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Homo sapiens Lactate Dehydrogenase c (*Ldhc*) Gene Expression in Cancer Cells Is Regulated by Transcription Factor Sp1, CREB, and CpG Island Methylation

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ABSTRACT: The human testis-specific lactate dehydrogenase c gene (*hLdhc*) is transcribed only in cells of the germinal epithelium. Recently *hLdhc* was reported to express in a broad spectrum of tumors with relatively high frequency in lung cancer, melanoma, and breast cancer, and in some prostate cancers. Two melanoma cell lines that express the *hLdhc* gene, A375M and C81-61, were identified and were used to characterize the *hLdhc* promoter and explore transcriptional regulation of this gene. A 110-bp core promoter, including a conserved GC box and cyclic adenosine monophosphate–responsive element (CRE), were identified as essential for basal promoter activity. The methylation status of the CpG island (CGI) in the *hLdhc* core promoter sequence was analyzed in *hLdhc*-expressing and nonexpressing cells and human prostate tumor tissues. The CGI in 2 cell lines expressing the gene was hypomethylated whereas the DNA from cells that did not express *hLdhc* was hypermethylated. The role of methylation in regulating this promoter was confirmed by experimental induction of *hLdhc* transcription with the methylation inhibitor 5′aza-deoxycytidine. Quantitative analyses of the methylation level in the CGI were performed in prostate tumor tissues by pyrosequencing. Overall, these experiments demonstrated that *hLdhc* expression in cancer cells was regulated by transcription factor Sp1 and CREB and promoter CGI methylation. In addition, these findings suggest the possibility of developing a biomarker for cancer diagnosis/prognosis based on DNA methylation of the *Ldhc* gene.

Key words: DNA methylation, cancer/testis antigen, promoter.

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Cancer/testis–associated genes (CTAs) are expressed only in the germinal epithelium of the testis and in some cancer cells, but not in noncancerous somatic tissues. The first CTA, MAGEA-1, was discovered in 1991 using T-cell epitope cloning (van der Bruggen et al, 1991). In 1997, Chen et al first introduced the concept of ‘‘cancer-testis antigen’’ for a pool of antigenic targets of cancer vaccination. In the last decade, there has been a great effort to search for this subgroup of tumor antigens, and many CTAs have been identified (Zendman et al, 2003). CTAs are currently considered promising candidates for diagnosis and immunotherapy of cancer (Zendman et al, 2003; Kalejs and Erenpreisa, 2005). Understanding the aberrant expression of these genes in cancer cells should provide insight into tumor development. One of the hypotheses for ectopic expression of testis-specific genes in cancer cells is the induction or activation of a gametogenic program in cancer (Old, 2001). A useful marker for such a program is the *Ldh* gene, which encodes an important glycolytic enzyme, lactate dehydrogenase, which converts pyruvate to lactate. This gene family includes 3 isozymes: *Ldha*, *Ldhb*, and *Ldhc*. *Ldha* and *Ldhb* are expressed in somatic tissues but *Ldhc* expression is strictly limited to the testis (Hintz and Goldberg, 1977). Recently, the germ cell–specific *hLdhc* gene was shown to express in a broad spectrum of tumors, with high frequency in lung cancer (47%), melanoma (44%), and breast cancer (35%) (Koslowski et al, 2002), but the mechanism for ectopic activation of this gene is unknown. As a matter of fact, because of lack of a reliable culture system, transcription regulatory mechanisms for germ cell–specific genes are poorly understood.

By screening a repertoire composed of 22 cancer cell lines, 2 melanoma lines were identified that express *hLdhc*. These cell lines provided a useful tool to characterize the promoter of *hLdhc* and to obtain an understanding of the regulatory mechanism for its ectopic expression in cancers. Previously, data describing the *hLdhc* promoter were obtained with a cell-free in vitro transcription assay (Bonny et al, 1998); the

