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Activation and Repression Domains Within the Promoter of the Rat Cathepsin L Gene Orchestrate Sertoli Cell-Specific and Stage-Specific Gene Transcription in Transgenic Mice[1]

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ABSTRACT

In murine testes, only Sertoli cells express the cathepsin L (*Ctsl*) gene, and this expression is restricted to stages V–VIII of the cycle. Our previous transgenic analysis of Tg (−2065/+977) demonstrated that this expression is regulated by a ~2-kb promoter. To begin to elucidate this regulation, we analyzed the in vivo expression of two new transgenes, Tg (−935/+977) and Tg (−451/+977). Tg (−935/+977) was expressed by Sertoli cells but, in contrast to Tg (−2065/+977), was expressed at all stages of the cycle, by spermatocytes, by the vascular endothelium, and by seven other organs. Tg (−451/+977) was not expressed by Sertoli cells but by spermatogenic cells and by the brain. Lack of expression of Tg (−451/+977) by Sertoli cells was not due to a lack of essential *cis*-acting elements. Transient transfection analysis of primary cultures of mature rat Sertoli cells demonstrated that in mature Sertoli cells, most of the activity of the *Ctsl* promoter is accounted for by one of two redundant upstream GC motifs and an Initiator that are within 100 bp of the transcription start site. We conclude that transcriptional repressors upstream from nucleotide −935 of the rat *Ctsl* gene restrict testicular expression of this gene to Sertoli cells at stages V–VIII. At these stages, transcriptional activators located between nucleotides −935 and −452 promote access of the transcriptional machinery to the two GC boxes and to the Initiator. Thus, upstream repressors and activators as well as *cis*-acting elements near the transcription start site control stage-specific *Ctsl* transcription by Sertoli cells.

cathepsin L, gene expression, gene regulation, promoter, Sertoli cells, spermatogenesis, transgenic, transgenic mice

INTRODUCTION

There is considerable evidence that extensive paracrine and juxtacrine interactions between the developing spermatogenic cells and their adjacent Sertoli cells are important for the fertility of male mammals. The normal functioning of Sertoli cells is essential for the replication and development of spermatogenic cells; mutations in a number of genes expressed by Sertoli cells reduce sperm production or cause infertility [1–5]. Additionally, there is substantial feedback of spermatogenic cells on Sertoli cells, and as the spermatogenic cells divide and develop, this feedback changes [6–9]. Consequently, as rat spermatogonia, spermatocytes, and spermatids progress in synchrony through the stages of the cycle of the seminiferous epithelium, the Sertoli cells with which they associate alter their expression of more than 150 genes, maximally expressing different genes at different stages [10]. Because Sertoli cells are the somatic cells whose in vivo functions are essential to spermatogenesis and fertility, it is important to understand the mechanisms that control their gene expression, and in particular how feedback from the spermatogenic cells with which they are associated controls their stage-specific gene expression.

An important experimental strategy by which to define how stage-specific gene expression is regulated in Sertoli cells is the identification of promoters that drive in vivo expression of a transgene in Sertoli cells in a manner that is comparable to the expression of the endogenous gene. A considerable strength of this strategy is that the function of the promoter is studied in vivo in Sertoli cells that are interacting normally with spermatogenic cells. Moreover, the successful execution of this strategy can provide a strong rationale for the use of transient transfection and DNA-protein interaction analyses to test the functions and transcription factor-binding activities of potential *cis*-acting elements within the identified promoter.

To date, researchers have identified promoters of only two genes—*Rhox5* and cathepsin L (*Ctsl*)—that drive reporter gene expression in the same stage-specific pattern as the endogenous gene [11, 12]. A 300-bp *Rhox5* promoter has been shown to drive transgene expression in Sertoli cells, and a GATA-binding site has been demonstrated to be essential for the activity of this promoter [11]. Our laboratory has focused on the *Ctsl* gene, which encodes a cysteine protease that, in the testes of mice and rats, is expressed only by Sertoli cells [5, 13, 14]. Mice that harbor a mutation that inactivates cathepsin L catalytic activity exhibit an increased incidence of seminiferous tubule atrophy and reduced numbers of germ cells in otherwise normal tubules, indicating that cathepsin L is required for quantitatively normal spermatogenesis [5]. In mice and rats, the expression of the *Ctsl* gene by Sertoli cells is highly stage specific; *Ctsl* mRNA levels are maximal at stages VI–VII and are low or undetectable at all other stages [5, 14]. We have shown that the *cis*-acting elements regulating transcription of the rat *Ctsl* gene by Sertoli cells are contained within a ~3-kb genomic fragment that spans nucleotides −2065 to +977, where +1 denotes the transcription start site (TSS; Fig. 1A). Our studies demonstrated that a transgene, Tg (−2065/+977), containing this genomic fragment is expressed in a Sertoli cell-specific manner, and the stage-specific expression of this transgene matches that of the endogenous gene [12].

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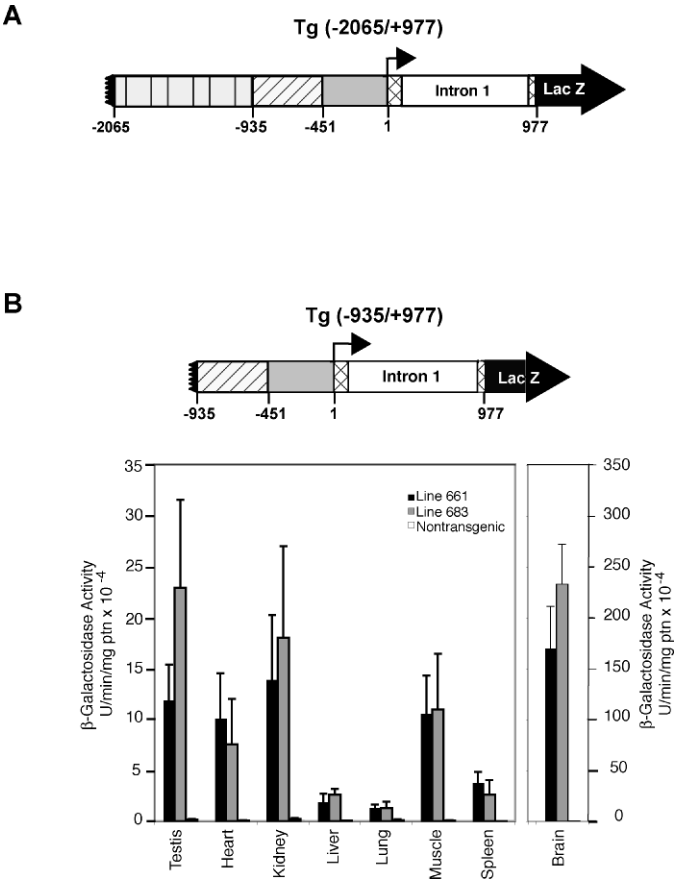

