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A-MYB (MYBL1) Stimulates Murine Testis-Specific *Ldhc* Expression via the cAMP-Responsive Element (CRE) Site[1]

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ABSTRACT

Generally, knowledge of the mechanism regulating gene expression in primary spermatocytes is incomplete. We have used the lactate dehydrogenase gene (*Ldhc*) as a model to explore these mechanisms during spermatogenesis. Its 100-bp core promoter contained two essential elements common to many genes, a GC box and a CRE site. Here we report results that support a model in which transcription factor MYBL1 acts as a coactivator directing tissue-specific expression via the CRE *cis* element. We hypothesize that this is a common mechanism involving activation of multiple genes in the primary spermatocyte. MYBL1 is expressed predominantly as a tissue-specific transcription factor in spermatocytes and breast epithelial cells. Our finding that LDHC expression is lost in 21-day testes of MYBL1 mutant mice supports our hypothesis. In the GC1-spg germ cell line exogenous MYBL1 induces activity 4- to 8-fold, although extracts from these cells do not show MYBL1 binding activity for the Myb consensus sequences in the *Ldhc* promoter by EMSA. Rather, MYBL1 stimulates expression from a synthetic promoter containing only CRE elements, suggesting MYBL1 activates the promoter by interacting with protein that binds to a CRE element. Mutation of three Myb sites does not affect *Ldhc* promoter activity significantly ($P > 0.05$). CREB-binding protein (CBP) is a coactivator that interacts with CRE-binding protein CREB. We show that the transactivation domain (TAD) in MYBL1 interacts with the KIX domain in CBP, and the TAD domain and DNA binding domain in MYBL1 each interact with the CREB N-terminal domain. MYBL1 also stimulated expression from testis-specific genes *Pgk2* (phosphoglycerate kinase 2) and *Pdha2* (pyruvate dehydrogenase alpha 2) promoters, each of which contains CRE promoter elements and is expressed in primary spermatocytes. We propose that MYBL1 directs germ cell-specific activation via the CRE site of certain genes that are activated specifically in the primary spermatocyte, although other, more indirect effects of MYBL1 remain a possible explanation for our results.

gene expression, gene regulation, sperm, spermatocyte, spermatogenesis

INTRODUCTION

Spermatogenesis is the three-stage process by which germ cells transform from progenitor cell spermatogonia to spermatozoa. Spermatogonia proliferate (mitosis), enter meiosis as spermatocytes, and become spermatids that differentiate into mature spermatozoa (spermiogenesis). These events are characterized by the expression of a group of genes that are testis specific. Deletion of these genes often leads to a defect in sperm production [1–3]. Therefore, precise control of where and when these genes are expressed is critical for normal spermatogenesis, but the regulatory mechanism for this tissue-specific expression is still not very clear. The two major germ cell types, spermatocytes and spermatids, each have unique sets of genes expressed that fulfill their distinctive morphological and functional requirements. Therefore, the transcriptional activation paradigm for genes in the spermatocyte and spermatid can be very different. In spermatids, a specific coactivator ACT (activator of CREM in testis) interacts with a CRE element binding factor, CREMτ, and is responsible for spermatid-specific gene expression [4]. This finding reveals the unique importance of tissue-specific coactivators in directing testis-specific gene expression. For genes expressed uniquely in spermatocytes, there is limited knowledge of regulatory mechanisms. However, a recent report indicated that a tissue-specific transcription factor MYBL1 is a master regulator for male meiosis [5]. A point mutation in the transactivation (TA) domain of MYBL1 leads to a blockage of spermatogenesis at pachytene and an aberrant transcriptome of 297 genes at 14 dpp and 623 genes at 17 dpp. Of particular relevance here is that among those downregulated genes, *Ldhc* is the one with the highest (7.4-fold) reduction, and *Pdha2* transcription is reduced 3.4-fold. However, a ChIP-chip assay, which hybridizes chromatin-immunoprecipitated DNA by MYBL1 antibody with Nimblegen 385K RefSeq Promoter arrays, reveals that MYBL1 does not bind directly to *Ldhc* and *Pdha2* gene promoter sequences [5]. The underlying mechanism by which *Ldhc* is regulated by MYBL1 remains to be elucidated.

We used *Ldhc* as our model to decipher the activation paradigm for spermatocyte gene expression. LDHC is a glycolytic enzyme that converts pyruvate to lactate; its expression is first detected at the onset of spermatogenesis and is exquisitely testis specific [6]. Targeted disruption of the *Ldhc* gene [7] destroys sperm function, possibly by creating a metabolic defect in the male gametes that rely on aerobic glycolysis to generate the ATP required for motility and capacitation. Because of its abundance, temporal specificity of expression, and importance for male reproduction, the *Ldhc* gene was, in our opinion, the appropriate choice for this study. We greatly simplified our system by limiting the transcription factor binding region to a 100-bp core promoter sequence that we confirmed as sufficient for activating expression in three independent transgenic experiments [6, 8, 9]. These studies demonstrated that a GC box and two CRE sites in the core promoter were involved. The transgene signal delivered by this core promoter was restricted to spermatocytes and not detected in spermatids even though the LDHC protein is observed in both cell types. We were unable to explain the lack of transgene expression in spermatids since the promoter sequence contained *cis* elements for binding SP1 and CREM,

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both of which are available in these cells. An answer may be forthcoming here in our description of a mechanism whereby MYBL1 synergizes with CRE element binding protein to direct cell-specific gene activation in the primary spermatocyte.

