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ARTICLE *in* JOURNAL OF ANDROLOGY · NOVEMBER 2003

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Journal of Andrology, Vol. 24, No. 6, November/December 2003

A Transgenic Analysis of Mouse *Lactate Dehydrogenase c* Promoter Activity in the Testis

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ABSTRACT: Transcription of the mouse testis-specific *lactate dehydrogenase c (mldhc)* gene is limited to cells of the germinal epithelium. Cloning and analysis of the *mldhc* promoter revealed that a 100-bp core promoter was able to regulate testis-specific transcription in vitro and in transgenic mice. Surprisingly, expression of the reporter in transgenic testes was limited to pachytene spermatocytes, whereas native LDH-C_4 was detected in pachytene and all later germ cells. To locate additional regulatory sequence that could recapitulate the native LDH-C_4 distribution pattern, we investigated the contribution of 5′ and 3′ flanking sequences to the regulation of LDH-C_4 expression. We found that transcription factor YY1 binds to the *mldhc* promoter, that the *mldhc* 3′ untranslated sequence does not permit a postmeiotic expression of a β-galactosidase reporter in transgenic mice, and that native *mldhc* mRNA is predominately meiotic, with only a low level of postmeiotic distribution. Our results suggest that the high level of LDH-C_4 in postmeiotic cells results from mRNA and protein stability.

Key words: Transcription, mRNA stability, protein stability.

J Androl 2003;24:843–852

The testis-specific mouse *lactate dehydrogenase c (mldhc)* gene is transcribed at high levels in germinal epithelial cells. LDH-C_4 protein appears at pachytene, becomes more abundant in round and elongating spermatids, and is present in mature spermatozoa. Cloning, sequencing, and analysis of the *mldhc* promoter revealed a 100-bp core region that was defined by its ability to direct testis-specific expression of a reporter gene in in vitro transcription assays (Zhou et al, 1994). This sequence was able to drive the testis-specific transcription of a reporter gene in transgenic mice as well (Li et al, 1998). The appearance of the β-galactosidase reporter was first seen in pachytene spermatocytes, as is the case with native LDH-C_4. Surprisingly, the reporter was not detected in later germ cell types. This result indicated that, although the 100-bp core promoter contains regulatory elements necessary for maintenance of testis-specificity, it does not contain sufficient sequence to recapitulate the native expression pattern of LDH-C_4. The addition of a 338-bp 5′ sequence to the 100-bp core promoter increased in vitro transcription by threefold (Zhou et al, 1994), which suggests the presence of regulatory elements in this region.

Other possible mechanisms for LDH-C_4 persistence into later germ cell types are mRNA and/or protein stability. Mouse *ldhc* mRNA is extraordinarily stable compared with mRNA from other genes (Salehi-Ashtiani and Goldberg, 1993). No degradation of *mldhc* mRNA was observed after incubation of dissociated germ cells in the presence of actinomycin-D for 24 hours. For comparison, *c-fos* mRNA was shown to degrade rapidly, and β-*tubulin* mRNA had a half-life of 10 hours.

For the present work, we demonstrated binding to the *mldhc* promoter by the transcription factor YY1. We also investigated the effect of 5′ and 3′ *mldhc* genomic sequences on reporter gene localization in the testes of transgenic mice. Neither the addition of approximately 4500 bp of 5′ flanking DNA to the 100-bp core promoter nor the inclusion of a *mldhc* 3′ untranslated region fragment containing the native polyadenylation signal allowed reporter expression in postmeiotic cell types. We also show that most native *mldhc* mRNA localizes to meiotic cells, with only a low level of postmeiotic mRNA present.

Materials and Methods

Nuclear Extract Preparation

Nuclear extracts were prepared from CD-1 mouse tissues by a modification of methods reported elsewhere (Gorski et al, 1986; Bunick et al, 1990; Zhou et al, 1994). All manipulations were done on ice in prechilled labware using prechilled solutions. Liv-

Supported by the National Institutes of Health (grant HD05863 to E.G.).

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Received for publication April 11, 2003; accepted for publication June 12, 2003.

er from 10–15 adult mice or testes from 50 adult mice were harvested and placed on ice. The aggregate weight of each tissue was approximately 15 g. Testes were decapsulated. Tissue was minced finely with a razor blade and then added to a 30-mL Teflon-glass homogenizer (Wheaton, Milville, NJ). The homogenizer was filled approximately two-thirds full with homogenization buffer (2 M sucrose, 10 mM HEPES [pH 7.6], 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% glycerol, and 0.5 mM DTT) that contained protease inhibitors (0.33 μg/mL aprotinin, 0.5 mM benzamidine, 1.14 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.1 mM PMSF). Tissue was homogenized by 5 strokes of a Teflon pestle attached to a drill. Tissue suspension was brought to 85 mL with additional homogenization buffer and then layered over 3 × 10 mL cushions of homogenization buffer in 40-mL polyallomar centrifuge tubes (Beckman, Palo Alto, Calif). Samples were centrifuged for 30 minutes at 76 221 × *g* and at 1°C in an SW 28 rotor (Beckman). Nuclei sedimented to the bottom of the tube, and cell debris remained suspended in the buffer. The buffer was carefully aspirated, and the pellet was resuspended in 10 mL of a 9 : 1 mixture of homogenization buffer : glycerol. Nuclei were transferred to a 15-mL Teflon-glass homogenizer (Wheaton) and gently homogenized 2 strokes by hand. The suspension was brought to 50 mL with additional 9 : 1 homogenization buffer : glycerol and layered over 2 × 10 ml cushions of buffer (without additional glycerol) in 40-mL tubes. Nuclei were centrifuged for 30 minutes at 76 221 × *g* and at 1°C, and the supernatant was aspirated as described above. Nuclei were resuspended in 10 mL of nuclei buffer (10 mM HEPES [pH 7.9], 10% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and 0.1 mM PMSF) and gently homogenized 2 strokes by hand in a 15-mL Teflon-glass homogenizer, to break up clumps. The homogenate was centrifuged for 15 minutes at 2988 × *g* in an SS-34 rotor (Sorvall, Newtown, Conn) at 4°C. The supernatant was decanted, and nuclei were resuspended in 10 mL of buffer, centrifuged as described above, and decanted. The pellet was resuspended in 0.3 to 0.5 mL of buffer, KCl was added to a final concentration of 400 mM, and the solution was gently mixed. Suspensions were incubated on ice for 1 hour, with periodic mixing. Debris was pelleted by centrifugation at 11 952 × *g* for 10 minutes at 4°C in an SS-34 rotor, and then the extract was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C. Extracts of nuclei prepared in this manner were found to retain binding activity for at least 6 months. Protein concentrations were typically 5 to 10 μg/μL, as determined by the Bio-Rad (Hercules, Calif) protein assay.