FIG. 1. Expression of Tg (−935/+977) in various organs of transgenic mice and schematic representations of Tg (−2065/+977) and Tg (−935/+977). **A**) Tg (−2065/+977) has been shown previously to drive both stage-specific and Sertoli cell-specific gene expression in transgenic mice [12]. The promoter contains 2065 nucleotides upstream of the TSS (black box), the first exon (74 nucleotides; hatched box), the first intron (892 nucleotides; white box), and the first 11 nucleotides of exon 2 (hatched box) up to the ATG initiation codon at nucleotide +978. The position of the TSS is indicated by the bent arrow. The numbering is relative to the *Ctsl* TSS, which is designated +1. For comparison with the results presented in this paper, the previously published data for expression of Tg (−2065/+977) in various organs are provided in Supplemental Table S2. **B**) β-Galactosidase enzymatic activity in testis and other organs of two lines of mice expressing the transgene Tg (−935/+977). Data (n = 3; means ± SEM) are shown for transgenic mice in line 661 and line 683, as well as for control, nontransgenic mice. In both transgenic lines, β-galactosidase activity in each organ was statistically greater than the activity in the same organ of control mice. The mice used for this analysis were 90 to 150 days old. ptn, protein.

The experiments described in the present study constitute the first major step in identifying the mechanisms that regulate Sertoli cell-specific and stage-specific transcription of the *Ctsl* gene. These experiments address three fundamental questions. What is the mechanism responsible for the expression of the endogenous *Ctsl* gene by Sertoli cells but not by other testicular cell types [5, 13, 14]? Does stage-specific expression of the *Ctsl* gene result from the activity of one or multiple *cis*-acting regulatory elements? Are these elements activators or repressors of transcription or, alternatively, are both types of elements required for stage-specific transcription of this gene? In the present study, we address these questions by analyzing the expression of two new *Ctsl* transgenes in which approximately 1000 bp and 1500 bp, respectively, were deleted from the 5′ end of Tg (−2065/+977).

MATERIALS AND METHODS

DNA Constructs

To increase our ability to identify the cells expressing the reporter gene, β-galactosidase, we fused the SV40 nuclear localization signal (NLS; P-K-K-K-R-K-V) to the N terminus of bacterial β-galactosidase, creating NLS-LacZ [15]. The Supplemental Methods (see also Supplemental Table S1; all Supplemental Data are available online at www.biolreprod.org) describe the assembly of this construct.

Two new transgenic constructs were assembled: Tg (−935/+977) and Tg (−451/+977). For Tg (−935/+977), the *Ctsl* promoter fragment was amplified by PCR using primers: −935-*Kpn*I-F and +977-*Sal*I-R (Supplemental Table S2). For Tg (−451/+977), the *Ctsl* promoter sequence was amplified by PCR using: −451-*Kpn*I-F and +977-*Sal*I-R (Supplemental Table S2). The PCR fragments were digested with *Kpn*I and *Sal*I and then subcloned into NLS-LacZ that had been digested with the same two enzymes. The sequences of the PCR fragments were verified by DNA sequencing. Transient transfection assays using the TM4 Sertoli cell line were performed to establish that the reporter gene LacZ was expressed and that β-galactosidase was localized to the nucleus. Supplemental Figure S1 documents that fusing the NLS to LacZ concentrates β-galactosidase in the nucleus but has no deleterious effect on enzymatic activity (Supplemental Results).

We also used transient transfection analysis to reevaluate the function of a previously described 110-bp proximal promoter of the *Ctsl* gene [16]. In these experiments, we studied the function of the proximal promoter when it was contained in a region spanning either 156 or 2065 bp upstream from the TSS. We generated a series of *Ctsl* reporter constructs containing specific mutations or deletions in the *Ctsl* promoter and tested their activities in Sertoli cells isolated from mature rats. To simplify their assembly, these constructs lacked the first intron but encoded the entire 5′ untranslated region (UTR) of *Ctsl* mRNA. *Ctsl* (−2065/5′ UTR)-Luc, whose assembly has been described previously [17], was used both in transient transfection analysis of mature Sertoli cells and as the template for the following constructs: *Ctsl* (−2065/5′ UTR/mut GC1)-Luc, *Ctsl* (−2065/5′ UTR/mut Inr)-Luc, *Ctsl* (−2065/5′ UTR/delete −156 to −13)-Luc, *Ctsl* (−156/5′ UTR)-Luc, *Ctsl* (−156/5′ UTR/mut GC1)-Luc, and *Ctsl* (−156/5′ UTR/mut GC2)-Luc. The relevant genomic fragments used in these constructs were generated by PCR. Mutations were introduced into the *Ctsl* promoter using oligonucleotides that altered the sequences of the DNA motif whose function in the *Ctsl* promoter was being tested. (See Supplemental Table S2 for sequences of the primers.) *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc was generated using *Ctsl* (−2065/5′ UTR/mut GC1)-Luc as the template and appropriate primers (Supplemental Table S2). *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc was used as the template for *Ctsl* (156/5′ UTR/mut GCs 1 and 2). Detailed descriptions of the assemblies of the new constructs are provided in Supplemental Methods.

Production of Transgenic Mice and Analysis of β-Galactosidase Activity in Organs and Cells

Plasmids containing Tg (−935/+977) and Tg (−451/+977) were digested with *Kpn*I and *Hin*dIII, and the 5470- and 4985-bp DNA fragments that contain the transgenes were purified and injected into single-cell B6SJL/F1 embryos, and the embryos were transferred to pseudopregnant females as described previously [12]. Transgenic offspring were identified by PCR analysis of DNA isolated from mouse tails using a forward primer (5′-GCGCCGGGTACCC TAGATTTTTCTAAATTCT-3′) and a reverse primer (5′-AGTATGTC GACGGTTCAAACACCTGGGGAA-3′) corresponding to nucleotides −89 to −67 and +103 to +122 of the rat *Ctsl* gene, respectively. Each founder was bred to wild-type B6SJL/F1 mice to obtain heterozygous F(1), F(2), and F(3) male transgenic offspring.

The Institutional Animal Care and Use Committee of Johns Hopkins University approved the use of animals for all of the studies described in this report.