In the MYB gene family (*Mybl1*, *Mybl2*, and *Myb*), MYBL1 is expressed predominantly in spermatocytes and breast epithelial cells, while *Mybl2* and *Myb* expression is ubiquitous [10, 11]. Low-level *Mybl1* expression has been reported in ovaries and brain [10, 12]. Targeted disruption of the *Mybl1* gene in mice leads to defective breast development and spermatogenic arrest at pachytene [11]. A single point mutation in the transactivation domain of MYBL1 has no phenotype in female but disrupts spermatogenesis [5]. In the present experiment we demonstrate that MYBL1 is able to activate the *Ldhc* promoter as well as the testis-specific genes *Pgk2* and *Pdha2*, which contain the CRE *cis* element(s); MYBL1 does not bind directly to the *Ldhc* promoter DNA sequence; instead, it activates the promoter through a CRE site. We provide evidence that MYBL1 interacts with CREB-binding protein (CBP) and CREB. It is additionally informative that MYBL1 is localized to the same cell type with the same expression pattern as the reporter gene driven by the *Ldhc* core promoter. These data support our hypothesis that MYBL1 is involved in the mechanisms of cell-specific gene expression in the primary spermatocyte.

MATERIALS AND METHODS

Plasmid Construction

Construct m*Ldhc*-425/20 was created by PCR using primer pair (forward 5′-CATAGATCTGCCTGGTCTACAGAGTTCCAGGACG-3′, reverse 5′-CATAAGCTTGCACAGGTAAGAAACCAGGATAACTGTTG-3′) and mouse genomic DNA. m*Ldhc*-88/10 used primer set (forward 5′-CATAG ATCTGGTACCTGAGCAGGCAGTG-3′, reverse 5′-CATAAGCTTGGAT AACTGTTGGGTCCAGGAGCCAACAG-3′). Construct h*Ldhc*-502/22 used PCR primer set (forward 5′-ATGGTACCCGCTCCAGGGCAGAAAA GAC-3′, reverse 5′-CATAAGCTTGCTGCTCCTTGACAGTTGACATTTGGAG AAC-3′) and human blood DNA. Construct m*Ldhc*-425/20 and m*Ldhc*-88/10 were cloned into the BglII/HindIII site of vector pGL4-luc2 (Promega). h*Ldhc*-502/22 was cloned into pGL4-luc2 at KpnI/HindIII site. Construct (CRE-Myb)*Ldhc*-miniP-pGL4 was created by annealing 4 phosphorylated olionucleotides 5′-CGGCTGCGTGCTGACGTTGACTTTGTGACGTTCC TTTTCCGTTATC-3′ (CRE-Myb S1), 5′-ATAACTGTTGGCTCCTGGACCC AACAGTT ATCCA-3′ (Myb S2), 5′-GATCTGGATAACTGTTGGGTCC AGGAGCCAACAGTTATGAT-3′ (Myb AS1), and 5′-AACGGAAAAGG AACGTCACAAAGTCAACGTCAGCACGCAGCCGGTT-3′ (CRE-Myb AS), then cloned into pGL4.24 in the BglI/BglII site (Myb site WT). The similar construct but with two Myb sites mutated (Myb site Mu2) was created by annealing oligonucleotides CRE-Myb S1, 5′-ATATCTG TTGG CTCCTGGACCCAA CAGAT ATCCA-3′ (MybMu S2), 5′-GATCT GGAT ATCTG TTGGGTCCAGGAGCCAA CAGAT ATGAT-3′ (MybMu AS1), and CRE-Myb AS. Another construct with all three Myb sites mutated (Myb site Mu3) was created by annealing 5′-CGGCTGCGTGCTGACGTT GACTTTGTGACGTTC CTTTTCCGATATC-3′ (CRE-MybMu S), MybMu S2, MybMu AS1, and 5′-ATCGGA AAAGGAACGTCACAAAGTCAA CGTCA GCACGCAGCCGGTT-3′ (CRE-MybMu AS). Construct 3xCRE$_{ldhc}$-miniP-pGL4 was created with three copies of *Ldhc* promoter CRE sequence in a tandem repeat (TGCGTGCTGACGTTGACTTTGTGACGTTCCTT); the insert was cloned into vector pGL4.24 (Promega), which contains a 30-bp minimal promoter. Construct 3xCREmu-miniP-pGL4 was created the same way except with two nucleotides mutation in CRE sites (TGCGTGC TGtgGTTGACTTTGTGtgGTTCCTT). PGK2-pGL4 was created with primer set (5′-CATGGTACCACTGCAGAGGATTTTCCACAGTATAATT-3,′ 5′-CATAGATCTAA GAGCCATCTTGATGGTATGCACAAC-3′); the 468-bp mouse *Pgk2* [13] and 187-bp *Pdha2* [14] promoter sequences were cloned into the pGL4 vector. Plasmid FLAG-A-Myb is a kind gift from Dr. Shunsuke Ishii (RIKEN Tsukuba Institute) [15]. MYBL1 mutant 1: truncated MYBL1 was PCR amplified (amino acids 1–408) with primer set (5′-GGTACCATGGC GAAGAGGTCGCGCAGTGAG-3,′ 5′-AAGCTTTTAACTAGTGTGCTCG TCAAGGCTATTTAG-3′) and cloned to vector pcDNA3.1; a negative regulatory domain (NRD) was deleted in this mutant1, as reported [16]. pRc/RSV-mCBP-HA (Dr. Richard Goodman) and pCMVb p300-HA (Dr. William Sellers) were purchased from Addgene. CREB-pcDNA3.1 is a gift from Dr. Kelly Mayo's lab.

Animal Tissue, Cell Culture, Transient Transfection, and Luciferase Assay

Twenty-one-day testis of mouse line *repro9*, which carries a missense point mutation in *Mybl1* allele, was obtained from Dr. John Schimenti at Cornell University [5]. Cell lines GC1-spg and Hela cells were obtained from ATCC. The Hela cell was used to confirm the interaction in an ectopic cell context. Cell culture, transfection, and luciferase assays have been described [17]. Briefly, culture medium was Dulbecco modified Eagle medium with 10% fetal bovine serum. Plasmid transfection reagent was Lipofectamine 2000 (Invitrogen); transfection was performed in triplicate following the manufacturer's protocol. Transfection efficiency was normalized by protein content of the lysate. Luciferase activity was measured by Bright-Glo Luciferase Assay System (Promega).