Generation of Labeled Polymerase Chain Reaction Probes

Prior to setting up polymerase chain reaction (PCR) reactions, 50 pmol of the 5′ oligonucleotide primer was end labeled by incubation with T4 polynucleotide kinase in the presence of γ-[^{32}P]ATP. Free nucleotides were removed by passage over a G-25 Sephadex spin column (Pharmacia, Peapack, NJ). The labeled oligonucleotide was used in a standard PCR reaction that contained PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% [w/v] gelatin), 200 μM dNTPs, 50 pmol of the 3′ oligonucleotide primer, 2.5 U Ampli*Taq* DNA polymerase (PE Biosystems, Foster City, Calif), and 20 ng plasmid DNA template containing the *mldhc* promoter fragment. The oligonucleotide pairs used were sense, MC −311 to −291 (5′-CCTACACACAGATGTAAGGGC-3′) and antisense, MC −221 to −241 (5′-TTAAAGGCCACTTTTCTGCTG-3′); sense, MC −272 to −252 RI (5′-ccggaattcCTCCTCAAACTCAAAGTT CTG-3′) and antisense, MC −181 to −201 (5′-GACCACA-CAGAAGATGGCAGC-3′); and sense, MC −239 to −219 (5′-GCAGAAAAGTGGCCTTTAAAG-3′) and antisense, MC −147 to −167 (5′-CCAAGGCCTCTAGTGTAAGAT-3′). Lowercase letters indicate nonhomologous sequence. Cycles were as follows: 94°C for 5 minutes; 25 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle of 72°C for 10 minutes. The labeled PCR product was purified by electrophoresis on a 1% agarose gel. The desired band was excised and recovered by spinning through glass wool, extracting with 25 : 24 : 1 phenol : chloroform : isoamyl alcohol, extracting with 24 : 1 chloroform : isoamyl alcohol, and ethanol precipitation. The pellet was washed briefly with 70% ethanol, air dried, and resuspended in 10 mM Tris-HCl (pH 8.0). The specific activity of probes was determined by scintillation counting.

Generation of Oligonucleotide Probes

Complementary oligonucleotides were annealed in STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA [pH 8.0]) by heating to 95°C for 5 minutes and then cooling to room temperature over several hours. Oligonucleotide pairs were labeled by incubation with Klenow fragment for 15 minutes at 25°C in the presence of 30 μCi of α-[^{32}P]dCTP and 33 μM each of unlabeled dATP, dGTP, and dTTP. Unlabeled dCTP was then added to a final concentration of 33 μM, and the reactions were incubated for an additional 10 minutes at 25°C. Unincorporated nucleotides were removed by passage over a G-50 Sephadex spin column (Pharmacia). The specific activity of each labeled probe was determined by scintillation counting.

Electrophoretic Mobility Shift Assays

Mouse testis or liver nuclear extract (7.5 μg) was incubated on ice for 15 minutes in the presence of binding buffer (13 mM HEPES [pH 7.9], 60 mM KCl, 0.13 mM EDTA, 2 mM DTT, 10% glycerol, and 0.2 mM PMSF), 1 μg poly dI-dC, and 1 μg boiled salmon sperm DNA. The KCl present in the nuclear extract was taken into account when calculating the final KCl concentration. A probe (50 000 cpm) was added, and the reactions were incubated on ice for an additional 30 minutes. Reactions were loaded onto 4% nondenaturing polyacrylamide gels that had been prerun at 150 V at 4°C for 45 minutes. Reactions were electrophoresed at 175 V at 4°C and then blotted onto paper support, dried, and exposed to film at −80°C for 5 to 24 hours. Anti-YY1 immunoserum (Santa Cruz Biotechnology, Santa Cruz, Calif) was added to some reactions. Immunoserum was either added initially or with the labeled probe. No difference in results were seen.

Transgenic Mice Construction

The constructs used to generate transgenic animals are shown in Figure 1. A 712-bp fragment of the mouse *ldhc* promoter, including 300 bp of 5′ artifactual DNA, was amplified by PCR using the primers sense, RI/*Sac*I 5′ (5′-ggaattcGAGCTCTGGG

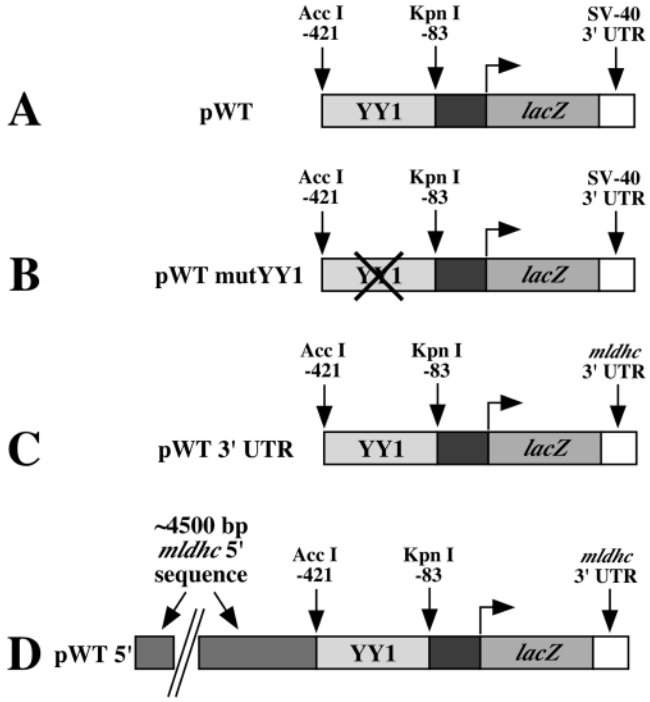

Figure 1. Constructs used to generate transgenic mice. Constructs A, B, and D correspond to panels A, B, and C in figure 6. **(A)** pWT contains a *mldhc* promoter fragment extending to the *Acc*I site at −421 bp 5′ to the transcription start site. **(B)** pWT mutYY1 is identical to panel A, except for a substitution mutation that eliminates binding to the YY1 site. **(C)** pWT 3′ UTR is identical to panel A, except for substitution of the native *mldhc* 3′ polyadenylation site and untranslated region in place of the SV-40 polyadenylation site and 3′ untranslated region. **(D)** pWT 5′ is identical to panel C, except for the addition of approximately 4500 bp of *mldhc* 5′ genomic sequence.