Measurement of β-Galactosidase Activity

β-Galactosidase enzymatic activity in testis, spleen, muscle, lung, liver, kidney, heart, and brain was quantified as described previously [12]. Reporter gene enzymatic activity was expressed as (relative light) units per minute per milligram of protein [12]. Enzyme activity was also measured in isolated seminiferous tubules, testicular interstitial tissue, a pool of spermatogenic cells, and freshly isolated mature Sertoli cells. Seminiferous tubules and interstitial tissue that included small blood vessels were isolated by manual dissection. Mixtures of spermatogenic cells were isolated as

described previously [18]; phase-contrast microscopy revealed that the purity of spermatogenic cells exceeded 95%. Sertoli cells were isolated from seminiferous tubules as described by Anway et al. [19]. Phase-contrast microscopy showed that Sertoli cells comprised 80%–90% of the isolated cell population, with the remaining 10%–20% being spermatogenic cells. Immediately after isolation, cells and seminiferous tubules, as well as a small fragment of the testis from which they had been isolated, were each homogenized in 150–250 μl of 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithiothreitol, 0.2 mM PMSF, and 5 μg/ml leupeptin. All organ, tissue, and cell homogenates were centrifuged at $12\,500 \times g$ and were then either analyzed immediately for β-galactosidase activity or were flash frozen in liquid nitrogen and stored at −80°C. Frozen samples were rapidly thawed in a 37°C water bath prior to analysis of enzymatic activity.

Cytochemical Localization of β-Galactosidase Enzyme Activity

β-Galactosidase enzymatic activity was localized to cells within testis sections using previously described cytochemical methods, and the tissues then were counterstained with nuclear fast red [12]. Images were captured using a Nikon Eclipse E800 (Melville, NY) microscope equipped with a digital camera (Princeton Instruments, Trenton, NJ) [12]. The stage of the cycle of cross-sections of seminiferous tubules containing Sertoli cells expressing β-galactosidase was determined [12]. Each β-galactosidase-positive Sertoli cell was counted, and the stage of the cycle of the tubule in which it resided was identified as stage I–V, VI–VIII, or IX–XII. Each of these three sets of stages represents approximately one third of the duration of one cycle of the seminiferous epithelium of the mouse [20]. Numbers of β-galactosidase-expressing Sertoli cells at each of these three sets of stages were divided by the total number of β-galactosidase-expressing Sertoli cells in each testis. The numbers of β-galactosidase-expressing Sertoli cells at stages I–V, VI–VIII, or IX–XII were expressed as the percentage of all β-galactosidase-positive Sertoli cells in each testis.

Immunocytochemical Localization of β-Galactosidase in Testis Sections

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Once the animals were completely unresponsive to a tail pinch, they were injected with 1000 units of heparin and then perfused via the left ventricle with PBS, and then 2.5% acrolein, 4% paraformaldehyde in PBS. The testes were postfixed with 4% paraformaldehyde and 30% sucrose and were embedded in egg gelatin, and the embedded tissue was postfixed again with 4% paraformaldehyde. Frozen sections (30 μm) were cut on a Leica SM200R frozen microtome (Bannockburn, IL), and sections were incubated in a 1:500 000 dilution of rabbit antibacterial β-galactosidase (no. AB986; Chemicon, Temecula, CA) or control rabbit immunoglobulin G (IgG). Antigen-bound IgG was detected using a Vectastain Elite-AP kit (Vector Laboratories, Burlingame, CA). The immunocytochemical signal was enhanced using a nickel sulfate-diaminobenzidine chromagenic solution. After development of the chromagenic reaction, the tissue sections were dehydrated and mounted in Histomount (National Diagnostics, Atlanta, GA).

Isolation of Sertoli Cells from Sexually Mature Rats and Transient Transfection Analysis of Reporter Constructs

Mature (60- to 70-day-old) Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Sertoli cells were isolated, cultured at a density of $\sim 1.6 \times 10^5$ cells/cm^2, and transfected as described previously [18, 21]. Transient transfections were performed using 15 μL of lipofectamine (Invitrogen Corp., Carlsbad, CA), 0.45 pmol of Firefly luciferase (Luc) reporter gene constructs that contained *Ctsl* promoter sequences, and 0.009 pmol of plasmid pRL-CMV (Promega Corp., Madison, WI). pRL-CMV was used to correct for variations in transfection efficiency. Firefly and *Renilla* luciferase activities were measured using the dual luciferase assay (Promega).

Statistical Analysis

Data were first analyzed by ANOVA, and then differences between individual means were established using Fisher protected least significant difference test (StatView 5.01; SAS Institute Inc., Cary, NC). Statistically significant differences were defined as $P < 0.05$.

RESULTS

Analysis of the Expression of Tg (−935/+977) and Tg (−451/+977) in Transgenic Mice

We have demonstrated previously that a region spanning −2065 to +977 of the rat *Ctsl* gene contains all of the *cis*-acting regulatory elements that are required to drive Sertoli cell-specific and stage-specific transcription. (To facilitate the comparison of the expression of the published transgene to the two transgenes described in the present study, the expression of Tg (−2065/+977) in different organs of transgenic mice is summarized in Supplemental Table S3.) Therefore, the goals of analyzing the expression of Tg (−935/+977) and Tg (−451/+977) in mice were to begin to identify domains within this ~3-kb fragment that contain these regulatory elements and to also gain insight into the functions of these elements. Furthermore, we sought to determine the mechanism by which the endogenous *Ctsl* gene is expressed by Sertoli cells but not by other testicular cell types, whether stage-specific expression of the *Ctsl* gene results from the activity of one or multiple *cis*-acting regulatory elements, and whether these elements are activators and/or repressors of transcription.

Expression of Tg (−935/+977)

We identified five independent lines of mice that carried Tg (−935/+977), three of which had male founders. Two of these males (661 and 683) founded lines that expressed Tg (−935/+977). Both lines produced similar numbers of transgenic males and females in the F(1) and F(2) generations, and the transgene was transmitted according to Mendelian laws. Biochemical analysis revealed that β-galactosidase enzymatic activity was 200- to 300-fold higher in testes obtained from the transgenic mice carrying Tg (−935/+977) than from nontransgenic mice (Fig. 1B). However, in contrast to Tg (−2065/+977), in which expression was exclusively in the testis (Charron et al. [12] and Supplemental Table S3), Tg (−935/+977) was also expressed in brain, heart, kidney, striated muscle, liver, lung, and spleen. Incubation of fixed testes from both lines of transgenic mice with the histochemical β-galactosidase substrate, X-gal, produced a robust reaction product in seminiferous tubules and in blood vessels. Within the seminiferous tubules of both lines of transgenic mice, the X-gal reaction product was concentrated near the nuclear membranes of Sertoli cells (Fig. 2, A, B, and D) and also in the nuclei of a subset of spermatogenic cells (Fig. 2, B and C). Additionally, some cells in the endothelium of testicular blood vessels expressed the transgene at very high levels (Fig. 2F). As expected, no X-gal reaction product was detected in testes of nontransgenic mice (Fig. 2E). In striking contrast, our previous studies demonstrated that Tg (−2065/+977) is only expressed by Sertoli cells [12]. The observation that Tg (−935/+977) is expressed by multiple cell types suggests that there must be a domain within the 5′ end of the *Ctsl* promoter (nucleotides −2065 to −935 of the rat *Ctsl* gene) that contains one or more *cis*-acting repressors whose function is to suppress transcription of the *Ctsl* gene in all testicular cells except Sertoli cells.