Immunohistochemistry

Consecutive 5-μm sections of adult testis from transgenic animals were used for MYBL1 and β-gal immunostaining. The transgene was a β-gal reporter driven by a 100-bp *Ldhc* promoter described previously [6]. Transgenic animals were generated at the Northwestern University Transgenic Core Facility and maintained according to Institutional Animal Care and Use procedures. IHC staining used in that report included addition of an antigen retrieval step for better nuclear antigen staining. After deparaffination, slides were immersed in a preheated 95–100°C citrate buffer (pH 6.0, 0.05% Tween) and microwaved with 10% power for 8 min. The slide dish was then allowed to cool down for 20 min at room temperature and rinsed in a tris-buffered saline (TBST) solution for 2 × 2 min. MYBL1 antibody was purchased from Sigma (catalog no. HPA008791). Antibody dilution was 1:35.

Electrophoretic Mobility Shift Assay

Cell nuclear extract was prepared from GC1-spg cells transfected with either MYBL1 or MYBL1 mutant 1 expression vector. Cultured cells were scraped from a Petri dish containing 100–200 μl of packed cells. After 15 min of incubation with 1 ml hypotonic lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCI, 5 μl Sigma proteinase inhibitor cocktail P8340), cells were centrifuged, resuspended in 1 ml hypotonic lysis buffer; and loaded in a 1-ml syringe with a No. 27-gauge needle, where it was drawn and ejected 10 times. The nuclei were centrifuged at 10 000 × *g* for 20 min, and the supernatant was discarded. A nuclear extract was prepared with 100 μl of high-salt buffer (20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCI, 0.2 mM ethylenediaminetetraacetic acid, 25% glycerol, 2 μl proteinase inhibtor cocktail) with shaking for 30 min at 4°C and centrifuged at 20 000 × *g* for 5 min. The supernatant was stored at −70°C until used. The gel shift assay kit from Promega was used according to the manufacturer's instructions. A 5% 0.75-mm PAGE gel was run in the cold room until the dye front reached the bottom and then dried using a Gel Dryer (BioRad) for 2 h before exposure to x-ray film overnight. Oligonucleotides used for the GMSA were described in Figure 5A.

Mammalian Two-Hybrid Assay

The CheckMate Mammalian Two-Hybrid System from Promega was used to interrogate the protein domain interactions between MYBL1, CBP, and CREB. The DNA binding domain (DBD), transactivation domain (TAD), and negative regulatory domain (NRD; see Fig. 5A) from MYBL1 were cloned into vector pACT that contains the HSP VP16 activation domain. The KIX domain sequence from CBP and three other domains from CREB (CREB1-99, KID, and CREB161-327; see Fig. 7A) were cloned into vector pBIND that contains the GAL4 DNA binding domain. pACT and pBIND vectors with fusion constructs were cotransfected with pG5luc containing multiple GAL4 binding sites. Transfection was performed in GC1-spg cells. Reporter luciferase activity was measured 36 h after by the dual luciferase assay system (catalog no. E1910; Promega). Vector pBIND contains an internal Renilla luciferase gene to control for transfection efficiency. Final luciferase activity was calibrated by Renilla lucfierase activity in each sample. Primers used for amplification of MYBL1 domains are 5′-GGATCCGTatggcgaagaggtcgcgcag-3′/5′-GCGGCCGCatgatccatagctgcacaaggtttg-3′ (DBD), 5′-GGATCCGTatgcaaaccc agaatcagttttacatacc/5′-GCGGCCGCttggcattccatggctccttc-3′ (TAD), and 5′-GGATCCGTtttaacgtcagtcttgtacttgaagg-3′/5′-GCGGCCGCcagtatgagagctcttgaa gtactac-3′ (NRD). Primers for CBP KIX domain are 5′-GGATCCGTaacac aattggttctgttggtgcag-3′/5′-cTCTAGA ttgagaaacctgcatgcgattcac-3′. Primers for

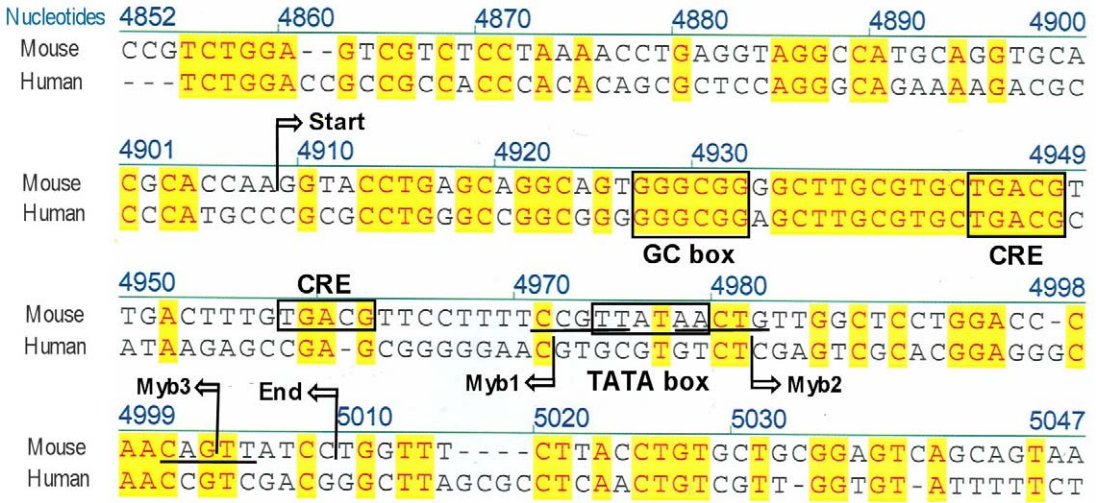


FIG. 1. Sequence alignment: murine and human *Ldhc* gene promoters. Sequence segments containing conserved elements are displayed. The GC box, CRE sites, and TATA box are annotated by rectangles. Three Myb binding sites are labeled as Myb1, Myb2, and Myb3, but the consensus sequences for Myb1 and Myb3 are located in the complementary strand. The labels "Start" and "End" specify the mouse core promoter region. The only conserved fragment between the murine and human promoters contains a GC box and a CRE site.