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transcription factor Sp1 was reported to play a major role in *hLdhc* expression. In addition and unlike the murine promoter, the proximal region of the human 5′ genomic sequence was found to contain a mini–CpG island (CGI). A CGI is a stretch of CpG dinucleotides sequestered within a short genomic DNA region, with the CpG dinucleotides as the target for DNA methylation. The activation of several human CTAs in tumor cells is reported to correlate with CGI hypomethylation in the gene promoter sequence (De Smet et al, 1999, 2004; Lim et al, 2005). Therefore, the role of CGI methylation in regulation of *hLdhc* gene expression was investigated in the melanoma cell lines in this study. Also, we took the opportunity to investigate prostate tumor tissues in view of a recent clinical study showing a correlation between gene regulatory region methylation and Gleason score, pathologic stage, and tumor occurrence of prostate cancer. CGI methylation was suggested to serve as a molecular biomarker for the detection and diagnosis of patients with prostate cancer (Bastian et al, 2004). Ellinger et al (2008) demonstrated that hypermethylation at a single locus did not correlate with any clinicopathologic variables; however, hypermethylation in promoters of 2 genes was significantly correlated with the pathologic stage and/or Gleason score. In this study the *hLdhc* CGI methylation level was examined in a small number of prostate cancer patients to evaluate its role as a potential biomarker. We report here that a 110-bp core promoter was able to drive robust reporter expression in cancer cells that express *hLdhc*, and that both the GC box and the CRE site in the core promoter are essential for basal promoter activity. In addition, promoter CGI methylation plays a role in controlling *hLdhc* expression in cancer cells.

Materials and Methods

Cell Culture, Drug Treatment, and Transient Transfection

Melanoma cell lines C8161, A375P, A375M, and Hela cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a 5% CO_2 humidified atmosphere. It was necessary to culture C81-61 (note that cell lines C8161 and C81-61 have a very similar nomenclature, with the only difference a single dash) in Ham F-10 with 15% FBS, 1× MITO+, and gentamicin sulfate because the low passage number has the cells behaving more like a primary culture, with poor growth in DMEM. Hela cells were obtained from the American Type Culture Collection, and the melanoma cell lines were provided by Dr Mary J. C. Hendrix, Northwestern Children's Memorial Hospital, Chicago, Illinois. Cells were plated at low density 12 hours prior to drug treatment; both 5′-aza-2′-deoxycytidine (5′Aza-dC) and trichostatin A (TSA) were purchased from Sigma-Aldrich (St Louis, Missouri). For 5′Aza-dC treatment, cell culture medium containing 5 μM 5′Aza-dC was changed every 12 hours, and RNA was extracted after 48 hours incubation. The treatment in triplicate with 5′Aza-dC + TSA was the same except that 300 nM TSA was added in the last 12 hours of incubation. Plasmid transfection reagent was Lipofectamine 2000 (Invitrogen, Carlsbad, California); 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, Madison, Wisconsin).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen) reagent. Two micrograms total RNA was used to synthesize first-strand cDNA by M-MLV reverse transcriptase (RT; Promega) according to the manufacturer's instructions. For *hLdhc* amplification, 1 μl RT product was used in 25 μl RT–polymerase chain reaction (PCR) mixture; PCR primers were 5′-GTCGTTGGTGTATTTTTCTGGTGTCA-3′ (sense) and 5′-CACAGCCTGCTATCTTCTGTTCTCC-3′ (antisense). PCR conditions were 1 minute at 94°C, 31 cycles of 15 seconds at 94°C, 30 seconds at 58°C, and 1 minute 30 seconds at 72°C. For human β-actin control, 0.5 μl RT product was used in 25 μl RT-PCR mixture; PCR primers were 5′-TGACGGGGT-CACCCACACTGTGC CCATCTA-3′ (sense) and 5′-CTA-GAAGCATTTGCGGTGGACGATGGA GGG-3′ (antisense); PCR conditions were 1 minute at 94°C, 25 cycles of 15 seconds at 94°C, 30 seconds at 63°C, and 1 minute 30 seconds at 72°C. The cycle number was adjusted for PCR amplification in the linear range. PCR products were run on an agarose gel, then stained by SYBR Green (Molecular Probes, Eugene, Oregon) for 30 minutes and imaged with a Kodak (Rochester, New York) Image Station 4000 System; for quantitative analysis and comparison of mRNA levels, Kodak Molecular Imaging software 4.0 was applied to obtain the net intensity for region of interest in the same gel. β-actin was used as a control for cDNA loading.