To determine whether Tg (−935/+977) was expressed in a stage-specific manner by Sertoli cells, we serially sectioned one testis from each transgenic line and enumerated Sertoli cells that expressed the transgene in seminiferous tubules at stages I–V, VI–VIII, and IX–XII. In marked contrast to the stage-specific expression of Tg (−2065/+977) that we reported previously [12], Sertoli cells at all stages of the cycle expressed Tg (−935/+977) (Fig. 3). Thus, although Tg (−935/+977)

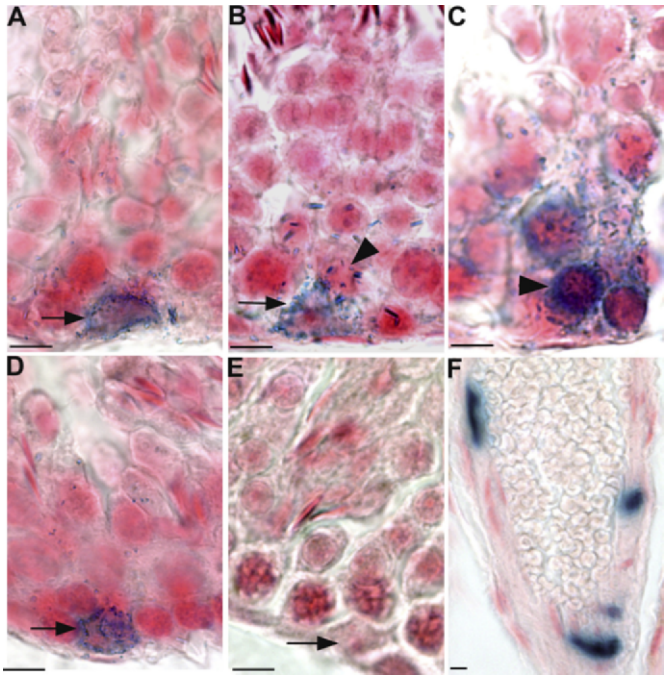

FIG. 2. Expression of Tg (−935/+977) in specific testicular cell types. To identify the cells expressing Tg (−935/+977), fixed testes were incubated in X-gal, which is metabolized by β-galactosidase to produce an insoluble blue reaction product. The testes were then embedded in paraffin, and 8-μm sections were cut and counterstained with nuclear fast red. **A**) Expression of the transgene at stage I or II. Arrow points to a Sertoli cell nucleus. **B**) Expression of the transgene at stage VII. Arrow points to a Sertoli cell nucleus; arrowhead points to a preleptotene spermatocyte nucleus. **C**) Expression of the transgene at stage X. Arrowhead points to a spermatocyte nucleus. **D**) Expression of the transgene at stage XI. Arrow points to a Sertoli cell nucleus. **E**) Lack of β-galactosidase activity in a stage IV or V seminiferous tubule of a control, nontransgenic mouse. Arrow points to a Sertoli cell nucleus. **F**) Expression of Tg (−935/+977) in a testicular blood vessel. Bars = 10 μm on the original tissue section.

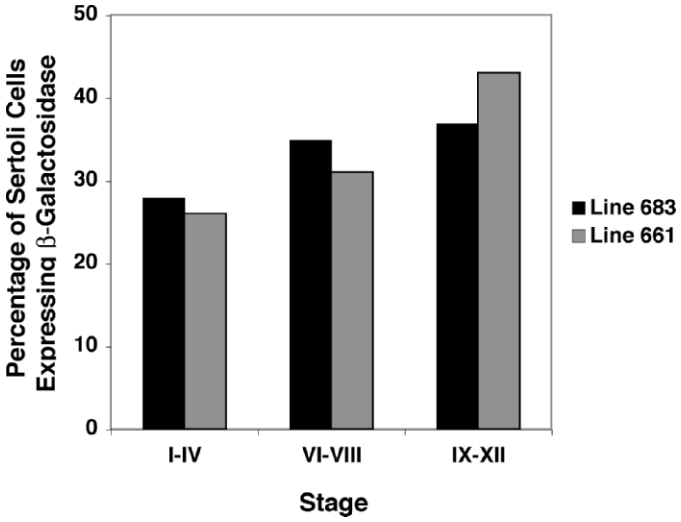

FIG. 3. The transgene Tg (−935/+977) is expressed by Sertoli cells at all stages of the cycle of the seminiferous epithelium. One testis from line 661 and one testis from line 683 were incubated in X-gal, embedded in paraffin, and serially sectioned. β-Galactosidase-positive Sertoli cells in stage I–V, VI–VIII, or IX–XII tubules were counted. These groups of stages occur with similar frequency within a mouse testis [21]. The numbers of β-galactosidase-expressing Sertoli cells at stages I–V, VI–VIII, or IX–XII were expressed as the percentage of all β-galactosidase-positive Sertoli cells in each testis. The mice used for this analysis were 90–100 days old.

contains the *cis*-acting elements that are required for Sertoli cell expression, this transgene lacks the regulatory elements required for stage-specific expression. These data lead us to conclude that these regulatory elements are *cis*-acting repressors that reside in the same domain (nucleotides −2065 and −935 of the rat *Ctsl* gene) as the repressors that restrict expression of the endogenous *Ctsl* gene to Sertoli cells.

Expression of Tg (−451/+977) in Transgenic Mice

We identified six independent lines of mice that expressed Tg (−451/+977), three of which had male founders. Two of these males (251 and 263) founded lines that expressed the transgene. There were similar numbers of transgenic males and females in the F(1) and F(2) generations in both of these lines, and the transgene was transmitted according to Mendelian laws. In both lines, β-galactosidase enzymatic activity was substantially higher in testes of transgenic mice than in testes of nontransgenic animals. However, in contrast to Tg (−935/+977), which was expressed in all organs that we tested, in mice carrying the Tg (−451/+977) transgene, β-galactosidase enzymatic activity was only detected in the testis and in the brain (Fig. 4, Supplemental Results, and Supplemental Fig. S2).