GGTGCTGGTTA-3′) and antisense, HPAS (5′-ATAACTGTTG GGTCCAGGAGCCAACAGTTATA-3′). Lowercase letters indicate nonhomologous sequences incorporating an *Eco*RI restriction site. The amplimer extended from a *Sac*I site at −702 bp 5′ to the transcription start site to +10 bp 3′ to the transcription start site. The amplimer was digested with *Eco*RI and ligated into the *Eco*RI and *Eco*RV sites of pBluescript II KS+ in which the *Hin*cII, *Sac*I, *Sal*I, and *Acc*I restriction sites had been deleted. This construct was digested with *Sac*I and *Acc*I, the single-stranded overhangs were blunted with mung bean nuclease (New England Biolabs, Beverly, Mass), and the construct religated, eliminating the 300 bp of artifactual DNA. The construct was digested with *Eco*RI and *Xho*I, and the resulting promoter fragment was cloned into the *Eco*RI and *Xho*I sites of pNAssβ (Clontech, Palo Alto, Calif), which resulted in pWildType (pWT) (Figure 1A). The YY1 site at −198 to −191 was mutated by converting nucleotides CAT to ACG using the USE Mutagenesis Kit (Pharmacia), according to manufacturer's instructions, which resulted in pWT mut YY1 (Figure 1B). A 722-bp fragment of the mouse *ldhc* 3′ untranslated region, including the native polyadenylation site, was amplified using primers MCpolyAsense, 5′-cgggatccACTCGCCACCTTCGACCGTGTGAC-3′ and McpolyAantisense, 5′-cgggatccGTCATGTTTCACCTGCAT GTATGCC-3′. Lowercase letters indicate nonhomologous sequence incorporating a *Bam*HI restriction site. The PCR product was gel-purified and digested with *Bam*HI. pWT was digested with *Bam*HI, the 200-bp SV-40 polyadenylation site was discarded, and the 722-bp *mldhc* 3′ untranslated region PCR amplimer was inserted in its place, which resulted in pWT 3′ UTR (Figure 1C). An approximately 4500-bp fragment of the *mldhc* 5′ genomic sequence was excised from a PAC clone that contained *mldhc* 5′ flanking sequence and exons 1 to 5 by digestion with *Eco*RI and *Kpn*I. pWT 3′ UTR was digested with *Eco*RI and *Kpn*I and the 5′ flanking fragment was added, which resulted in pWT 5′ (Figure 1D). The PCR-produced portions of all constructs were sequenced on an Applied Biosystems ABI310 sequencer (ABI, Foster City, Calif) using BigDye chemistry to confirm that no mutations had been introduced.

Table 1. *Transgenic constructs*

Construct (see Figure 5)	Number of lines	Number β-galactosidase positive
A	4	1
B	8	2[a]
C	4	1
D	3	2[a]

[a] Very weak staining in one of these lines.

The *mldhc* promoter–*lacZ* fusion fragments from the above constructs were released by digestion with *Eco*RI and *Pst*I, purified on a 1% agarose gel, excised from the gel under ultraviolet illumination, spun through glass wool, extracted 3 times using phenol : chloroform : isoamyl alcohol (25 : 24 : 1), extracted 2 times using chloroform : isoamyl alcohol (24 : 1), ethanol precipitated, and resuspended in 10 mM Tris-HCl (pH 8.0). Transgenic animals were generated by pronuclear injection at the Northwestern University/Children's Memorial Institute for Education and Research transgenic facility. Animals were screened for transgenesis by Southern hybridization of genomic DNA to a fragment of the *lacZ* gene. The number of transgenic lines obtained for each construct and the number of lines in which β-galactosidase was detectable using immunohistochemistry are indicated in Table 1.

Paraffin Embedding and Sectioning

Testes were harvested from dead mice, the capsule was punctured, and testes were placed in Bouin's fixative from 4 to 5 hours to overnight. Fixed tissue was washed in multiple changes of phosphate-buffered saline (PBS). Tissue was incubated 2 times for 30 minutes in 50% ethanol, 4 times for 15 minutes in 70% ethanol, 2 times for 30 minutes in 95% ethanol, 4 times for 15 minutes in 100% ethanol, 3 times for 20 minutes in a 1 : 1 mixture of 100% ethanol and xylene, and 3 times for 20 minutes in xylene. Excess xylene was gently blotted with a Kimwipe, and the tissue was incubated 3 times for 1 hour at 60°C in Paraplast Plus embedding medium (Oxford Labware, St Louis, MO). Tissue was placed into an embedding mold with fresh embedding medium and cooled quickly by floating in a 4°C water bath. Sections were cut at a thickness of 6 μm, floated in a 50°C water bath, mounted onto Superfrost Plus slides (VWR, West Chester, Penn), and incubated for 1 hour at 60°C. Embedded tissue and sections were stored at room temperature.

