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Molecular and functional characterization of the murine *ldh2* promoter region: Sp-binding GC-box domains are the key cis-elements regulating *ldh2* gene expression during spermatogenesis☆

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ABSTRACT

The goal of the present study was to elucidate the specific transcriptional mechanisms that regulate *ldh2* gene expression during the early stages of spermatogenesis. DNA sequence analysis of the 1.0-kb *ldh2* promoter region directly upstream of the transcriptional start site indicated the presence of three SP-protein binding GC-box elements and the absence of TATA and CAAT boxes. Functional characterization studies of the mouse *ldh2* promoter were performed in the SV40 transformed mouse spermatogonial cell line, GC-1 spg. Transfection/transient expression studies using full-length and truncated *ldh2* promoter/luciferase reporter constructs revealed that all three of the SP-binding cis-regulatory GC-box elements are required for optimal *ldh2* promoter activity. Additional site-directed mutagenesis studies indicated that the two most proximal GC-box sites play essential regulatory roles in mediating basal *ldh2* promoter activity. These studies suggest that the expression of the *ldh2* gene in spermatogonia and early spermatocytes are regulated by SP-mediated transcriptional mechanisms.

1. Introduction

The pathways involved in energy metabolism that produce ATP in the mitochondria are of vital importance for survival of all cells including germ cells of the testis. Glycolysis is one of the major metabolic pathways involved in the production of ATP. The endpoint of glycolysis is pyruvate, which can either be reduced to form lactate, or oxidized to CO_2 and H_2O depending on metabolic conditions that exist within the cells. Although our current knowledge about the biochemical aspects of carbohydrate and fatty acid metabolism are extensive, details involved in regulation of these metabolic pathways in the respective cell types still remain unknown. Published studies have shown that changes in the metabolic conditions occur as germ cells in the seminiferous tubules of the testis migrate from the basement membrane into the hypoxic environment of the adluminal compartment (Russell, 1977; Boussouar and Benahmed, 2004; Grootegoed et al., 1984). Elucidation of the molecular mechanisms that determine the pattern of expression of the lactate dehydrogenase isoenzymes in differentiating germ cells can provide further insights into how these cells are able to maintain their metabolic energy requirements as they migrate towards the lumen.

The lactate dehydrogenase isoenzymes [L-lactate NAD + oxidoreductase (LDH) EC 1.1.1.27] are tetrameric enzymes responsible for the reversible conversion of pyruvate to lactate in the glycolytic cycle. Five isoforms of LDH are expressed in most somatic tissues and represent a combination of the individual A (muscle) and B (heart) subunits that are designated; LDH-1 (B_4), LDH-2 (A_1, B_3), LDH-3 (A_2B_2), LDH-4 (A_3B) and LDH-5 (A_4) (Markert, 1963). Homotetrameric LDH (C_4) isoforms are found mainly in the meiotic and postmeiotic germ cell lineages in the testis (Hawtrey and Goldberg, 1968). The three LDH protein subunits (A, B and C) are encoded by separate genes that dis-

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play unique tissue-specific expression patterns (Blackshaw and Elkington, 1970; Fine et al., 1963; Li et al., 1989). Studies performed in our laboratory have shown that the three LDH subunit genes are expressed with specific developmental patterns in differentiating mouse male germ cell lineages (Thomas et al., 1990). Further molecular studies that were performed in our laboratory and others, have shown that the stage and cell-type-specific expression patterns of the *ldh3* gene observed in male germ cells are regulated at the transcriptional level (Li et al., 1998; Yang and Thomas, 1997; Yang et al., 1998; Kroft et al., 2003).