To identify the testicular cell type(s) expressing Tg (−451/+977), we initially incubated testes from both transgenic lines with X-gal and processed the testes for histology. However, this procedure, which we successfully used to identify the cells in transgenic testes expressing Tg (−2065/+977) and Tg (−935/+977), failed to detect expression of Tg (−451/+977) in any testicular cell type. We therefore employed a potentially more sensitive immunocytochemical method. Analysis of both transgenic lines revealed that β-galactosidase was present in the

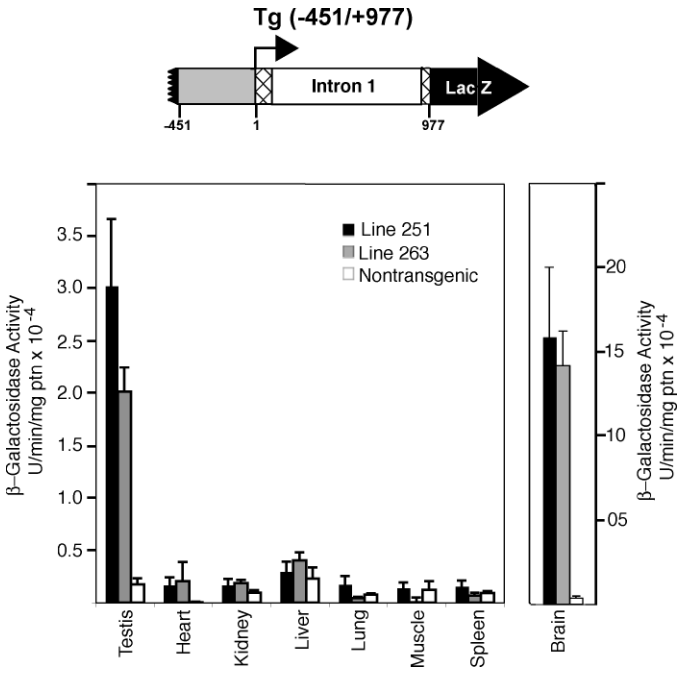

FIG. 4. β-Galactosidase enzymatic activity in testis and various other organs of two lines of mice expressing the transgene Tg (−451/+977). Data (n = 3; means ± SEM) are shown for transgenic mice in lines 251 and 263 as well as for control, nontransgenic mice. In both transgenic lines, β-galactosidase activity was statistically greater in testis and brain than in the comparable organs of control mice. ptn, protein.

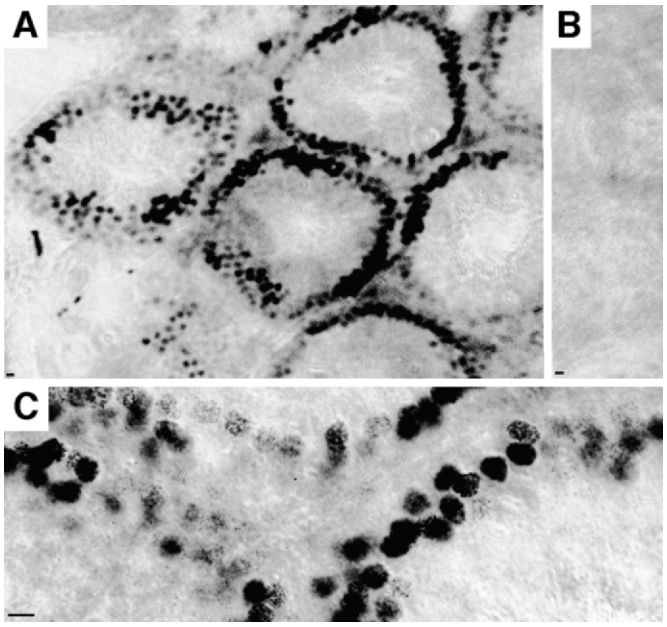

FIG. 5. Immunocytochemical localization of β-galactosidase in a testis of a Tg (−451/+977) mouse. **A**) Immunocytochemical localization of β-galactosidase in a testis section of a transgenic mouse. **B**) Lack of β-galactosidase antigen in a testis of a control, nontransgenic mouse. **C**) Higher magnification of cells expressing β-galactosidase in a transgenic testis. Intensely staining cells are pachytene spermatocytes. The binding of antibody to its antigen in the tissue sections was detected by development of a peroxidase reaction product. Images were taken using phase-contrast optics. Bars = 10 μm on the original tissue section.

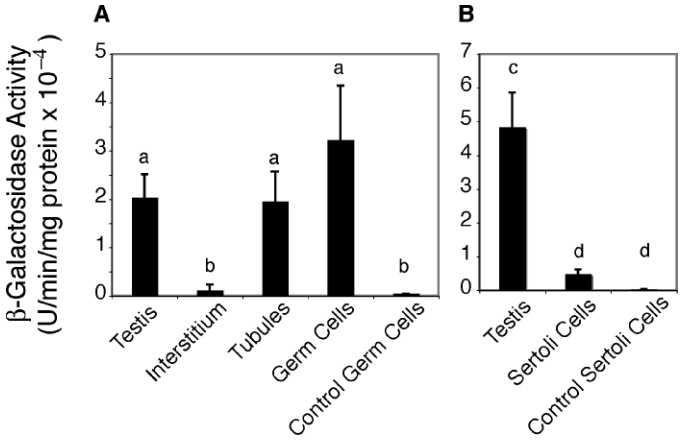

FIG. 6. β-Galactosidase activity in testes, interstitium, seminiferous tubules, germ cells, and Sertoli cells of mice carrying the Tg (−451/+977) or nontransgenic, control mice. Seminiferous tubules, spermatogenic cells, and interstitium were isolated and analyzed in a separate experiment from Sertoli cells. Tubules and cells were isolated from lines 251 and 263, and data (mean ± SEM) were pooled. **A**) Measurement of β-galactosidase activity in testes, interstitium, seminiferous tubules, and germ cells of transgenic mice. β-Galactosidase activity in germ cells isolated from control, wild-type mice was also measured. **B**) Measurement of β-galactosidase activity in testes of transgenic mice, in Sertoli cells of transgenic mice, and in Sertoli cells isolated from testes of control, nontransgenic mice. Data were obtained from cells isolated from two mice of line 263 and from cells isolated from one mouse of line 251. Data from both lines were similar, and data were pooled for purposes of statistical analysis. Means labeled by different letters differ statistically from the other means in that part of the experiment.

nuclei of spermatogenic cells, particularly pachytene spermatocytes, but not in Sertoli cells or in any other testicular somatic cell type, including the vascular endothelium (Fig. 5). To confirm these results, we compared β-galactosidase enzymatic activity in total testis, seminiferous tubules, interstitial tissue that included blood vessels, and a pool of spermatogenic cells. As negative controls, enzymatic activity was assayed in spermatogenic cells isolated from nontransgenic mice. The results from the two transgenic lines were similar; significant enzymatic activity was detected in total testis, in seminiferous tubules, and in spermatogenic cells, but not in interstitial tissue (Fig. 6A). Figure 6B shows the results of a second experiment in which we compared β-galactosidase enzymatic activity in Sertoli cells isolated from transgenic and control (nontransgenic) mice. As a positive control, we also measured enzymatic activity in a fragment of the transgenic testes from which the Sertoli cells were isolated. Although robust β-galactosidase activity was detected in the testis of both lines of transgenic mice, the low level of activity measured in Sertoli cells did not differ statistically from the activity in Sertoli cells isolated from control, nontransgenic mice. To expand this analysis, we also isolated Sertoli cells and spermatogenic cells from one mouse of each transgenic line and then measured β-galactosidase activity in the four preparations of cells. Results from both lines were identical. β-Galactosidase enzymatic activity in spermatogenic cells was ~7-fold higher than the activity in Sertoli cells, and the residual activity in the Sertoli cell fraction could be explained by a low level of contamination with spermatogenic cells. Taken together, our results indicate that unlike Tg (−2065/+977) and Tg (−937/+977), Tg (−451/+977) is not expressed by Sertoli cells, suggesting that the in vivo activity of the *Ctsl* promoter in Sertoli cells requires one or more *cis*-acting elements within a domain that spans from −935 to −452 of the rat gene.

