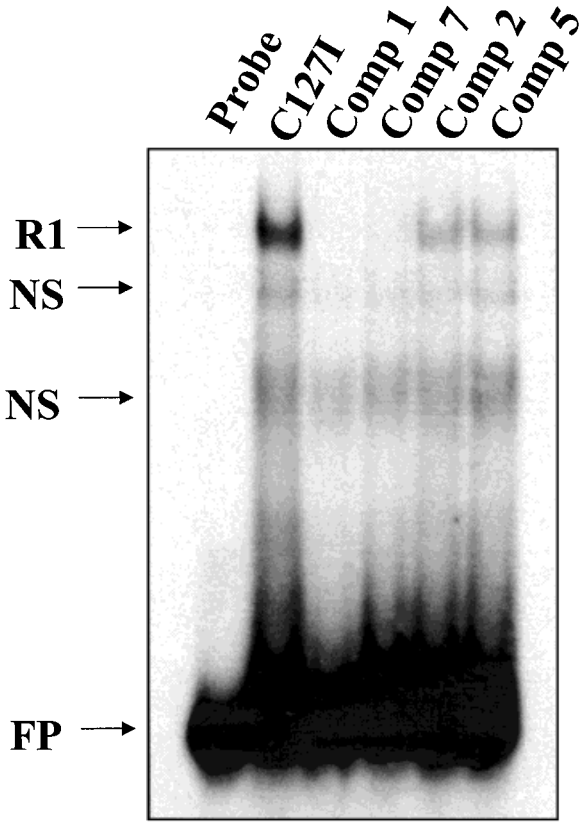

Fig. 4. Protein binding to the competitor 1 probe. The labeled 29 bp competitor 1 probe was incubated with 12.8 μg of C127I nuclear proteins and used in gel shift assays. Incubation of competitor 1 probe with nuclear proteins yields three major bands in EMSA (**lane C127I**). Incubation with an additional 80-fold molar excess of unlabeled competitor DNA is indicated above the lanes labeled Comp1, Comp7, Comp2, and Comp5. A band that is specifically competed is designated R1 and bands not specifically competed are indicated by NS. Unbound probe is labeled as FP.

sequence from −1,472 to −875 (pGL3B *Acc* I) resulted in a dramatic increase in luciferase activity (Fig. 2). This increase in transcription appears to occur with the elimination of a repressor's DNA binding site. Additional support for the ability of the region from −948 to −875 to silence transcription was shown by placing the putative silencer upstream from the somatic histone H1d proximal promoter [Wolfe et al., 1999]. Changing the position of the element did not eliminate its ability to repress transcription.

To examine nuclear factors that mediate H1t silencing and to identify their cognate binding sites we performed EMSAs with a number of probes within this region. One probe used was a 29 bp DNA fragment designated Comp 1 from this region between the *Pvu* II and *Xba* I restriction sites (Figs. 4, 5). Previous protein binding studies indicated a footprint in the upstream region located between the *Pvu* II site (−948) and the *Xba* I site (−930) [Wolfe et al., 1999]. In EMSAs using the 29 bp competitor 1 probe a single major shifted band designated R1 was formed (Fig. 4) that was specifically eliminated with cold competitor 1. It is interesting that the band designated R1 forms with extracts from