Immunohistochemistry

A modification of the method described by Morales and Hecht (1994) was used. Paraffin embedded sections were deparaffinized by incubating 2 times for 5 minutes in xylene and rehydrated by incubating 2 times for 3 minutes in 100% ethanol, 3 minutes in 95% ethanol, 20 minutes in 70% ethanol that contained 1% H_2O_2 (to inactivate endogenous peroxidase activity), 20 minutes in 70% ethanol saturated with Li_2CO_3 (to neutralize picric acid from the fixative), 3 minutes in 50% ethanol, 10 minutes in ddH_2O, 10 minutes in PBS (pH 7.2), and 5 minutes in 300 mM glycine in PBS. The remainder of the immunostaining was done using reagents from the Zymed Histostain-plus kit (Zymed Laboratories Inc, South San Francisco, Calif). All operations were done at room temperature, except where indicated. Tissue sections were encircled with PAP Pen (Research Products International, Mount Prospect, Ill), to provide a well for incubations. Sections were blocked for 10 minutes with blocking solution and then incubated overnight at 4°C with primary antiserum diluted in PBS. Sections were washed 2 times for 5 minutes and 1 time for 10 minutes in PBS. Sections were incubated for 30 minutes with secondary antiserum and then washed 2 times for 5 minutes and 1 time for 10 minutes in PBS. Sections were incubated for 10 minutes with enzyme conjugate, followed by 2 times for 5 minutes and 1 time for 10 minutes in PBS. Staining was visualized by incubation with 3,3′-diaminobenzidine (DAB)/chromogen for 1–3 min. Slides were then rinsed well with ddH_2O, counterstained for 4 minutes with hematoxylin, rinsed briefly in 2 changes of ddH_2O, dipped 5 times into acid alcohol (1% HCl in 70% ethanol), rinsed in running tap water, incubated for 30 seconds in Li_2CO_3-saturated ddH_2O, and rinsed again in ddH_2O. Sections were dehydrated by incubation for 3 minutes in 50% ethanol, 3 minutes in 70% ethanol, 3 minutes in 95% ethanol, and 2 times for 3 minutes in 100% ethanol. Sections were incubated for 3 minutes in xylene, air dried, and mounted with Cytoseal (VWR Scientific).

In situ hybridization

In situ hybridization was done as described elsewhere (Lin et al, 1997), using *mldhc* cDNA as the probe.

Results

Localization of Protein Binding to Fragments of the Mouse ldhc Promoter

Previous experiments demonstrated that a 100-bp core promoter was able to regulate reporter gene expression in an in vitro assay (Zhou et al, 1994). β-galactosidase expression in transgenic testes was limited to germ cells in the pachytene stage of meiotic prophase I. The addition of 338 bp 5′ to the core promoter increased in vitro transcription by threefold. The increase in in vitro transcription could result from either increased transcription in the population of pachytene nuclei or a greater percentage of nuclei in the population (ie, round and elongating spermatids in addition to pachytene spermatocytes) was now supporting transcription. To investigate whether factors binding to the 338-bp promoter fragment could be responsible for increased meiotic and/or postmeiotic *mldhc* transcription, an electrophoretic mobility shift assay (EMSA) was done using radiolabeled PCR products corresponding to fragments of this region as probes. Probes corresponding to −272 to −181 and −239 to −147 bp 5′ to the transcription start site were bound by factors present in testis nuclear extract and produced a single shifted band in each case, whereas a probe corresponding to −311 to −241 bp produced no shifted complexes (Figure 2). A double-stranded oligonucleotide probe within the region where the −272 to −181 and −239 to −147 bp probes overlap, corresponding to −203 to −173 bp, was synthesized, along with a series of probes containing 6-bp mutations along the −203 to −173 bp fragment (Figure 3B). In EMSAs, mutations 1 and 5 had little effect on protein binding, whereas mutations 2 and 3 completely eliminated binding and mutation 4 eliminated binding to a great extent (Figure 3A).

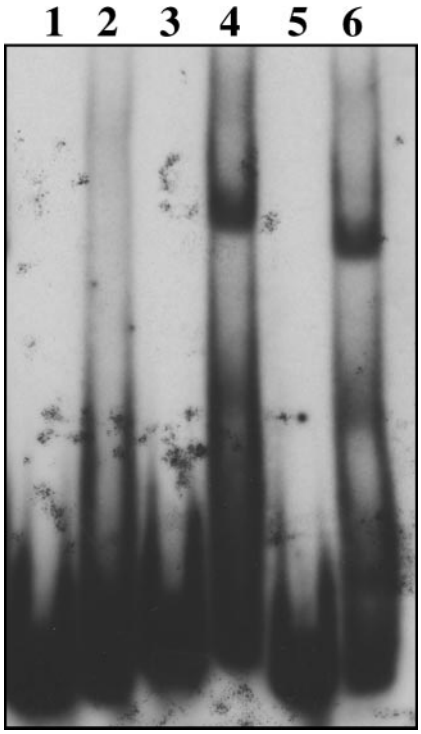

Figure 2. Electrophoretic mobility shift assays to determine the location of protein binding within the *Acc*I to *Kpn*I region of the *mldhc* promoter. Lanes 1, 3, and 5 contain free probes corresponding to −311 to −241, −272 to −181, and −239 to −147 bp, respectively, 5′ to the transcription start site. Lanes 2, 4, and 6 contain the same probes as lanes 1, 3, and 5, with the addition of mouse testis nuclear extract. Shifted bands were observed for the −272 to −181 and the −239 to −147 bp probes, indicating that factor(s) from mouse testis nuclear extract recognize sequences within these probes.

YY1 Is the Factor that Recognizes the −203 to −173 Region

Computer analysis of the sequence covered by mutations 2, 3, and 4 (GCG package, Madison, Wis) revealed a near-

B

WT	TGG CTG CCA TCT TCT GTG TGG TCC CAG GGC T
1	GAT GCA CCA TCT TCT GTG TGG TCC CAG GGC T
2	TGG CTG GAT GAC TCT GTG TGG TCC CAG GGC T
3	TGG CTG CCA TCT GAC TCA TGG TCC CAG GGC T
4	TGG CTG CCA TCT TCT GTG GAT CAG CAG GGC T
5	TGG CTG CCA TCT TCT GTG TGG TCC AGT AGT T

Figure 3. EMSA to determine sequences important for binding within the −203 to −173 fragment. **(A)** Probes are indicated above each set of lanes and correspond to sequences in panel B. Reactions run in lanes 1, 3, 5, 7, 9, and 11 contain free probes, and reactions run in lanes 2, 4, 6, 8, 10, and 12 contain probes and mouse testis nuclear extract. Boxed probe sequences **(panel B)** indicate mutated nucleotides. The near-consensus YY1 binding site is underlined. Mutations 2 and 3 eliminated binding almost completely, and mutation 4 eliminated binding to a great extent.

consensus recognition site for the transcription factor YY1 (Hariharan et al, 1991; reviewed in Shi et al, 1997), from −198 to −191 (Figure 3B, underlined). To investigate the possibility that YY1 was responsible for the shifted complexes in the EMSAs, a Southwestern blot was done using the wild type −203 to −173 bp probe and one in which the CAT within the YY1 site was mutated to ACG (Figure 4). The wild-type probe was bound by a factor of 65 kD, the molecular weight of YY1 (Figure 4, lane 1), whereas the mutant probe was not (Figure 4, lane 2).