During spermatogenesis, dramatic changes occur in the profile of metabolic gene expression as mouse male germ cells migrate from the basement membrane into the adluminal compartment. For the *ldh* genes, the transcriptional switch from *ldh2* to *ldh3* and *ldh1* gene expression is directly related to the physiological change from mitochondrial-based aerobic oxidative metabolism to glycolysis. To date, a detailed molecular analysis of the promoter region of the mouse *ldh2* subunit gene has not yet been performed to identify the cis-regulatory elements responsible for its transcriptional activation in spermatogoina and early spermatocytes. However, the promoter regions of the mouse and rat *ldh1* gene have been extensively studied and characterized in a number of previously published studies (Fukasawa and Li, 1986; Short et al., 1994, 1991). Collectively, these studies indicated that in somatic cells, *ldh1* gene expression patterns are regulated by two elements present in the 5′ upstream promoter region, a cAMP-dependent regulatory element (CRE) and a single SP-binding GC-box element. Similar molecular and genetic studies of the human and mouse *ldh3* promoters have demonstrated that in germ cells the *ldh3* subunit gene is regulated by a single SP-binding GC-box element present in the 5′ upstream region (Li et al., 1998; Yang and Thomas, 1997; Yang et al., 1998; Kroft et al., 2003).

The goal of this study was to determine whether SP factor mediated molecular mechanisms are responsible for expression of the *ldh2* gene in mouse germ cells. The immortalized mouse germ cell line, GC-1 spg (Hofmann et al., 1992) were utilized for these studies since the *ldh2* gene is expressed in spermatogonia and early spermatocytes (Thomas et al., 1990). Initial genomic analysis of the *ldh2* gene performed in this study has indicated that three Sp-factor binding GC-box sites occur in the promoter region. The molecular characterization of these GC-box cis-regulatory sites performed in this study are important since several SP factors that can interact at these sites have been identified in mouse germ cells. Persengiev et al., 1996 have identified 8.8- and 8.2-kb transcripts that encode 110-kDa SP1 transcription factors that are expressed exclusively in spermatogonia. Furthermore, our laboratory has demonstrated that alternatively spliced SP1 transcripts encoding 60-kDa and 90-kDa SP transcription factors are developmentally expressed during spermatogenesis (Thomas et al., 2005). In addition, Wilkerson et al., 2002 have shown that SP3 transcription factors are also expressed in differentiating germ cells during spermatogenesis.

2. Materials and methods

2.1. Genomic clones

Three BAC genomic clones were isolated after screening a mouse BAC genomic library (Research Genetic Inc., Huntsville, AL) with mouse *ldh2* cDNA probes. These genomic clones were initially characterized by EcoRI restriction followed by Southern blot analysis. A 16-kb EcoRI fragment was subsequently isolated and partially sequenced using polymerase chain reaction (PCR) primers that were designed from the mouse *ldh2* cDNA sequence information (Hiroaki et al., 1990). These PCR primers were utilized to completely sequence a 2.5-kb region of the *ldh2* promoter in both directions. DNA sequence information for the 1.0-kb region upstream from the transcriptional start site of the mouse *ldh2* promoter was submitted to GenBank (Accession No. AF174288).

2.2. Plasmid constructs

Truncated mouse ldh2 promoter constructs: The truncated mouse *ldh2* promoters used in this study were produced by Polymerase Chain Reaction. The PCR reaction mixture consisted of 200 ng of the 1.0-kb mouse *ldh2* promoter construct (pCAT3-E/*ldh2*), PCR primers (10 pM), dNTP mix (10 μM) and 2.5 units TAQ polymerase (Invitrogen, Carlsbad, CA). PCR conditions were 27 cycles of denaturation at 95 °C, annealing at 65 °C for 15 s, and extension at 72 °C for 1 min. Three *ldh2* gene-specific 5′oligo primers designed for this study are as follows:

- Bp3, 5′-GGTGACCTCTAACTTTAGAG-3′ (−220/−201);
- Bp4, 5′-CTTGAAGGAGATTGAACGAG-3′ (−165/−146);
- Bp1, 5′-GTCTCGGGTTTCCAATCACA-3′ (−125/−106);
- Bp2, 5′-CCAGAAAAGAGGAGGCTCTG-3′ (+45/+26) represented the 3′ *ldh2* gene-specific oligo primer. The PCR products representing the three different truncated *ldh2* promoters were purified (QIAquick Gel Extraction kit, Qiagen, Valencia, CA), and blunt end ligated into Xho1 digested luciferase reporter pGL3 basic vector (Promega, Madison, WI) was filled-in using the Klenow Fragment of DNA polymerase. The recombinant plasmids were designated as pGL3 *ldh2* ABC (−220/+45) for the full-length mouse *ldh2* promoter construct, pGL3 *ldh2* AB (−165/+45) and pGL3 *ldh2* A (−125/+45), respectively, for the two truncated mouse *ldh2* promoter constructs. Isolated plasmid constructs were sequenced to determine the correct orientation of the ldh2 promoter fragments.