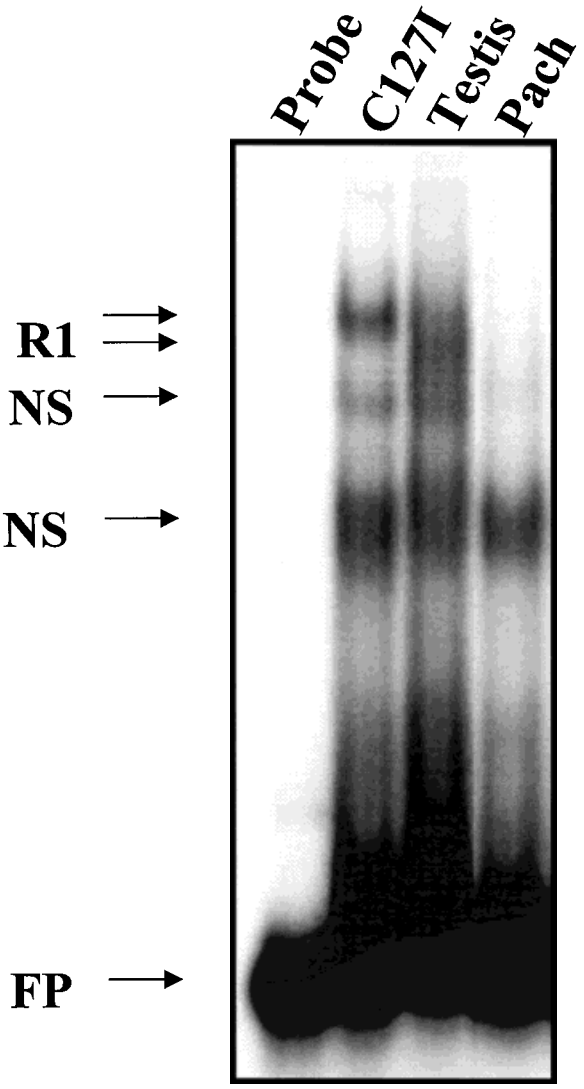

Fig. 5. Comparison of binding of nuclear proteins from C127I cells and various rat tissues to the 29 bp probe. EMSAs were performed using the 29 bp probe (**lane 1**). A low mobility band designated R1 was produced using C127I nuclear extracts. EMSAs using rat testis nuclear extracts produced a band with slightly faster mobility (shown by the second R1 arrow). R1 was not present in nuclear extracts from rat pachytene spermatocytes. The labels NS and FP indicate nonspecific bands and free radiolabeled probe.

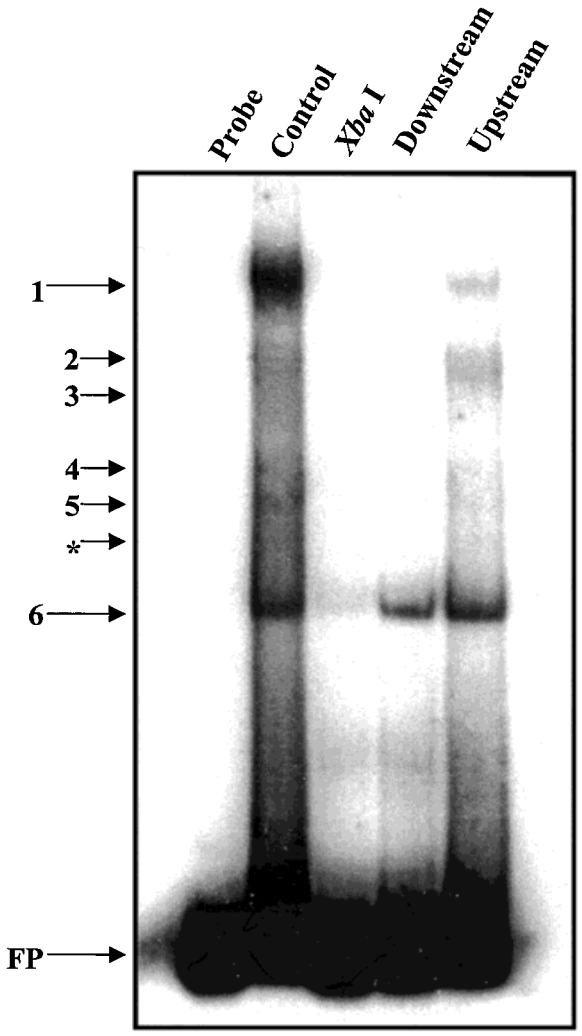

Fig. 6. Competition of protein binding to the 150 bp *Xba* I restriction fragment. EMSAs were conducted using a labeled 150 bp *Xba* I probe (**lane 1**) bound to nuclear proteins from mouse C127I cells (**lane 2,** Control). Competition was performed with a 7.5-fold molar excess of the 150 bp *Xba* I fragment (**lane 3**). EMSA competitions were performed with a 60-fold molar excess of either the 141 bp *Acc* I—*Mae* III restriction fragment downstream from the *Acc* I site (**lane 4,** Downstream) or the 144 bp *Mae* III—*Acc* I restriction fragment upstream from the *Acc* I site (**lane 5,** Upstream). Major complexes 1 and 6 are numbered and indicated by long arrows, and minor complexes 2 through 5 are numbered and indicated by short arrows. FP indicates free radiolabeled probe.

all tissue sources and cell types except those derived from enriched pachytene primary spermatocytes, the population of germinal cells where the histone H1t gene is expressed (Fig. 5). Thus, the formation of band R1 correlates with nuclear extracts from cells that do not express the histone H1t gene.