Plasmid Constructs

Primers Used for Constructs—Forward primers carry a KpnI restriction site and reverse primer a HindIII restriction site for all human promoter constructs. hldhc5′793F: 5′-ATGGTACC-CTACCTGTAATGGAAAGCCACGGAAG-3′ (construct −1718/+22); hldhcSeqF: 5′-ATGGTACC CGCTCCAGGG-CAGAA AAGAC-3′ (construct −502/+22); hldhc148F: 5′-ATGGTACCACGGAGGGCAACCGTCGAC-3′ (construct −393/+22); hldhc265F: 5′-ATGGTACCTGATTGCGCCA-AGCAAAGC-3′ (construct −278/+22); hldhc364F: 5′-ATGG-TACCTCCCATTGCCTGGTAGACT-3′ (construct −180/+22); hldhc452F: 5′-ATGGTACCGGCTCCAACATT-CTGCAAACTG-3′ (construct −92/+22). Common reverse primer is hldhc5′LR2: 5′-CATAAGCTTGCTGCTCCTTGA-CAG TTGACATTTGGAGAAC-3′; human blood genomic DNA (Promega) was used as template to amplify the region of interest by Pfu polymerase (Stratagene, La Jolla, California). PCR products were purified on agarose gel and digested by restriction enzyme pair (KpnI/HindIII) and cloned into the pGL4-luc2 (Promega).

Primers Used for Deletion Constructs—Common forward primer was hldhcSeqF; reverse primer is shown as follows: hldhc148Re: 5′-CATAAGCTTCGACTCGAGACACGCAC-G-3′ (construct −502/−393); hldhc265Re: 5′-CATAAGCTT-TACAGATGACCCACAGGGAC-3′ (construct −502/−278); hldhc364Re: 5′-CATAAGCTTGCTGGGGCACCAGCG-3′ (construct −502/−180); hldhc474Re: 5′-CATAAGCTTCAG-TTTGCAGAATGTTGGAGC-3′ (construct −502/−92). A short 40-bp human ldhc promoter (−462/−422) construct was prepared by cloning an annealed synthetic oligonucleotide pair hLdhc40promoterS (5′-CGGCGGGGGCGGAGCTTGC-GTGCTGAC GGGGCGGAGCTTGCGTGCTGACGCAT-AAGAGCCA-3′) and hLdhc40promoterAS (5′-AGCTTG-GCTCTTATGCGTCAGCACGC AAGCTCCGCCCCCCG-CCGGTAC-3′) into pGL4-luc2 vector. Three mutated promoter constructs were prepared by using an overlapping primer pair that contained mutations in either GC box or cyclic adenosine monophosphate–responsive element (CRE) sites in the *hLdhc* core promoter region (−502/−393). For the hSpmu construct, PCR1 (primer hldhcSeqF and hSpmuFor 5′-GGG**TT**GGAGCTTGCGTGCTG-3′) and PCR2 (primer hSpmuRe 5′-ACGCAAGCTCC**AA**CCCCCGCCGGCCC-AG-3′ and hLdhc148Re) were performed separately, then the PCR products were combined and diluted 200-fold for a further PCR using primers hLdhcSeqF and hLdhc148Re. This final PCR product was digested by KpnI/HindIII, cloned into vector pGL4-luc2, and confirmed by sequencing. A similar procedure was used to create the hCREmu construct by using 2 overlapping primers containing mutation: hCREmuFor 5′-GCGTGCT**G**T**GG**CATAAGAGCCGAGCG-3′ and hCRE-muRe 5′-CTTATGC**C**A**C**AGCACGCAAGCTCC-3′. The double mutant construct Sp/CREmu was created by using hSpmu as template, but the PCRs were performed using the primer set for creating hCREmu construct.

Electrophoretic Mobility Shift Assay

The gel shift kit supplied by Promega was used according to the manufacturer's instructions with the following modifications. The labeled probe was purified on a G-50 micro column (Amersham Pharmacia, Piscataway, New Jersey). Electrophoretic mobility shift assay (EMSA) on a 5% 0.75-mm-thick acrylamide gel was performed in the cold room until the dye front reached the bottom of the gel. The following oligonucleotides were used for EMSA: GC box probe hldhc-GC (5′-GGGCCGGCGG **GGGGCGG**AGCTT-3′); CRE site probe hldhc-CRE (5′-GCTTGCGTGC**TGACG**CATAAG-3′); hldhc-GCmu1 (5′-GGGCCGGCGGGG**TT**GGAGCT-3′); hldhc-GCmu2 (5′-GGGCCGGC**TA**GGGGCGGAGCTT-3′); hldhc-CREmu (GCTTGCGTGCT**G**T**GG**CATAAG-3′). Sp1 antibody (sc-59) for supershifts was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California), and antibody recognizing CREB was obtained from Rockland Immunochemicals (Gilbertsville, Pennsylvania).