To confirm that YY1 was the protein binding the −203 to −173 bp probe, a supershift analysis was done. Anti-YY1 immunoserum was added to EMSAs using mouse testis or liver (representative somatic tissue) nuclear extract. Because YY1 is ubiquitously expressed (Shi et al, 1997), we wanted to determine whether the binding activity observed using testis nuclear extract was also present in somatic tissue. Binding to the −203 to −173 probe was observed in reactions that contained either testis or liver nuclear extract (Figure 5A and B, respectively). The addition of α-YY1 immunoserum resulted in the elimination of the shifted complex and the appearance of 2 supershifted bands with lower mobility (Figure 5A and B, arrows). The addition of a 100-fold excess of unlabeled −203 to −173 bp probe eliminated the shifted bands (in a normal exposure; see Figure 5 legend). However, the addition of excess unlabeled probe containing the CAT → ACG mutation within the YY1 binding site mentioned above was unable to compete away the shifted complex. The YY1 antiserum was validated by doing a Western blot on testis nuclear extract (Figure 5C). A single band of the expected size, 65 kD, was observed, demonstrating that the antiserum recognizes only YY1 in testis extract.

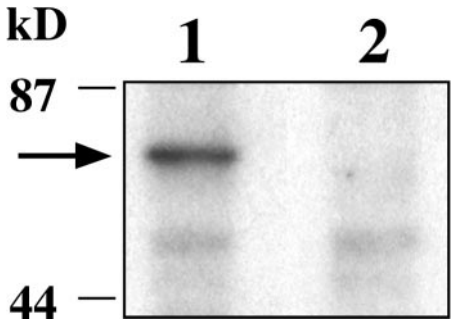

Figure 4. Southwestern blot analysis of mouse testis nuclear extract using either wild-type **(lane 1)** or YY1 mutant **(lane 2)** oligonucleotides corresponding to −203 to −173 bp as probes. In the YY1 mutant probe, the CAT within the YY1 binding site (see Figure 3B) was changed to ACG. A protein at 65 kD (arrow) bound the wild-type but not the mutant probe. This molecular weight was identical to that reported for YY1.

An mldhc *Promoter Fragment Extending to −421 bp Regulates the Expression of β-Galactosidase Only in Pachytene Spermatocytes*

Transgenic mouse lines were generated from 2 constructs, to investigate the physiological relevance of the observed in vitro binding of the *mldhc* promoter by YY1. The first construct contained the *mldhc* promoter from −421 to +10 bp fused to the bacterial *lac Z* gene (figure 1A). The second construct was identical, except for the 3-bp CAT → ACG mutation within the YY1 site that eliminated the ability of the site to compete for binding in the gel-shift assays (Figure 1B). Testes from male offspring of founder mice were fixed, embedded in paraffin, sectioned, and labeled with α-β-galactosidase immunoserum. In transgenic mice harboring the wild-type construct, β-galactosidase was detected only in pachytene primary spermatocytes (Figure 6A), which is identical to the localization reported for a promoter fragment extending to the *Kpn*I site at −83 bp (Li et al, 1998). Native expression also begins at the pachytene cell stage, but LDH-C_4 appears to increase during all later germ cell stages (Hintz and Goldberg, 1977) (Figure 6D). β-galactosidase localization in lines harboring the 3-bp YY1 mutation construct was identical to that of the wild-type construct (Figure 6B).

The Addition of 5′ and 3′ Flanking Sequences Does Not Extend Reporter Expression to Postmeiotic Cell Types

The lack of reporter expression in postmeiotic cell types of mice bearing the −421 to +10 bp promoter fragment led to 2 hypotheses. First, regulatory elements 5′ to −421 bp or in the 3′ untranslated region could be necessary for transcription in postmeiotic cell types. Second, *mldhc* may not be transcribed in postmeiotic cell types, but, instead, the mRNA persists because of mRNA stability conferred by sequence in the 3′ untranslated region. To investigate these hypotheses, an additional approximately 4500 bp of genomic DNA was added 5′ to the *Acc*I site at −421 bp, and a 722-bp fragment of *mldhc* 3′ untranslated region containing the native polyadenylation site was substituted for the SV-40 polyadenylation site present in the expression vector (Figure 1D). As was observed with the previous constructs, strong β-galactosidase expression was observed in pachytene cells. No β-galactosidase was detected in postmeiotic cell types (Figure 6C) above the background observed in nontransgenic control animals (Figure 6E). A similar reporter expression pattern was observed for mice bearing a transgene containing the mldhc 3′ untranslated region and polyadenylation site but not the 4500-bp 5′ sequence (Figure 1C; immunohistochemistry data not shown). None of the transgenic lines exhibited a high level of postmeiotic reporter expression, as is seen with native LDH-C_4 expression (Figure 6D). These results suggest that, in the context of the integrated transgenes, the additional 5′ and 3′ sequences neither permit postmeiotic transcription nor confer stability on the chimeric mRNA.

Mouse ldhc mRNA Is Present Predominately in Meiotic Cell Types

The apparent discrepancy between localization of native LDH-C_4 and β-galactosidase expressed from our transgenic constructs suggested that the increased level of LDH-C_4 in postmeiotic cell types may result from mechanisms distinct from increased *mldhc* mRNA abundance. In situ hybridization was done on mouse testis sections, using *mldhc* cDNA as the probe, to investigate this possibility. The hybridization signal was predominately meiotic (Figure 7A and B, periphery of tubules), whereas a lower level of mRNA was detectable in postmeiotic cells at higher magnification (Figure 7B, center of tubules). The *mldhc* mRNA abundance profile throughout spermatogenesis closely matches the level of β-galactosidase reporter seen at each stage.