CREB domains are 5′-GGATCCGTatgaccatggactctg gagc-3′/5′-cTCTAGA ctcctgtgaatcttcactttctgc-3′ (CREB1-99), 5′-GGATCCGT tctgtggatagtgtaactg attccca-3′/5′-cTCTAGAaatcggggttggcactgttacag-3′ (KID), and 5′-GGATCCGT taccaaactagcagtgggcag-3′/5′-cTCTAGAatctgacttgtggcagt aaaggtcc-3′ (CREB161-327). The cloning site is BamHI/NotI for pACT and BamHI/XbaI for pBIND. Expression vectors listed in the *Plasmid Construction* section were used as PCR template.

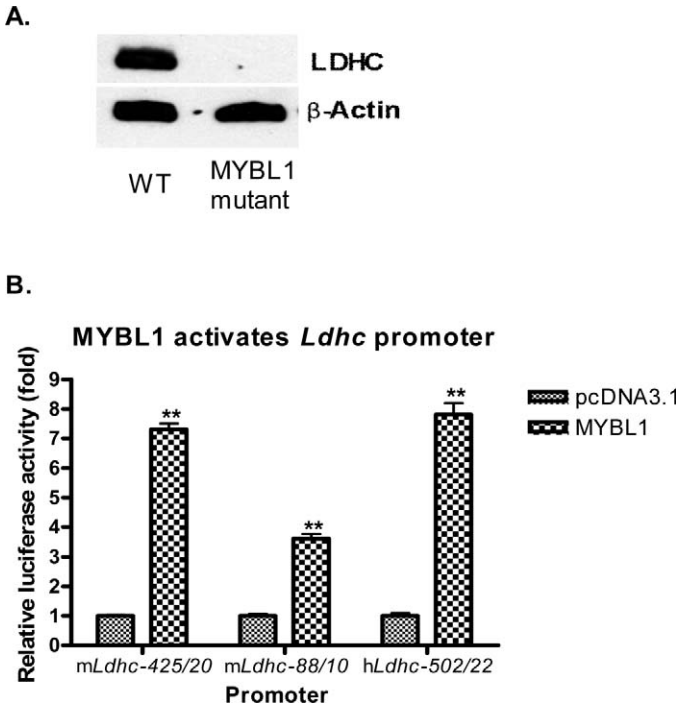


FIG. 2. MYBL1 regulates *Ldhc* gene expression. **A**) By Western blot, LDHC expression was not detected in 21-day male testis of MYBL1 mutant mouse. **B**) MYBL1 activates both mouse and human *Ldhc* promoters. Long (−425/+10) and short (−88/+10) murine and human promoter sequences (−502/+22) were tested for activation by cotransfection of promoter-reporter constructs and MYBL1 expression vector in a GC1-spg germ cell line. A corresponding 7-, 4-, and 8-fold increase with mouse long and short promoter and human promoter was observed in the MYBL1 treatment groups (**$P < 0.01$). Values are shown as the mean ± SEM.

Statistics

Statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software Inc.). One-way analysis of variance and Bonferroni multiple comparison test or pairwise *t*-test were used to evaluate data. Differences were considered significant when P was less than 0.05.

RESULTS

The murine 100-bp *Ldhc* core promoter contains a GC box, two CRE sites, three Myb binding sites, and a TATA box [6]. Sequence alignment analysis using Vector NTI software was performed on 5-kb 5′genomic DNA of the mouse and human *Ldhc* in a search for conserved regions. The genomic sequence is very different between these two species. The only conserved fragment is located within the 100-bp mouse core promoter region that contains a GC box and CRE site (Fig. 1).

LDHC expression was examined in the 21-day testis of MYBL1 *repro9* mutant mice. Expression was not detectable by Western blot, while the wild-type counterpart presented a robust signal (Fig. 2A).

Because three Myb consensus sequences (A/T)AAC (T/G)G were identified in the mouse *Ldhc* core promoter and since MYBL1 is expressed predominantly in spermatocytes, a functional assay was designed to determine whether MYBL1 interacts with the promoter. Mouse long (−425/+10) and short (−88/+10) promoter and human *Ldhc* (−502/+22) promoter activity increased 7-, 4-, and 8-fold, respectively ($P < 0.01$) when these constructs were cotransfected with the MYBL1 vector in GC1-spg germ cells (Fig. 2B).

The cellular localization and expression pattern of MYBL1 was compared to that of the reporter gene driven by a 100-bp *Ldhc* core promoter in transgenic mice. The reporter gene expression pattern is similar to MYBL1 (Fig. 3). Immunohistochemical analysis of consecutive testis sections reveals unambiguously that both proteins are localized in the spermatocyte, albeit MYBL1 distributes in the nucleus compared to cytoplasmic localization of the β-gal reporter representative of LDHC.

MYBL1 interaction with the *Ldhc* promoter and three Myb consensus sequences was interrogated by a gel shift assay. No gel shift was detected, indicating that MYBL1 does not bind to the Myb consensus sites (Fig. 4). In contrast, a truncated