Identification of cis-Acting Regulatory Elements That Confer Ctsl Promoter Activity in Mature Sertoli Cells

Previously conducted transient transfection analysis using Sertoli cells isolated from mature rats had demonstrated that a reporter construct that contained only the *Ctsl* proximal promoter (−87 to +33 relative to the TSS) had the same activity as a construct that contained ~2 kb of upstream sequence. The activity of this proximal promoter was completely abolished upon mutation of a single GC motif, designated GC1, that bound SP3 in nuclear extracts prepared from mature Sertoli cells [16]. Those results led us to conclude that the binding of SP3 to the proximal promoter drives expression of the *Ctsl* gene in Sertoli cells. However, the observation that Tg (−451/+977) was not expressed in vivo in Sertoli cells was not consistent with this conclusion. Why did the results obtained by transgenic analysis disagree with results obtained by transient transfection analysis? In our previous experiments, the proximal promoter was studied outside of its normal molecular context, the ~2 kb of sequence upstream from the TSS that is required for proper in vivo function of the *Ctsl* promoter in Sertoli cells. We therefore decided to test the function of the proximal promoter when it was present within a larger fragment of the *Ctsl* gene. Three experiments analyzed the activities of a series of *Ctsl* reporter constructs that were transfected into Sertoli cells that had been isolated from mature (60- to 70-day-old) rats.

The first experiment tested the function of GC1 in the context of 156 bp of the *Ctsl* gene immediately upstream from the TSS. We introduced a mutation into GC1 that had previously been shown to abrogate the activity of a reporter

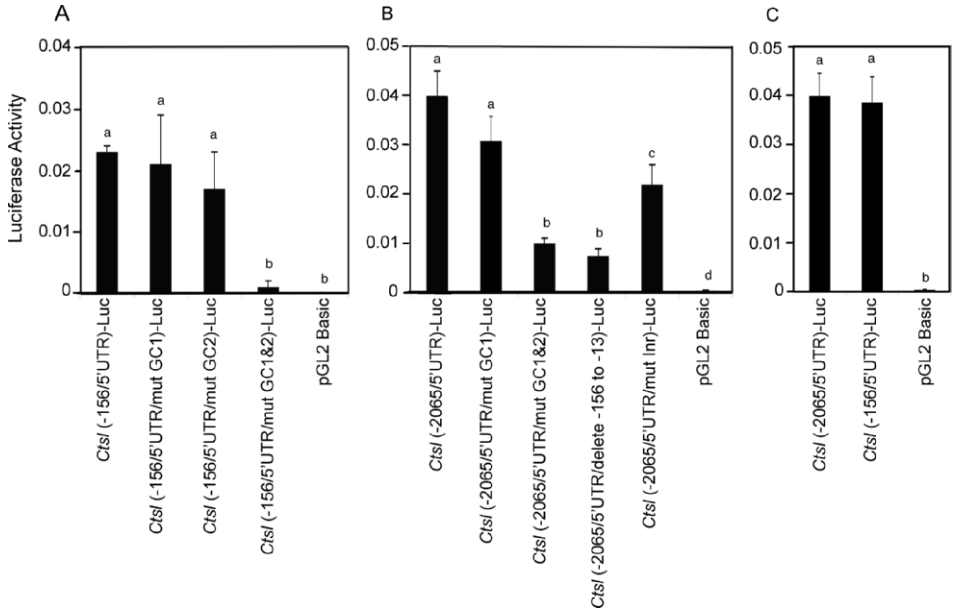

FIG. 7. Identification of *cis*-acting regulatory elements that confer *Ctsl* promoter activity when transfected into Sertoli cells isolated from sexually mature rats. **A**) Demonstration that GC1 and GC2 have redundant activities when tested in the context of 156 bp of *Ctsl* promoter immediately upstream from the TSS. Mature rat Sertoli cells were transfected with one of the following reporter constructs: *Ctsl* (−156/5′ UTR)-Luc, *Ctsl* (−156/5′ UTR/mut GC1)-Luc, *Ctsl* (−156/5′ UTR/mut GC2)-Luc, *Ctsl* (−156/5′ UTR/mut GCs 1 and 2)-Luc, or the negative control plasmid, pGL2 Basic. **B**) Demonstration that GC1 and GC2 have redundant activities when tested in the context of 2065 bp of *Ctsl* promoter immediately upstream from the TSS and the demonstration that the Initiator (Inr) is required for full promoter activity. Mature Sertoli cells were transfected with one of the following *Ctsl* reporter constructs: *Ctsl* (−2065/5′ UTR)-Luc, *Ctsl* (−2065/5′ UTR/mut GC1)-Luc, *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc, *Ctsl* (−2065/5′ UTR/delete −156 to −13)-Luc, *Ctsl* (−2065/5′ UTR/mut Inr)-Luc, or pGL2 basic. **C**) The 156 bp of *Ctsl* promoter immediately upstream from the TSS exhibits maximal activity. Mature Sertoli cells were transfected with *Ctsl* (−2065/5′ UTR)-Luc, *Ctsl* (−156/5′ UTR)-Luc, or pGL2 basic. In all three experiments, potential differences in transfection efficiency were corrected by cotransfecting the Sertoli cells with pRL-CMV, which encodes *Renilla* luciferase. At 24 h after transfection, Sertoli cells were collected, luciferase activities were measured, and Luc activity was defined as firefly luciferase activity/*Renilla* luciferase activity. Data (mean ± SEM) were obtained from four independent replicates of each experiment. Means labeled with different letters differ statistically. GC1&2, GCs 1 and 2.

construct that contained only the *Ctsl* proximal promoter [16] and compared the activity of this reporter construct with a similar reporter construct with an intact GC1. Surprisingly, in the context of 156 bp upstream from the TSS, mutation of GC1 did not have a significant effect on *Ctsl* promoter activity (Fig. 7A, compare *Ctsl* (−156/5′ UTR/mut GC1)-Luc with *Ctsl* (−156/5′ UTR)-Luc). The presence of a second, upstream GC box might explain this unexpected outcome, and computational analysis of nucleotides −2065 to +1 of the rat *Ctsl* gene identified an additional 24 potential GC boxes (www.cbil.upenn.edu/cgi-bin/tess/tess). One GC box that spans nucleotides −98 to −92 (hereafter referred to as GC2) is present in *Ctsl* (−156/5′ UTR/mut GC1)-Luc. GC2 was of interest because it was only 13 nucleotides upstream from GC1 and because its location is conserved between the rat and mouse genes (data not shown). However, mutation of GC2 also did not affect promoter activity (Fig. 7A, compare *Ctsl* (−156/5′ UTR/mut GC2)-Luc with *Ctsl* (−156/5′ UTR)-Luc). In contrast, when both GC1 and GC2 were mutated, *Ctsl* promoter activity was lost (Fig. 7A, compare *Ctsl* (−156/5′ UTR/mut GC1 and GC2)-Luc with pGL2 Basic).