We also performed EMSAs with a 150 bp *Xba* I DNA fragment from the silencer region (Figs. 6, 7). Nuclear extracts from C127I cells gave reproducible binding to the *Xba* I fragment. Typically, two major bands (bands 1 and 6; Fig. 6) were observed along with several minor bands (bands 2–5). Competitions with several oligonucleotides shown in Figure 3 allowed us to examine sites of these interactions. All of the bands detected, including major bands 1 and 6, were effectively competed using 50 ng of competitor 8 (Fig. 7), which spans the region from 887 to 838 bp upstream from the rat H1t transcriptional initiation site. It should be noted that that band 1 was competed by a downstream *Acc* I—*Xba* I competitor and competed with an upstream *Xba* I—*Acc* I competitor (Fig. 6). In addition, a band with the same

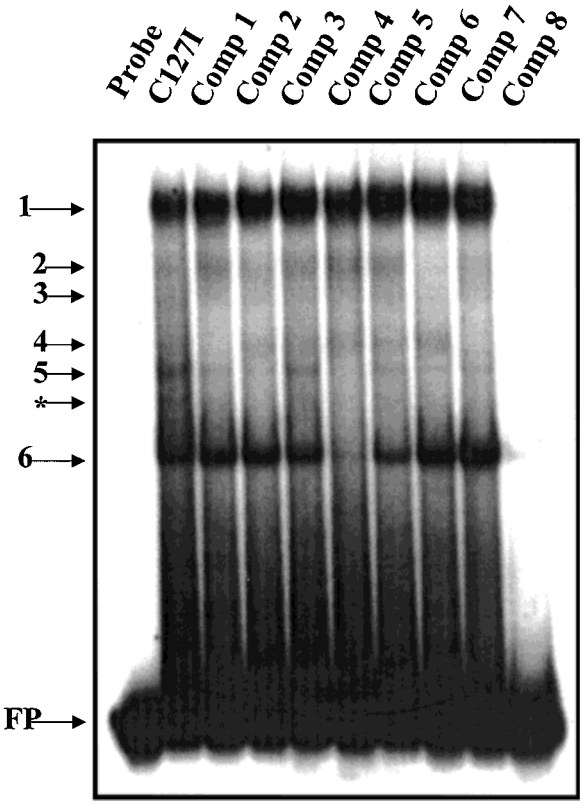

Fig. 7. Localization of binding sites within the 150 bp *Xba* I restriction fragment. EMSAs were performed with 3.2 μg of C127I nuclear proteins and double-stranded synthetic oligonucleotide competitors to examine protein-DNA interactions within the 150 bp *Xba* I restriction fragment. The labeled *Xba* I restriction fragment was electrophoresed in **lane 1** (Probe). Incubation with C127I nuclear proteins in **lane 2** yielded two major bands indicated by long arrows (bands 1 and 6) and four minor bands indicated by short arrows (bands 2–5). The minor band designated by an asterisk appears to be a spurious band. Competitions included a 60-fold molar excess of specific double-stranded oligonucleotides as indicated above each lane. FP indicates free radiolabeled probe.