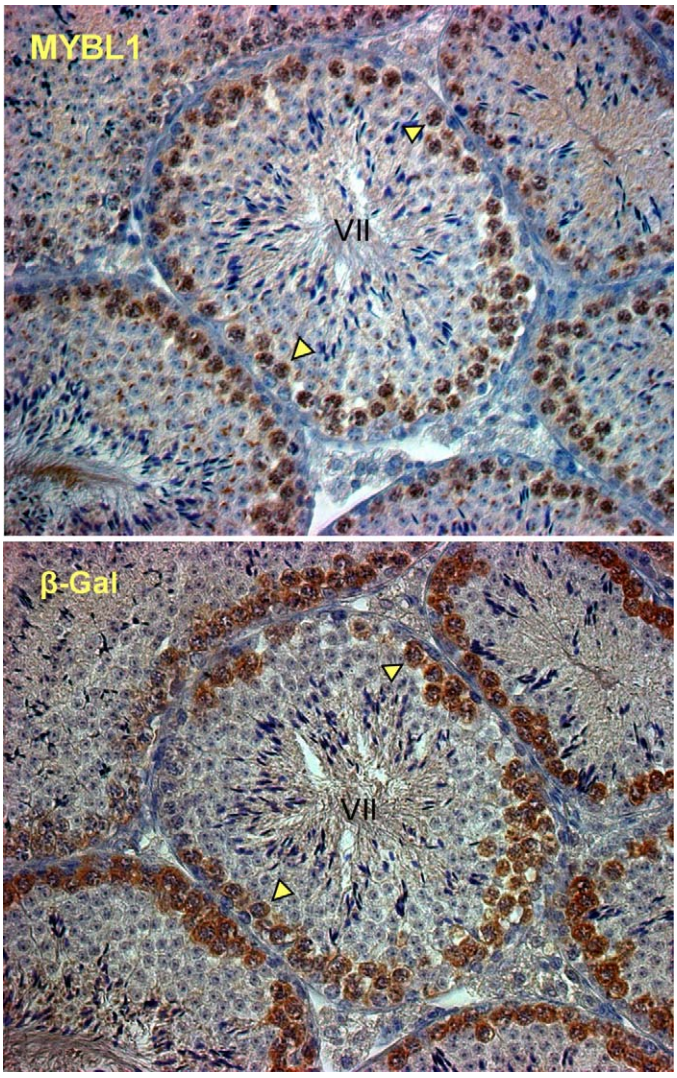


FIG. 3. Testis sections showing immunohistochemical distribution of MYBL1 and of a β-gal reporter transgene. In transgenic animals, the β-gal reporter was driven by a 100-bp *Ldhc* promoter. A positive cell is marked with an arrowhead. Both proteins are localized in the same cell type (spermatocytes). Consecutive testis sections from the transgenic animal were used for immunohistochemical analyses. Original magnification ×400.

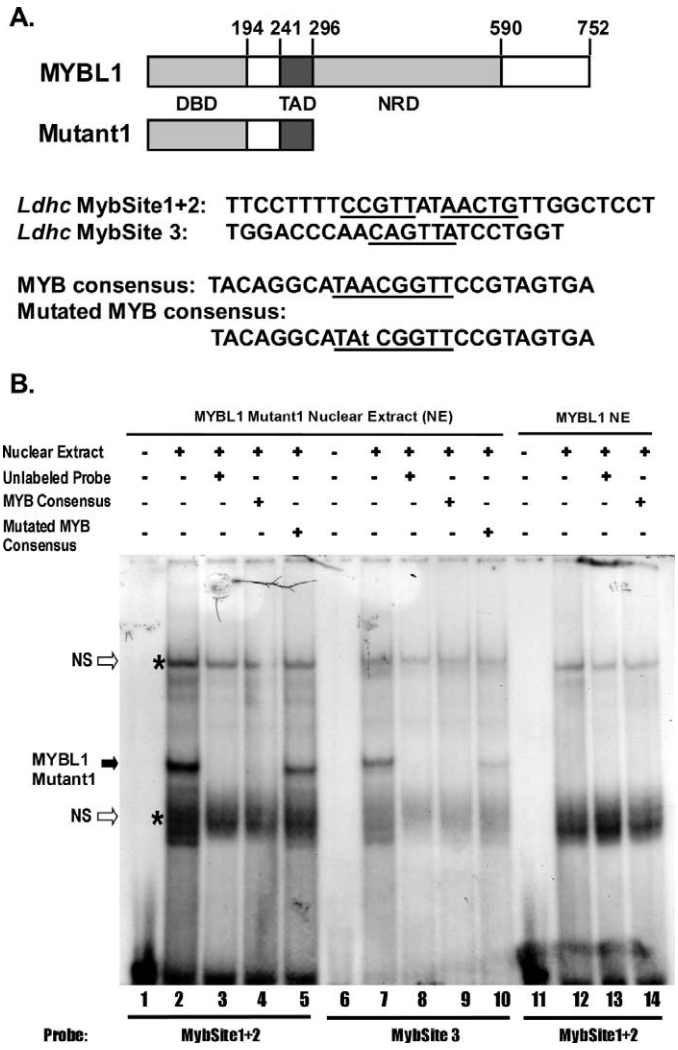
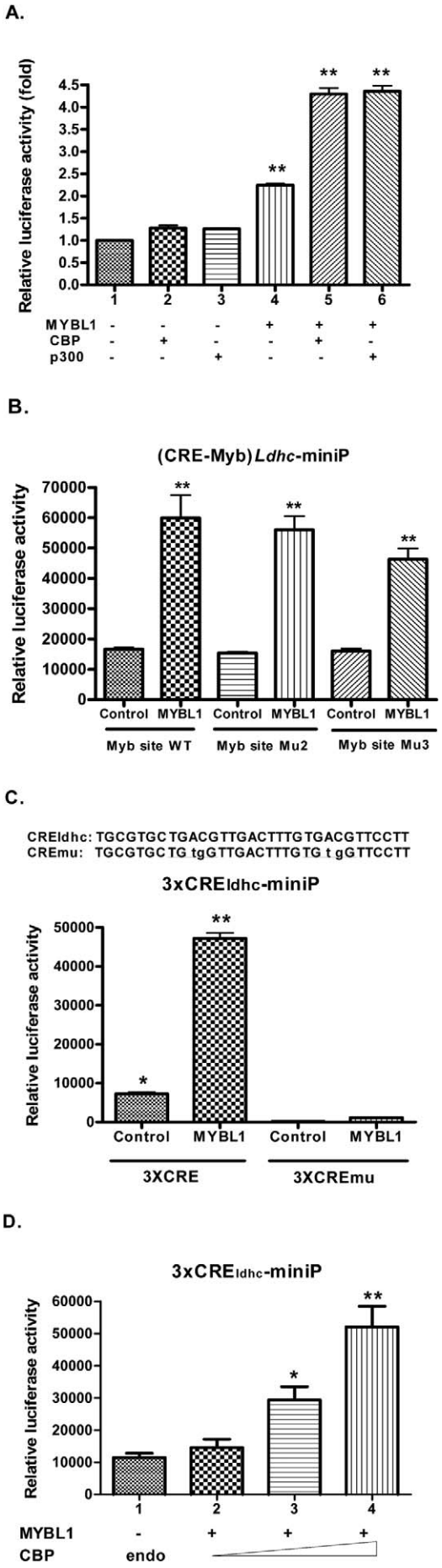