Bisulfite Genomic Sequencing

Genomic DNA was extracted from cultured cells or tissue by either DNeasy Tissue Kit (Qiagen, Valencia, California) or Wizard Genomic DNA Purification Kit (Promega). The EZ DNA Methylation-Gold kit (Zymo Research, Orange, California) was used for bisulfite treatment. The PCR amplification procedure was as described previously (Clark et al, 1994) with the following modifications: Seminested PCR amplified the CGI region of the *hLdhc* promoter; the first PCR primer set was MET600F: 5′GGTTAGGTTGTTTTAAATTTTTGATTT-TAGG and MET1161Re: 5′CAAAAACAAAACAC-CACCTTTAAA; after the first PCR the reaction was diluted 100 times and 1 μl used for the second PCR amplification. The second PCR primer set was MET873F: 5′GTTTTGTA-TATTTTTTGGGTTAGTTTAGAA and MET1161Re. PCR conditions were 2 minutes at 94°C; 5 cycles of 1 minute at 94°C, 2 minutes at 51°C, and 2 minutes at 72°C; followed by 30 cycles of 30 seconds at 94°C, 2 minutes at 51°C, and 90 seconds at 72°C; with a final 6-minute extension at 72°C. PCR products were separated on an agarose gel, excised, and purified by QIAquick Gel Extraction Kit (Qiagen). TA cloning was used to insert the PCR products into pGEM-T Easy Vector (Promega). The insert was confirmed by restriction digestion and sequenced with the T7/SP6 universal primer in the cloning vector. About 10 subclones were picked for each DNA sample.

Pyrosequencing Analysis

Prostate tumor DNA samples were provided by Dr Karen Kaul's Molecular Diagnosis Laboratory in Evanston Northwestern Healthcare, Evanston, Illinois. Prostate samples were obtained from patients according to Prostate Tissue Bank protocol. Tissue slices were embedded in optimum cutting temperature medium and frozen, then stored at −80°C. Frozen sections were hematoxylin and eosin–stained and examined microscopically; tumor percentages were recorded by a clinical pathologist. Samples for this study were obtained by cutting three 10-μm sections from each tissue block for DNA extraction and purification using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota). DNA was treated by the bisulfite modification kit as mentioned previously. Bisulfite-treated DNA was then amplified by PCR with a pair of methylation DNA primers; the PCR products were used for pyrosequencing analysis. The pyrosequencing service was offered by EpigenDx Inc (Worcester, Massachusetts). The assay design package is available upon request.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism for Windows version 4.0 (GraphPad Software Inc, San Diego, California). One-way analysis of variance and Bonferroni's multiple comparison test were used to evaluate data. Differences were considered significant when P was less than .05.

Results

Melanoma C81-61 and A375M express *hLdhc*, but their isogenic counterparts C8161 and A375P do not express this gene (Figure 1). Multiple splicing variants were found in both melanoma cell lines, consistent with the