The goal of the second experiment was to determine whether the redundant functions of GC1 and GC2 were conserved in the context of the 2065-bp region located upstream from the TSS of the *Ctsl* gene. Consistent with the first study, mutation of GC1 had no effect on promoter activity, whereas mutation of both GC1 and GC2 reduced *Ctsl* promoter activity in mature Sertoli cells by 75% (Fig. 7B, compare *Ctsl* (−2065/5′ UTR)-Luc with *Ctsl* (−2065/5′ UTR/mut GC1)-Luc and with *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc). Additionally, deletion from −156 to −13 bp had the same effect as the mutations GC1 and GC2 together, indicating that these two motifs were the only functional elements within this 143-bp region of the *Ctsl* promoter (Fig. 7B, compare *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc with *Ctsl* (−2065/5′ UTR/delete −156 to −13)-Luc). We identified only one other functional *cis*-acting element in the proximal promoter, the Initiator (−1 to +6) that recruits the core transcription apparatus to the start site [22]. Mutation of the Initiator reduced promoter activity by 50% (Fig. 7B, see *Ctsl* (−2065/5′ UTR/mut Inr)-Luc). Thus, almost all of the transcriptional activity of the 2065 bp of the *Ctsl* gene upstream from the TSS can be explained by the activities of either GC1 or GC2 and the activity of the Initiator. Other GC boxes that sequence analysis predicts are upstream from GC1 and GC2 might explain the residual function of *Ctsl* (−2065/5′ UTR/mut GCs 1 and 2)-Luc. However, a third experiment demonstrated that the activity in mature Sertoli cells of *Ctsl* (−156/5′ UTR)-Luc, which contains only the two proximal GC boxes, GC1 and GC2, was identical to the activity of *Ctsl* (−2065/5′ UTR)-Luc (Fig. 7C). Therefore, these putative upstream GC boxes do not have a significant effect on *Ctsl* promoter activity when GC1 or GC2 is intact. Taken together, the data in Figure 7 indicate that no upstream *cis*-acting elements replace the function of the proximal promoter of the *Ctsl* gene when it is within its normal genomic context. Therefore, the lack of expression of Tg (−451/+977) by Sertoli cells in vivo must not be due to a lack of required transcription factor-binding sites within the *Ctsl* promoter fragment. Rather, in vivo, those transcription factors must be unable to access their cognate binding sites in the transgene.

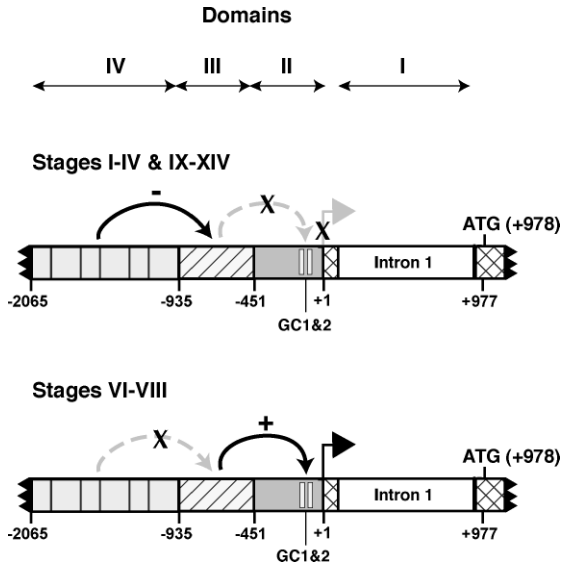

FIG. 8. A proposed model to explain how stage-specific expression of the *Ctsl* gene in rat Sertoli cells is regulated. Research described in this paper and preceding papers [12, 16, 17] identifies four functional domains within the region that spans −2065 to +977 of the *Ctsl* gene. Domain II contains two redundant GC boxes, GC1 and GC2. We propose that access of SP transcription family members to these sites is regulated by *cis*-acting elements within domains III and IV. This model predicts that at stages VI–VIII, domain III recruits transcription factors that alone or in conjunction with coactivators relax the chromatin structure of domain II. We propose that this relaxation allows access of SP family members to GC1 and GC2. Expression of the *Ctsl* gene at stages VI–VIII is then enhanced by the action of domain I, the first intron. This model also predicts that at stages I–IV and IX–XIV, *cis*-acting elements in domain IV block the ability of domain III to relax the chromatin structure of domain II, rendering the GC boxes inaccessible to SP family members. Thus, we propose that stage-specific expression of the *Ctsl* gene by Sertoli cells results from the activities of upstream elements in domains III and IV that regulate the chromatin structure of domain II. GC1&2, GCs 1 and 2.

DISCUSSION

The major objectives of the studies reported in the present study were to determine why the testicular expression of the *Ctsl* gene is restricted to Sertoli cells, and why its expression is stage specific. The data presented in this paper support the conclusion that there are one or more repressor elements upstream from −935 of the rat gene that suppress *Ctsl* expression in spermatogenic cells and in the vascular endothelium, as well as in all of the other organs that we examined. Our data also indicate that Sertoli cell expression of the *Ctsl* gene is regulated by multiple *cis*-acting elements that are distributed across a number of functional domains within the promoter. One or more of these elements is a *cis*-acting repressor that suppresses expression of this gene at all stages except stages V–VIII. Therefore, we suggest that the episodic activity of these repressors is the principal cause for stage-specific expression of the *Ctsl* gene by Sertoli cells.

A more complete view of the regulation of expression of the *Ctsl* gene in murine Sertoli cells is obtained when the data described in this paper are combined with data from our previous publications [12, 16–18]. Taken together, the data identify four functional domains in the *Ctsl* gene that interact to precisely control its expression in Sertoli cells (Fig. 8).