FIG. 4. Analysis of the DNA-protein interaction between three Myb consensus sequences in the *Ldhc* promoter and MYBL1 or truncated mutant (mutant1) proteins by electrophetic mobility shift assay (EMSA). **A**) Schematic depicting structure of wild-type MYBL1 and mutant1 construct; oligonucleotide sequences for EMSA shown below: DBD, DNA binding domain; TAD, transactivation domain; NRD, negative regulatory domain. Myb sites are underlined; mutated nucleotides were labeled with lowercase letters. **B**) EMSA showing DNA-protein interactions with the three Myb consensus sequences of the *Ldhc* promoter, MYBL1 and the truncated mutant1. Lanes 1–10 demonstrated a protein-DNA interaction between mutant1 and Myb consensus sequences; gel shifts in lanes 2, 5, 7, and 10 were marked with arrows or NS (nonspecific). Lanes 11–14 show the interaction for intact MYBL1 protein. Nuclear extracts were prepared from GC1-spg germ cells transfected with MYBL1 or mutant1. The intact MYBL1 protein did not yield a specific shifted band with the Myb probe from the *Ldhc* promoter, while mutant1 produced distinctive band shifts that could be competed off by Myb consensus but not mutated sequence.

MYBL1 without a negative regulatory domain (NRD) was able to bind to the Myb oligonucleotides and produce a distinctive band shift. The domain structure of MYBL1 protein was reported previously [10–12, 18], and a schematic diagram of MYBL1 wild type and mutant1 is shown in Figure 4A. The lack of a band shift suggested that MYBL1 may bind indirectly to the *Ldhc* promoter. Considering that it has a binding partnership with MYB, a coactivator CBP was thought to be an excellent candidate to bridge a similar interaction [19] with MYBL1. This has been confirmed [15, 20] with the demonstration that MYBL1 physically binds to CBP. Therefore, we cotransfected a CBP or p300 expression vector with *Mybl1* and an *Ldhc* promoter construct. Both CBP and p300 enhance promoter activity ($P < 0.05$), while there is no significant difference without MYBL1 (Fig. 5A). To further confirm that there is not any DNA protein interaction between MYBL1 and Myb binding sites, a construct with an *Ldhc* promoter fragment containing CRE and Myb sites only [CRE-Myb]*ldhc*-miniP-pGL4 and a similar construct with Myb site mutations were created for a functional assay (Fig. 5B). In this construct, CRE sites were kept because we had shown previously that *Ldhc* promoter activity was abolished if both the GC box and the CRE sites were mutated [6]. Promoter activity for the construct with Myb site mutations is not significantly different from the nonmutated construct ($P > 0.05$) (Fig. 5B).

To confirm the specific interaction between MYBL1 and CRE binding protein (CREB), three copies of CRE elements from the mouse *Ldhc* promoter were cloned as tandem repeats into a luciferase reporter vector with a 40-bp minimal promoter



(3xCRE$_{ldhc}$-miniP-pGL4). A similar construct was prepared, except a two nucleotide mutation was introduced into all the CRE sites (3xCREmu-miniP-pGL4). Cotransfection of these synthetic promoter-reporters with the MYBL1 expression vector in Hela cells increased the promoter activity 5-fold, while the mutated construct remained at background level, indicating that there is a specific interaction between CREB and MYBL1 via the CRE site (Fig. 5C). CBP was cotransfected with 3xCRE$_{ldhc}$-miniP-pGL4 and MYBL1 vector; the promoter activity increase was CBP dosage dependent (Fig. 5D).

A physical interaction between CBP and MYBL1 was reported previously by coimmunoprecipitation [15] and gel shift [20]. The KIX domain in CBP was responsible for such interactions [20]. To explore which domain in MYBL1 is involved in the interactions with CBP and CREB, a mammalian two-hybrid assay was performed. The interaction between the TAD in MYBL1 and the CBP KIX domain is significantly higher than in the control ($P < 0.01$; Fig. 6B). Similar results were reported for MYB and CBP [19, 21]. For the CREB protein, the N terminal (CREB 1–99) interacted with both DBD and TAD domains ($P < 0.01$; Fig. 6C). The KID domain in CREB, known to interact with KIX domain in CBP, had no measurable association with any domain in MYBL1 ($P > 0.05$).

CRE is a common promoter element for many genes that express in the primary spermatocyte [6]. The testis-specific *Pgk2* [22] and *Pdha2* [14] genes each contain a CRE site and show similar temporal expression as *Ldhc*. *Pgk2* and *Pdha2* promoter activity was increased 3- to 4-fold when cotransfected with *Mybl1* (Fig. 7).

DISCUSSION

The data presented in this report reveal a novel transcriptional regulatory mechanism whereby MYBL1 activates the *Ldhc* promoter independent of binding to a specific DNA sequence. MYBL1 stimulates promoter activity via the CRE site involving CREB binding to the promoter. However, whether this effect is direct or indirect cannot be stated unequivocally. MYBL1 may be tethered in a transcriptional initiation complex with both coactivator CBP and CREB. We base this interesting model on the two-hybrid assay described