Mutated mouse ldh2 promoter constructs: The full-length mouse *ldh2* promoter construct, pGL3 *ldh2* ABC (−220/+45), was employed as template to make mutated constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For pGL3 *ldh2* mA, the first GC-box (−75/−68) was mutated from GCCCCGCC to GAAAAGAA; for pGL3 *ldh2* mB, the second GC-box (−146/−139) was mutated from CCGAGGGG to AAGTTGTT. The double mutated mouse *ldh2* promoter construct was designated pGL3 *ldh2* mAB.

2.3. SP1 and SP3 plasmid expression constructs, transfections into drosophila SL2 cells

SP1 and SP3 cDNAs were cloned upstream of the V5 tag sequence in pAC 5.1 V5-His plasmids (Invitrogen) and these plasmids used for the expression of SP1 and SP3 transcription factors. The pGL3 basic vector (Promega) containing the *ldh2* promoter (pGL3 *ldh2*) was used to assay for luciferase activity. The day before transfection, Drosophila Schneider SL2 cells were incubated in a complete Schneider Drosophila medium (Invitrogen Corp., Carlsbad, CA) containing 10% heat-inactivated FBS (Gibco-BRL, Rockville, MD) and 1% penicillin–streptomycin (Sigma–Aldrich, St. Louis, MO) at room temperature. The SL2 cells were washed with Phosphate Buffered Saline (PBS), resuspended in a complete medium containing fetal bovine serum (FBS), and seeded in 6 well plates (3 × 10 cells/well). Transfection of pAC 5.1/V5-His SP3 (0.2 μg) and co-transfections with the pGL3 *ldh2* construct (0.2 μg) were performed using Effectene transfection reagent (10 μl per 15 μl total reaction vol) (Effectene Transfection Reagents kit, Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.4. GC-1 spg cell culture and transfection assays

The GC-1 spg cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The GC-1 spg cells were seeded into 24 well plates at 30–50% confluence and incubated at 37 °C in 5% CO_2 for 24 h to about 80% confluence. For transient transfection, Effectene reagents (Qiagen) were used to deliver the plasmid DNA (pGL3 ldh2 constructs) into the GC-1 spg cells. For each well, 200 ng of plasmid DNA and 10 μl of Effectene reagent added were delivered to the cells in 350 μl culture medium according to manufacturer's protocol. Thirty nanograms of the GFP expression plasmid (pEGFP-C1, Clontech, Palo Alto, CA) was co-transfected as a control for transfection efficiency.

2.5. Western blot analysis

Thirty micrograms of nuclear extract from the GC-1 spg cells or Drosophila SL2 were fractionated by SDS polyacrylamide gel (SDS-PAGE; Criterion Precast Gel, Bio-Rad laboratories, Richmond, CA). The separated proteins were transferred onto nitrocellulose membranes (Hybond-C Super, Amersham Life Science, Arlington, CA). For detection of the SP proteins, the GC-1 spg nuclear extract membrane was incubated with primary antibodies for SP3 (Panomics Inc., Redwood City, CA) and SP1 (Santa Crutz Biotechnology Inc., Santa Crutz, CA), diluted (1:500) with 1× TBS plus 0.1% Tween 20 (Bio-Rad laboratories). Subsequently, the membrane was washed with 1× TBS and incubated with second antibody, goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc.) diluted (1:1000) in 1× TBS plus 0.1% Tween 20. Immunoreactive proteins were visualized using Western Blotting Luminol Reagents (Santa Cruz Biotechnology Inc.). For detection of the SP3 fusion proteins expressed in the Drosophila SL2 cells, the membrane was incubated with anti V-5 antibody