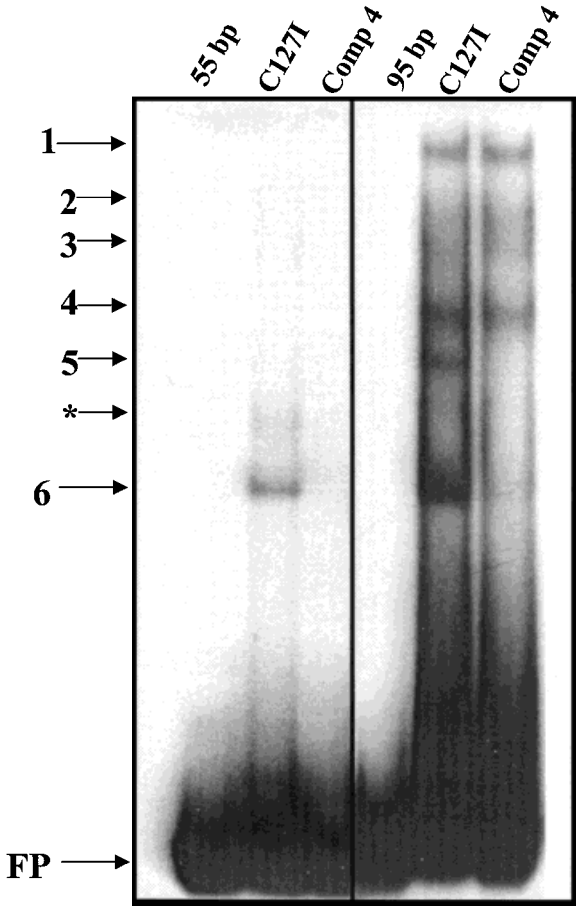

Fig. 8. Protein binding to subfragments of the 150 bp *Xba* I restriction fragment. EMSAs were conducted using either an upstream 55 bp *Xba* I-*Acc* I probe (**left**) or a downstream 95 bp *Acc* I-*Xba* I probe (**right**) and C127I nuclear proteins. The large arrows on the left side of the figure indicate the two major bands (1 and 6), the small arrows indicate the four minor bands (2–5), and the asterisk indicates a spurious band. Competitions with a double-stranded oligonucleotide, competitor 4, which overlapped the central *Acc* I restriction site were performed (lanes "Comp4"). Competitor 4 was added at a 30- or 80-fold molar excess to the binding reactions with the 55 bp *Xba* I-*Acc* I probe (left) or the 95 bp *Acc* I-*Xba* I probe (right), respectively. FP indicates free radiolabeled probe.

mobility as band 1 formed with a downstream 95 bp DNA probe but not with an upstream 55 bp probe (Fig. 8).

Note that band 6 was competed with 100 ng of competitor 4, a 30 bp double-stranded oligonucleotide that covers the region from 887 to 858 bp upstream from the rat H1t transcriptional initiation site (Fig. 7). Data from these EMSAs indicated that the *Acc* I restriction site may be located within the binding site of one of the DNA binding factors that forms band 6. It should be noted that previous protein binding studies indicated a weak footprint over the entire region represented by the 150 bp *Xba* I probe (−930 to −780) and strong footprints over the regions −902 to −888 and −854 to −831) [Wolfe et al., 1999]. Although we see band 6 binding in vitro, it is not clear what happens in vivo when the promoter is truncated at the *Acc* I site. Protein binding to the site is likely lost and protein-protein interactions over the region surrounding the *Acc* I site are disrupted.

We are attempting to identify factors that bind to the silencer sequence. Some potential factors were identified with the aid of TRANSFAC, a program to aid in identification of consensus binding elements [Heinemeyer et al., 1998]. Potential binding sites identified in the region from −948 to −930 include Sp1 (positive strand). Potential binding sites identified in the region from −903 to −887 include C/EBP beta [Chang et al., 1990] (positive strand) and AR [Chang et al., 1988] and HiNF-A [van Wijnen et al., 1988] (negative strand). Sites identified in the region from −855 to −835 include Oct-1 [Fletcher et al., 1987], YY1 [Shi et al., 1991], C/EBP1 [Chang et al., 1990], and SRF [Graham and Gilman, 1991] (positive strand) and cMyc [Halazonetis and Kandil, 1991] (negative strand). The Oct-1 binding site spans the *Acc* I restriction site used to delete the silencer region. It is possible that the silencer region extends through the region adjacent to and downstream from the *Acc* I site.

In conclusion, this study reveals a region upstream from the rat H1t gene that contains potential sites for repressor protein binding. Eukaryotic repressors work in one of several ways [Johnson, 1995]. By competing for specific activator binding sites on DNA they disrupt activator binding [MacDonald et al., 1995; Small et al., 1995]. By co-occupying sites on DNA with activators they interfere with the activity of the DNA-bound activator (quenching) as seen with cytokine transcriptional control [Scheinman et al., 1995] and insulin gene regulation [Robinson et al., 1995]. By interfering with the general transcriptional machinery they directly inhibit transcription [Fondell et al., 1993; Wahi and Johnson, 1995]. Protein binding data in this study are consistent with a model where a repressor protein complex binds to the silencer region between the *Pvu* II and *Xba* I restriction sites. A protein complex also binds to the region near the *Acc* I restriction site. Removal of the

promoter upstream from the *Acc* I restriction site eliminates binding to the *Pvu* II – *Xba* I region and alters binding to the silencer region relieving transcriptional repression as demonstrated in the luciferase expression assays shown in Figure 2.

ACKNOWLEDGMENTS

This research was supported by a Merit Review grant from the Department of Veterans Affairs (S.R.G.) and by NIH grant HD29381 (S.R.G.).

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