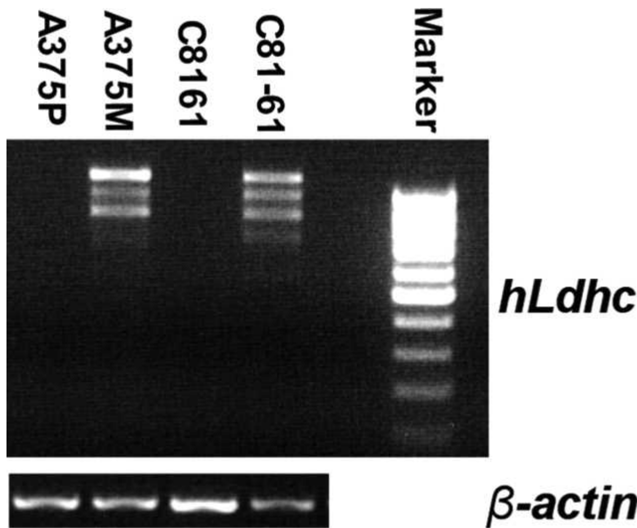

Figure 1. Reverse transcriptase polymerase chain reaction (RT-PCR) screening for *hLdhc* expression in 4 melanoma cell lines (see text for description). Upper panel: RT-PCR for *hLdhc*; bottom panel: β-actin control. A375P and A375M melanoma cell lines are isogenic. C8168 and C81-61 are isogenic. Multiple transcripts of *hLdhc* expression were detected in A375M and C81-61 cells. The top (slowest-migrating) band on the gel was the full-length cDNA based on sequencing data. The other bands are cancer-specific splicing variants of the gene.

report by Koslowski et al (2002) describing expression of several CTAs in a panel of neoplastic cells. The top (slowest-migrating) band on the gel was the full-length cDNA based on sequencing data. The other bands are cancer-specific splicing variants of the gene.

The C81-61 cultured cells have a low passage number (5) and grow very slowly with low transfection efficiency for plasmid DNA. Therefore the A375M cell line was chosen for characterization of the *hLdhc* promoter. The 5′ proximal promoter sequence of the *hLdhc* gene is illustrated in Figure 2. Twenty-four CpG dinucleotides were found to sequester in a 200-bp promoter region. Some important transcription factors, including Sp1, CRE, and HIF1, all have binding sites in this region. To identify the core promoter, a progressive 5′ deletion of the flanking sequence of *hLdhc* led to a series of constructs with promoters from 100 bp to 1.7 kb of DNA (Figure 3). All the tested promoter fragments have a common 3′ end (position +22). These constructs were used for transient transfection of the A375M melanoma cell line. Reporter luciferase activity was not significantly different between construct −1718/+22 and −502/+22 ($P > .05$), indicating that the first 1.2 kb fragment (from position −1718 to −502) does not contribute to the promoter activity. However, a further 100-bp deletion (region from −502 to −393) resulted in a more than 10-fold reduction of reporter activity ($P < .001$). Any further deletion did not seem to affect activity, indicating that the region from −502 to −393 is essential for *hLdhc* promoter activity (Figure 3). Next we asked whether downstream *cis* element(s) are required for promoter activity when the core region (−502 to −393) is retained. All of the promoter sequences for the 5 constructs in this assay have a common 5′ start position −502 (Figure 4). However, compared to the core promoter construct (−502/−393), activity did not increase when the length of the sequence was increased stepwise to the translation start site. In contrast, reporter activity decreased significantly ($P < .05$), suggesting negative regulatory elements embedded in the region from position −393 to +22.

In the 110-bp core promoter region, a GC box and a CRE binding site were identified adjacent to each other

Figure 2. 5′ proximal promoter region of *hLdhc* gene. This sequence region contains the CpG island with 24 CpG dinucleotides (underlined). Sequence inside the first box is GC box, the second box is cyclic adenosine monophosphate–responsive element, and the third box is HIF1 binding sites. Transcription initiation site is at −375 nt.