Discussion

We show that the zinc-finger transcription factor YY1 binds to the *mldhc* promoter in both testis and liver (representative somatic tissue), consistent with its ubiquitous expression pattern. YY1 has been implicated in both positive and negative regulation of a growing list of genes (Shi et al, 1997). The question of how a ubiquitously expressed transcription factor can be involved in both the activation and repression of specific genes in a tissue- and temporal-specific manner is answered by YY1's interactions with numerous coactivators and corepressors (Shi et al, 1997; Thomas and Seto, 1999). Recent evidence has suggested that many of YY1's interaction partners may function in chromatin modification through histone acetyltransferase (coactivator) or histone deacetylase (core-

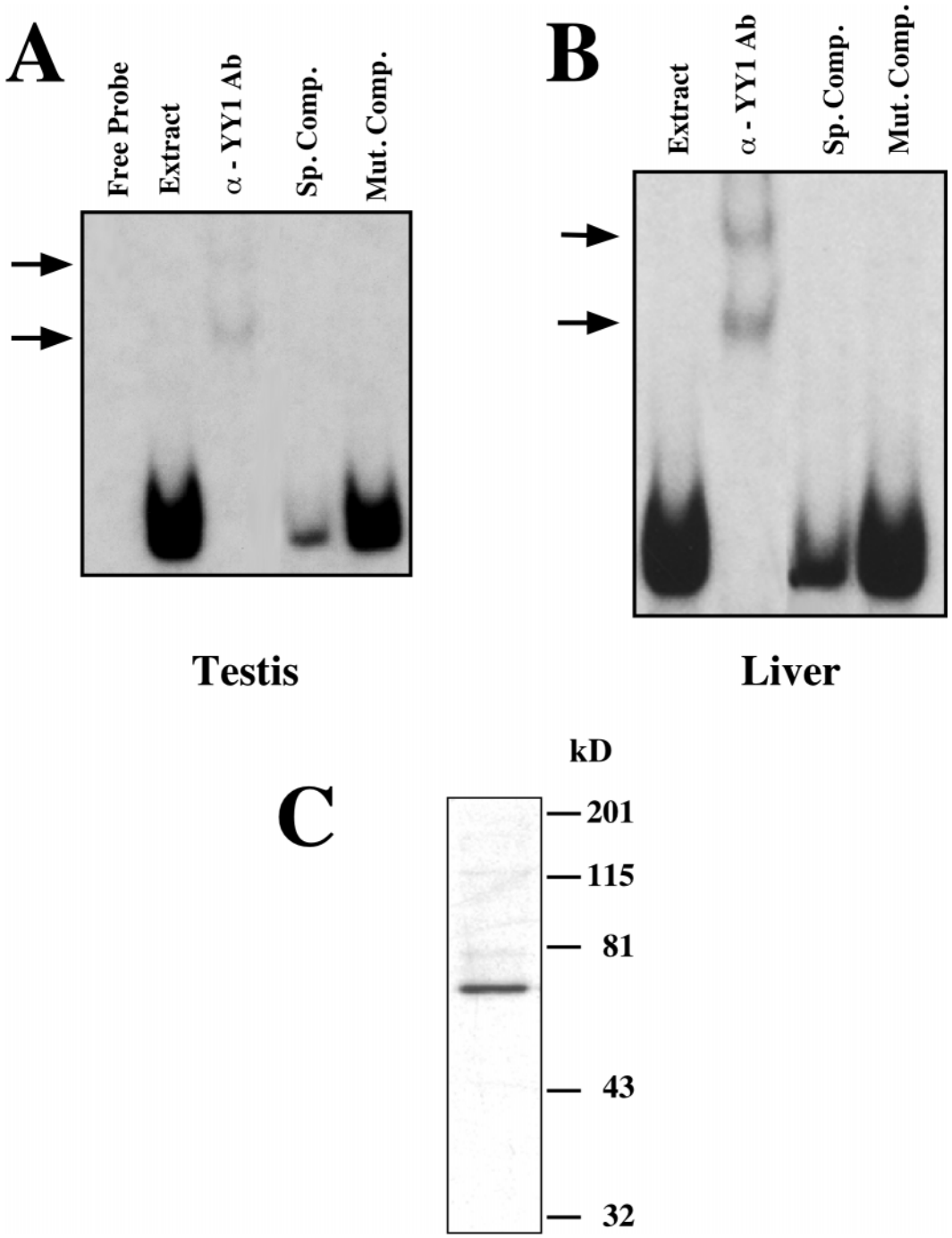

Figure 5. To confirm the factor binding to the −203 to −173 probe was in fact YY1, a supershift assay was done. Radiolabeled probe containing the YY1 binding site from the *mldhc* promoter was bound by proteins from mouse testis **(A)** and liver **(B)** nuclear extract. The addition of YY1 antiserum caused the appearance of 2 supershifted bands (arrows). A 100-fold excess of unlabeled probe (Sp. Comp.) competed away binding, but unlabeled probe with a mutation in the YY1 binding site (Mut. Comp.) was unable to compete binding. Films were deliberately overexposed, to visualize the faint supershifted bands. In a normal exposure, competition by the unlabeled native probe would be more complete. **(C)** To demonstrate the specificity of the YY1 antiserum, mouse testis nuclear extract was probed with YY1 antiserum in a Western blot. A single band was detected at 65 kD, the molecular weight of YY1. The positions of molecular-weight markers are indicated.

pressor) activity. YY1's mechanism of activation or repression would involve recruiting cofactors that either acetylate or deacetylate histones in the genomic DNA surrounding the target gene, leading to active or inactive chromatin states, respectively. The above data may explain why strong in vitro YY1 binding to the *mldhc* promoter did not translate to a noticeable effect in our transgenic study. Because our transgenes integrated into ran-