FIG. 5. **A**) Synergistic effects of coactivator CBP/p300 with MYBL1 and the interaction with Myb and CRE *cis* elements. Coactivator CBP/p300 enhances *Ldhc* promoter activity (mouse *Ldhc* −88/+12) when cotransfected with MYBL1. *Ldhc* promoter activity was significantly higher with CBP/p300 overexpression (**$P < 0.01$) in treatments 5 and 6 compared to treatments 1–4; activity in treatment 4 is significantly different than treatments 1–3 (**$P < 0.01$). **B**) A *Ldhc* promoter fragment containing CRE and Myb sites only (CRE-Myb). *Ldhc* was cloned into vector pGL4 with a 40-bp minimal promoter (miniP). The Myb sites are either wild type (Myb site WT) or mutated (Myb site Mu 2 or Mu 3; two Myb sites mutated, or all three Myb sites mutated). The promoter activity was significantly different between control and MYBL1 treatment (**$P < 0.01$) but not significantly different between the three constructs (WT, Myb site Mu 2, and Mu 3) when cotransfected with MYBL1 expression vector ($P > 0.05$). **C**) A synthetic promoter with three copies of *Ldh* CRE sites (3xCRE$_{ldhc}$) and a miniP was activated significantly by MYBL1 (**$P < 0.01$) with 7-fold increase of promoter activity. A similar construct with two nucleotides mutated in the CRE sites (3xCREmu) showed only background activity that was significantly lower than that of the (3xCRE$_{ldhc}$) with (**$P < 0.01$) or without MYBL1 (*$P < 0.05$). **D**) Dose-dependent upregulation by CBP on (3xCRE$_{ldhc}$) activity. endo, endogenous. Values are shown as the mean ± SEM, *$P < 0.05$, **$P < 0.01$.

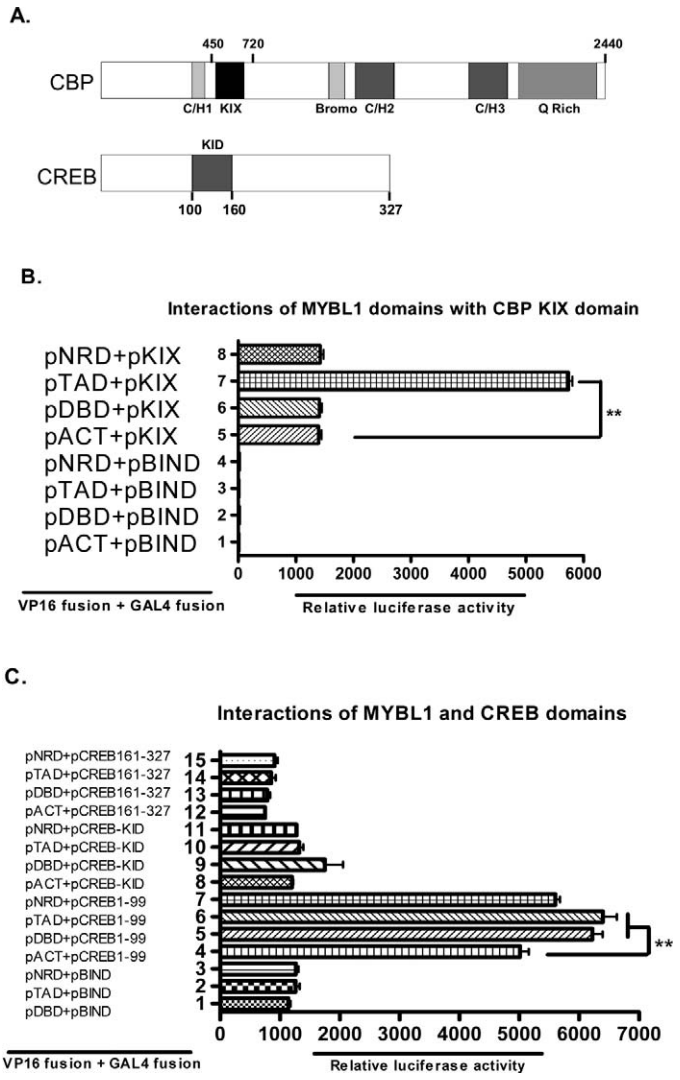


FIG. 6. Protein-protein interactions between MYBL1 and CBP and CREB with the two-hybrid assay system. **A**) Schematic depicting domain structure of CBP and CREB proteins. Numbers refer to amino acid position. **B**) Interactions of three MYBL1 domains with the CBP KIX domain. Vectors pDBD, pTAD, and pNRD contain the DBD, TAD, and NRD domain from MYBL1, while pKIX contains the KIX domain from CBP. Empty vector pACT and pBIND contains VP16 and GAL4 fusion protein and was used as control for transfection of GC1-spg germ cells. Interaction between TAD and KIX domain was detected ($^{**}P < 0.01$). **C**) Interactions of MYBL1 and CREB domains. Vectors pCREB1-99, pKID, and pCREB161-327contain 3 CREB domains. The KID and CREB161-327 domains did not interact with any of MYBL1 domains ($P > 0.05$), but the CREB N-terminal CREB1-99 domain interacted with both DBD and TAD from MYBL1 ($^{**}P < 0.01$) but not the NRD domain ($P > 0.05$). Values are shown as the mean ± SEM.

above as well as protein-protein interactions between MYB protein and CBP and CBP and CREB described elsewhere [15, 19, 20, 23, 24]. However, the interaction between MYBL1 and CREB by the two-hybrid assay is modest, and an effort to coimmunoprecipitate these two proteins was unsuccessful, presumably because of the weak interaction shown in Figure 6C. Therefore, we will not rule out an alternative scenario in which MYBL1 stimulates CREB indirectly; for example, overexpression of MYBL1 may in turn affect CREB expression or its phosphorylation level, although we did not observe a difference in CREB phosphorylation state (data not shown).

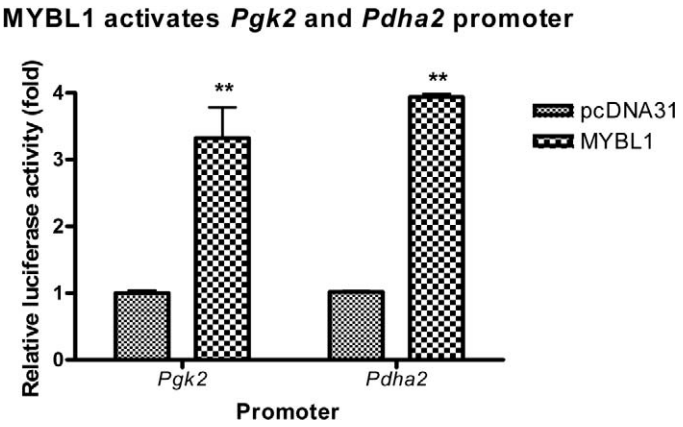


FIG. 7. The 468-bp testis-specific *Pgk2* promoter and 187-bp *Pdha2* promoter were activated 3- to 4-fold when cotransfected with MYBL1 into GC1-spg cells. Both *Pgk2* and *Pdha2* promoters contain a CRE *cis* element. Values are shown as the mean ± SEM, $^{**}P < 0.01$.