Domain I: The First Intron

Transient transfection analysis of mature Sertoli cells led us to conclude that the *Ctsl* first intron stimulates gene expression approximately 5-fold by increasing the total amount of transcript that is produced and by enhancing transcript polyadenylation and translation [17]. Therefore, once stage-specific transcription of the *Ctsl* gene is induced, the high amount of cathepsin L protein synthesized by Sertoli cells in stage VI and VII seminiferous tubules is due in part to activity of the first intron. It should be noted, however, that results from the present study clearly show that the first intron does not encode the *cis*-acting elements essential to stage-specific transcription, because Tg (−935/+977), which contains the first intron, is expressed at all stages of the cycle of the seminiferous epithelium.

Domain II: The Proximal Promoter Plus GC2 (Nucleotides −98 to +6)

Transient transfection analysis employing Sertoli cells isolated from sexually mature rats clearly established that almost all of the promoter activity of the 2-kb fragment of the *Ctsl* gene upstream from the TSS is due to either of two GC boxes within 100 bp of the start site as well as to an Initiator element. We predict that in Sertoli cells, SP3 is the candidate transactivator of domain II. This prediction is based on our having shown that this is the transcription factor in extracts of cultured Sertoli cells that binds GC1 in vitro, and because we have localized SP3 to Sertoli cells in vivo by immunocytochemistry (Charron et al. [16] and Wright, unpublished results). Therefore, in vivo, Sertoli cells express the transcription factor required for transactivation of Tg (−451/+977). Nonetheless, in vivo, Sertoli cells do not express this transgene. This lack of expression must result either from an inability of SP3 to access the critical GC boxes or to an inability of the bound transcription factor to transactivate the transgene.

One potential mechanism for the inhibition of SP3 binding is methylation of CpG dinucleotides in the transgene, followed by recruitment of a transcriptionally repressive multiprotein complex that compacts the chromatin [23–30]. However, although the rat *Ctsl* gene contains a CpG island (spanning −528 to +719; http://cpgislands.usc.edu), the endogenous *Ctsl* gene is expressed in many different cell types, suggesting that the CpG island that exists both within the endogenous *Ctsl* gene and within Tg (−451/+977) is not effectively targeted for DNA methylation [14].

A second mechanism potentially responsible for silencing of Tg (−451/+977) by Sertoli cells in vivo is the recruitment of histone-modifying enzymes that catalyze posttranslational modifications, such as deacetylation of lysines 9 or 14 and methylation of lysines 9, 27, or 36 of histone 3. Such modifications cause chromatin compaction and constrain nucleosome mobility, possibly rendering the proximal promoter inaccessible to SP3 in Sertoli cells [28, 31]. Alternatively, such histone modifications may not block SP3 binding, but rather render this transcription factor incapable of interacting with the core transcriptional machinery. For example, whereas Sp family members are bound to the promoter of a silenced luteinizing hormone receptor promoter, the transcription factor forms a complex with enzymes that catalyze histone deacetylation, and thereby promotes chromatin compaction [32].

Domain III: A Transcriptional Activator Is Recruited to cis-Acting Elements Located Between Nucleotides −935 and −452

The expression of Tg (−935/+977) by Sertoli cells in two independent lines of transgenic mice identifies a functional domain III (nucleotides −935 to −452) that is upstream from

domain II. In the *Ctsl* promoter incorporated into a chromosome, domain III may recruit transcription factors or histone-modifying complexes that relax the compact chromatin domain II, rendering it accessible to SP3 and/or to the core transcriptional machinery. Given the size of the domains II and III—451 and 483 bp, respectively—the recruited factors must have the capacity to remodel hundreds of base pairs of tightly compacted chromatin, thereby allowing access of the transcriptional machinery to the gene's proximal promoter. The SWI/SNF family of ATP-dependent, nucleosome-remodeling complexes and pioneering transcription factors have this capability [33–35].

The SWI/SNF complex is directed to specific DNA sequences by binding to one of a number of different transcription factors [36–38]. Once brought to the promoter by these transcription factors, the SWI/SNF complex can displace histones, slide nucleosomes along the chromatin fiber and, consequently, relax chromatin equal to thousands of base pairs of DNA, making this DNA sequence accessible to the transcriptional machinery [39–41]. Additionally, pioneering transcription factors, such as members of the forkhead factor (FOX) family and GATA-4, can bind to DNA sequences within highly compacted chromatin, thereby relaxing the chromatin and allowing other transcription factors to gain access, including those that recruit members of the SWI/SNF family [42–44].

Domain IV: A Transcriptional Repressor Is Recruited to cis-Acting Elements Located Between Nucleotides −2065 and −936

In contrast to Tg (−2065/+977), Tg (−935/+977) is expressed in many different organs and by Sertoli cells at all stages of the cycle of the seminiferous epithelium. Therefore, we suggest that domain IV, spanning from −2065 to −936, interacts with domain III to direct both Sertoli cell-specific and stage-specific *Ctsl* promoter activity. It is noteworthy that the effect of domain IV is to repress promoter activity in all cells but Sertoli cells. Additionally, in Sertoli cells, domain IV represses promoter activity at all stages but stages V–VIII. This function of domain IV is physiologically relevant because stage-specific expression of the rat *Ctsl* gene is regulated primarily by signals from spermatocytes and/or spermatids that suppress transcription at stages I–IV and IX–XIV [7].

In conclusion, the data in this and previous papers begin to generate a model that explains how Sertoli cell-specific and stage-specific expressions of the *Ctsl* gene are regulated (Fig. 8). This model posits that domain III recruits one or more transcription factors that singly or in cooperation with the SWI/SNF complex relax the chromatin structure of domain II. This relaxed chromatin permits the binding of SP3 and/or the core transcription apparatus to the proximal promoter in domain II. However, the stage-specific expression of Tg (−2065/+977) indicates that the function of domain III is constrained to stages V–VIII. Our data indicate that this constraint is due to action of the domain IV that we predict responds to stage-specific suppressive signals from spermatogenic cells at stages I–IV and IX–XIV of the rat (stages I–V and IX–XII of the mouse). We propose that these suppressive signals cause domain IV to inhibit the function of domain III. We also propose that at stages VI and VII, the suppressive signals from spermatogenic cells cease, and the repressor no longer binds to its DNA motif in domain IV. Domain III is then free to recruit the remodeling factors that relax the chromatin of domain II, which in turn allows binding of SP3 and/or the core transcription apparatus. Transactivation of the *Ctsl* promoter ensues. Once transcription is initiated, domain I, the first intron, amplifies transcription and ensures efficient polyadenylation and translation of the transcript. Consequently, although the synthesis of cathepsin L protein in rats is undetectable at stages I–VI and IX–XIV, Sertoli cell synthesis of cathepsin L at stages VI and VII represents approximately 1.6% of all of the protein synthesized by both the Sertoli cells and the spermatogenic cells within intact seminiferous tubules [45].

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