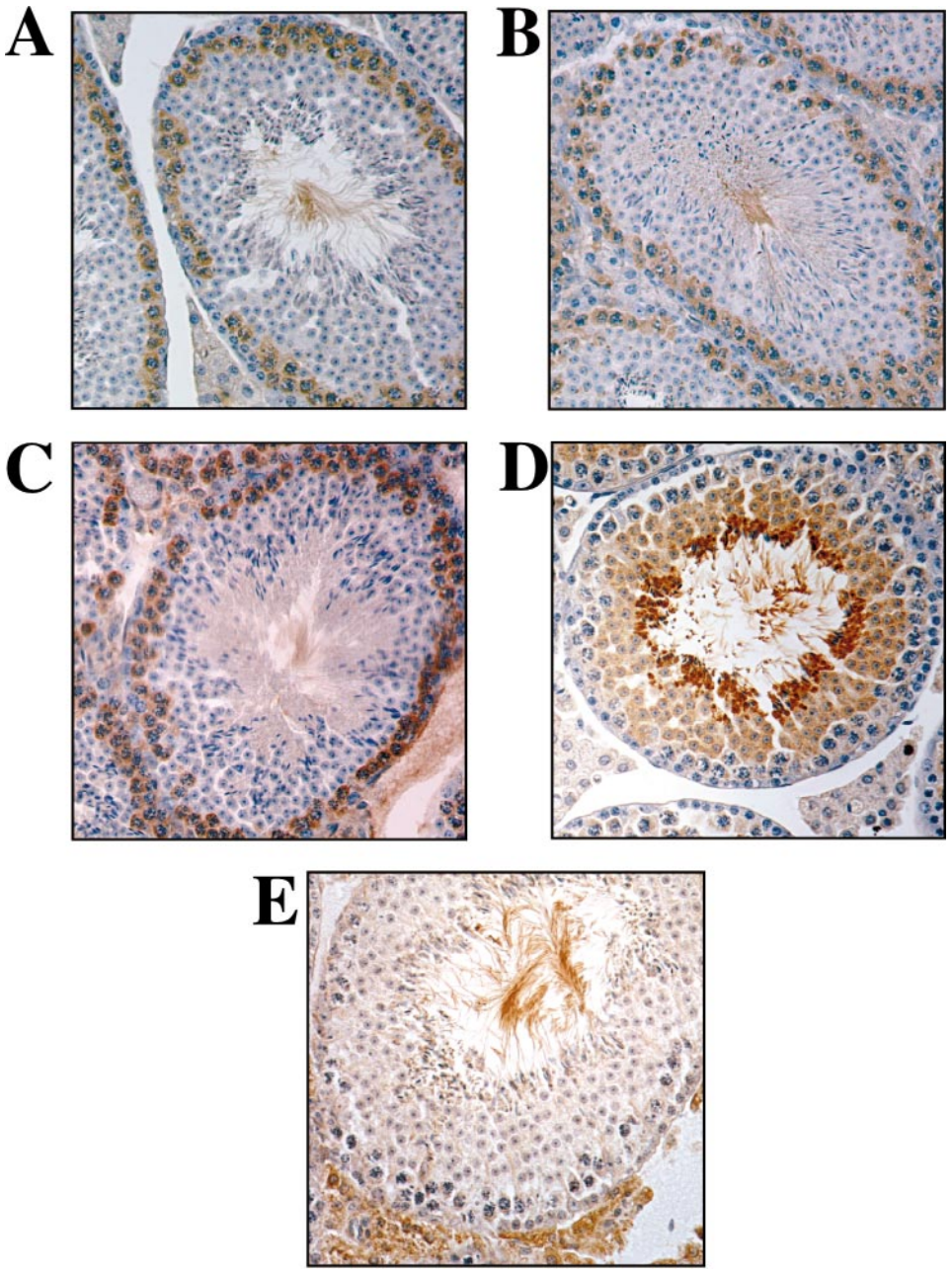

Figure 6. **(A, B, and C)** Testis sections from mice harboring *mldhc* transgenic constructs are as depicted in Figure 1A, B, and D, respectively. Sections were immunostained with β-galactosidase antiserum and detected with DAB/chromogen, which deposits a red-brown precipitate at the site of antibody binding. Sections were counterstained with hematoxylin, which stains nuclei blue. **(D and E)** Wild-type testis sections were immunostained with LDH-C_4 and β-galactosidase antiserum, respectively. All panels are 200× magnification.

dom sites instead of the native *mldhc* chromatin context, any effect that YY1-mediated chromatin modification would have had on transcription would be lost. The expression of our transgenes would, therefore, be dependent on integration into a chromatin context that was accessible to the transcriptional machinery. The low percentage of lines positive for each of our transgenes that actually expressed a detectable level of the β-galactosidase reporter (Table 1) strengthens this contention.

YY1 has been implicated in the regulation of the testis-specific *proacrosin* gene (Schulten et al, 1999, 2001) which, like *mldhc*, is first transcribed in pachytene spermatocytes (Kashiwabara et al, 1990). The cores of the YY1 sites in both promoters are of identical sequence, GCCATNTT, although the site in the *proacrosin* promoter is on the minus strand and the site in the *mldhc* promoter is on the plus strand. The YY1 binding site in the *proacrosin* promoter is located within the first intron. Consen-