The coactivator CBP plays important roles in transcriptional activation. It binds specifically to the PKA-phosphorylated form of CREB and functions as a link between CREB and the transcription preinitiation complex to activate the CRE-containing promoter [23]. In addition to an adaptor function, CBP and its paralogue p300 have powerful histone acetyltransferase activity to acetylate chromatin histone tails and relax the nucleosome structure, thereby enhancing transcriptional activity [25]. In fact, CBP was reported to function as a coactivator for the *Myb* gene by direct binding to the transcriptional activation domain in a phosphorylation-independent manner; CBP utilizes the same domain for binding CREB and interacting with MYB [19]. CBP also cooperates with oncoprotein v-Myb to regulate hematopoiesis [24]. CREB in the testis, unlike the restricted expression of CREM in spermatids, is expressed in early germ cells only through the midpachytene stage of spermatogenesis and therefore may be considered an important inducer of gene expression in these early stage germ cells [26, 27]. We reported elsewhere [6] CREB binding to the CRE sites in the *Ldhc* promoter.

According to a developmental study of the murine male germ cell transcriptome, there is a dramatic upregulation of 3322 and 3210 unique transcripts at Postpartum Days 14 and 20, respectively, when spermatocytes transit to spermatids [28]. Chromatin decondensation [29, 30] and genomic demethylation [31] are likely prerequisites for global gene activation at the onset when spermatogonial cells commit to meiosis. After the repression from condensed chromatin and methylated DNA has been released, chromatin DNA becomes accessible to transcription factors; a unique combination of transcription factors for each gene is responsible for tissue-specific expression [22, 32]. A number of testis-specific gene promoters have been identified using transgenic animals [33–40]. The reporter genes driven by these promoters either are silent or express at a basal level in somatic tissues. Therefore, tissue-specific transcription factor/coactivator assembly would be expected to play an important role in directing tissue-specificity of gene expression. It is very likely that common tissue-specific activators are involved in the mechanisms responsible for the production of more than 3000 unique transcripts in spermatocytes or spermatids. For example, spermatid-specific coactivator ACT interacts with CREMτ via a CRE site directing spermatid-specific gene expression [4]. But we still have limited knowledge about the spermatocyte-specific activation

mechanism(s) even though some key regulatory elements have been identified for several testis-specific genes [32].

In our model system, a GC box and two CRE sites were identified as the key elements for *Ldhc* gene promoter activity. Many testis-specific genes share GC box and CRE sites [6]. Our previous study indicated that mutation of the GC box did not abolish tissue specificity of the promoter [6]. Therefore, the CRE site could be equally important for both spermatocyte- and spermatid-specific gene activation. It is puzzling why a 100-bp core promoter drives reporter expression only in the spermatocyte but not spermatid stages in this study [6]. There is abundant CREM in spermatids that should be able to bind to CRE sites in the *Ldhc* promoter. Our results here allow us to propose that MYBL1, which is restricted to the primary spermatocyte, plays an important role in regulating expression of the *Ldhc* gene via the CRE *cis* element. This is not unlike the mechanism described above for spermatid-specific gene activation [4]. MYBL1 does activate transcription by a mechanism independent of binding to a specific DNA sequence. This would explain why *Ldhc* and *Pdha2* were not detected as targets for MYBL1 in the ChIP-chip assay [5]. A similar phenomenon was reported for v-Myb in which a truncated v-Myb lacking the DNA binding domain was still capable of activating the *Hsp70* promoter [41].

Previously, we demonstrated that the transgene consisting of reporter β-gal driven by a 100-bp *Ldhc* promoter is sufficient for spermatocyte-specific expression [6, 8]. Extending the promoter by 4.5 kb of 5′genomic sequence and utilizing the native 3′UTR of *Ldhc* did not change the expression pattern [9]. Transcription factor SP1/SP3 and CREB/CREM in spermatids and other somatic tissues do not seem to be sufficient to stimulate promoter expression. Based on a review of multiple tissue-specific expression scenarios in which tissue-specific coactivators are involved, Spiegelman and Heinrich [42] proposed a model for the transcriptional activation mechanism: Coactivator is required to drive transcriptional activity over a threshold needed for full gene activation. In the *Ldhc* promoter, MYBL1 does not bind to DNA directly and so may behave as a coactivator able to enhance promoter activity 4- to 8-fold. A similar assembly of factors could regulate the testis-specific genes *Pgk2* and *Pdha2*. *Pgk2* is transcribed in the primary spermatocyte [13], and its expression is lost in MYBL1 knockout mice [11]. *Pdha2* was first detected in primary spermatocytes [14], and its transcription was reduced 3.4-fold in MYBL1 mutant mice [5]. Both the *Pgk2* promoter [22] and the *Pdha2* promoter [14] contain a CRE *cis* element, and activities are increased 3- to 4-fold with MYBL1. These observations tend to support our hypothesis that the tissue-specific factor MYBL1 is involved in the cAMP signaling pathway to impose a unique mechanism for gene activation in the primary spermatocyte. Isolation of an MYBL1 transcriptional complex and identification of the assembly of these cofactors from testis would be the next step to understand more completely this tissue-specific regulatory paradigm. The fact that *Mybl1* deletion [11] leads to arrest of spermatogenesis at pachytene supports this effort. Gaining knowledge about how MYBL1 is regulated and identification of its downstream gene targets will add to our understanding of spermatogenesis at the molecular level.

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