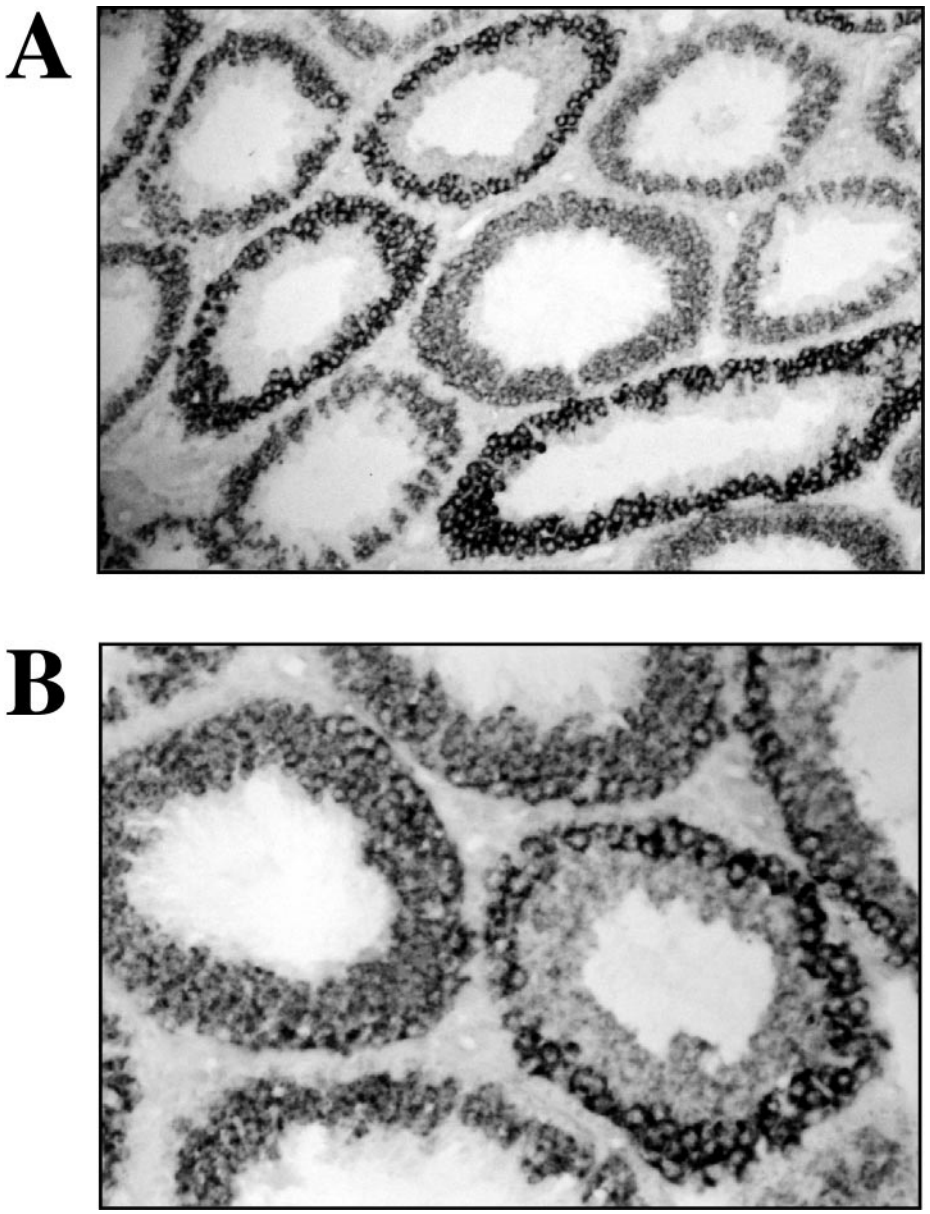

Figure 7. Mouse *ldhc* in situ hybridization. Testis sections were probed with digoxigenin-labeled *mldhc* cDNA. Although the majority of hybridization was associated with premeiotic cell types (periphery of tubules) **(panel A)**, a small amount of hybridization was seen in postmeiotic cells (center of tubules) in a higher resolution image **(panel B)**. Magnification, panel A 200×, panel B 400×.

sus YY1 binding sites have been found in the promoters of the testis-specific *protamine 1, 2,* and *3* genes and the *transition protein 1* and *2* genes (Johnson et al, 1988; Kleene et al, 1990; Yelick et al, 1991; Schluter et al, 1996). However, the consensus sequence used for the search (CCATNT) was sufficiently simple that YY1 sites were found in all promoters examined. Whether YY1 plays a role in the regulation of any of these genes will have to be determined experimentally.

The substitution of the *mldhc* polyadenylation site and 3′ untranslated region for the SV-40 polyadenylation site present in the reporter construct did not allow β-galactosidase expression in postmeiotic germ cells. In addition, the majority of *mldhc* mRNA observed in the in situ hybridization experiment was in meiotic cell types, whereas a lower level was detected in postmeiotic cells. These results suggest that the mechanism for conferring stability on *mldhc* mRNA is not contained within the 3′ untranslated region. The *mldhc* mRNA was found to be very stable, with no measurable degradation after a 24-hour incubation in isolated germ cells that had been treated with actinomycin-D, compared with *β-tubulin* mRNA which, had a half-life of 10 hours, and *c-fos* mRNA, which decayed rapidly (Salehi-Ashtiani and Goldberg,

1993). It is possible that the postmeiotic mRNA results from transcription and not mRNA stability, but the fact that none of the 3 *mldhc* promoter fragments assayed to date regulates expression of the β-galactosidase reporter in postmeiotic cells suggests that this is not the case. Formal proof of *mldhc* transcriptional activity in postmeiotic cell types is difficult to obtain without a stable cell line for such measurements. Examples of other testis-specific genes in which mRNA is stored for later translation include *proacrosin* (Florke et al, 1983), *phosphoglycerate kinase-2* (Gold et al, 1983), *protamine 1* (Kleene et al, 1984; Braun et al, 1989), and *transition protein 1* (Yelick et al, 1989). For *mldhc*, however, the fact that *mldhc* mRNA decreases in abundance postmeiotically whereas LDH-C_4 expression increases suggests that protein stability plays the major role in postmeiotic LDH-C_4 accumulation. LDH-C_4 is extremely heat-stable, retaining 65% activity after incubation at 65°C for 20 minutes (Goldberg, 1972). The LDH-C_4 half-life at 65°C is 43 minutes, giving it a much greater heat stability than LDH-B_4 (heart), which has a half-life of 10 minutes, and LDH-A_4 (muscle), which has a half-life of 9 minutes under the same conditions. The heat stability of LDH-C_4 is used to purify it from the other LDH isozymes. LDH-C_4 activity persists after heat treatment, while the other LDH isozymes are inactivated.

The data presented here, along with those of previously published reports, suggest the following model for LDH-C_4 expression. Testis specificity is maintained by a relatively short core promoter sequence (Zhou et al, 1994; Zhou and Goldberg, 1996; Li et al, 1998), and LDH-C_4 accumulation in postmeiotic germ cells is accomplished through mRNA (present article; Salehi-Ashtiani and Goldberg, 1993) and protein (Goldberg, 1972) stability. YY1 may also play a role in *mldhc* regulation through the control of chromatin accessibility.

Acknowledgments

We thank Dr. Virginia Carr for excellent advice on embedding, sectioning, and immunostaining, Drs. Jorg Bungert and Scott Ness for helpful discussions concerning EMSAs, and Dr. Teresa Woodruff for access to her digital microscopy equipment. We also thank John Eme for performing some of the immunostaninig procedures.

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