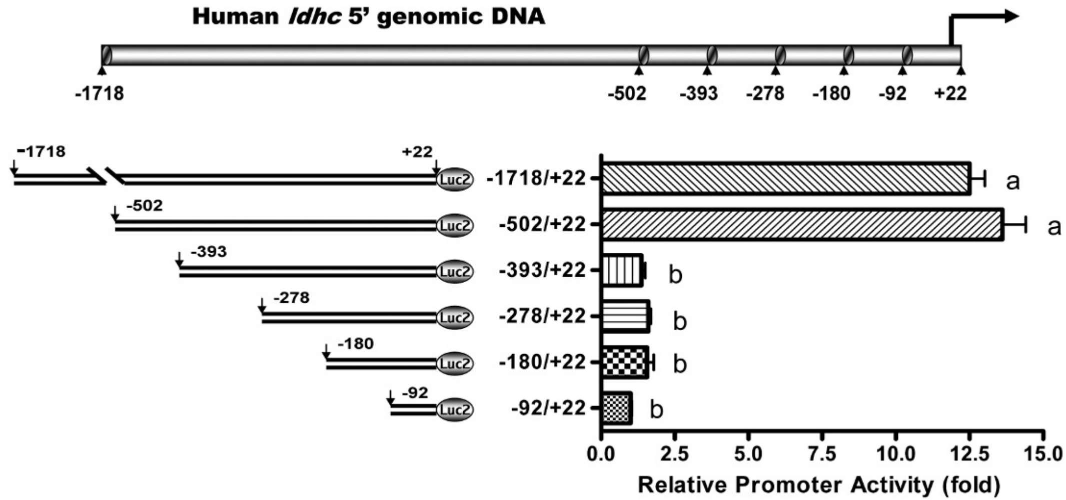

Figure 3. Deletion analysis of promoter. A luciferase reporter assay identified a core promoter between −502 and −393 of the *hLdhc* gene 5′ flanking sequence. Reporter activity was reduced more than 10-fold from construct −502/+22 to −393/+22 by deleting the core promoter sequence. Luciferase value are shown as fold increases compared with the shortest promoter construct (−92/+22). Bars bearing different letters (a, b) are statistically different at $P < .05$.

(Figure 5). A 2-bp mutation in the GC box (hSpmu) or CRE site (hCREmu) caused an 87% and a 91% reduction in promoter activity, whereas a double mutation Sp/CREmu abolished promoter activity. To test whether a GC box and CRE site were sufficient for core promoter activity, a 40-bp promoter construct (construct −462/−422) containing the pure GC box and CRE site was prepared (Figure 5). However, the reporter activity from this 40-bp promoter was only 25% of that from the 100-bp core promoter ($P < .001$).

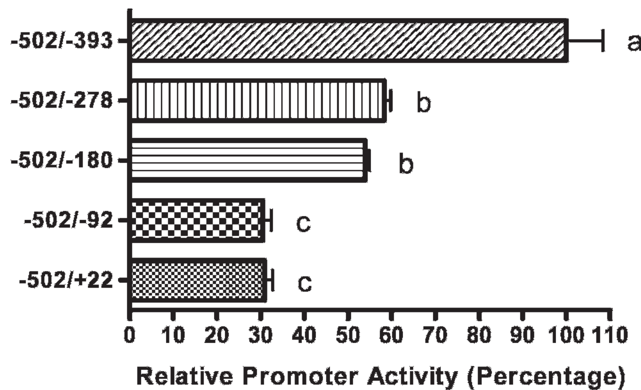

Figure 4. Analysis of the 3′ promoter sequence. A *cis* element required for gene activation was not detected in the downstream sequence of the core promoter (−502/−393). Reporter activity did not increase as sequence was added to the core promoter stepwise to the translation start site. All promoter fragments started from the same 5′ position. Reporter activity in the core promoter was labeled as 100% and other constructs were expressed as percentage of core promoter activity. Different letters (a, b, c) indicate that the groups were significantly different ($P < .050$). a is significantly different from b and c.

A gel shift assay was performed to examine the specific DNA-protein interactions between GC-box and CRE sites of the *hLdhc* promoter and DNA binding proteins from the A375M cell nuclear extract (Figure 6). The *hLdhc* promoter contains a consensus GC box (GGGGCGG) that incidentally is conserved between mouse (Tang et al, 2008) and human, but also an overlapping potential binding site (GGCGGGG-GGCGG) for Egr (early growth response) transcription factor family (Egr consensus: GCGGGGGCG). Multiple protein-DNA complexes were identified for the fragment *Ldhc*-GC that contained a GC box (Figure 6). One hundred–fold cold *Ldhc*-GC oligonucleotide was able to compete off all the shifted bands. But oligonucleotide *Ldhc*-GCmu1 with a mutated GC box could partially compete off the shifted bands. Presumably the remaining GC-rich sequence in the potential Egr site is able to bind to GC-box binding protein but with lower affinity. Oligonucleotide *Ldhc*-GCmu2 contained a mutation in the Egr site but not in the GC box, and was able to completely compete off all the shifted bands because of its intact GC box. A supershift band was observed when Sp1 antibody was added to the reaction. Multiple shift bands were also identified for the *Ldhc*-CRE probe. One hundred–fold cold *Ldhc*-CRE oligonucleotides can compete with shifted bands, but the oligonucleotides with a CRE mutation could not compete with the top shift band. Addition of a CREB antibody led to a supershift band. The supershift data indicated that transcription factors Sp1 and CREB in the A375M cell nuclear extracts bind specifically to the GC box and CRE site in *hLdhc* promoter.

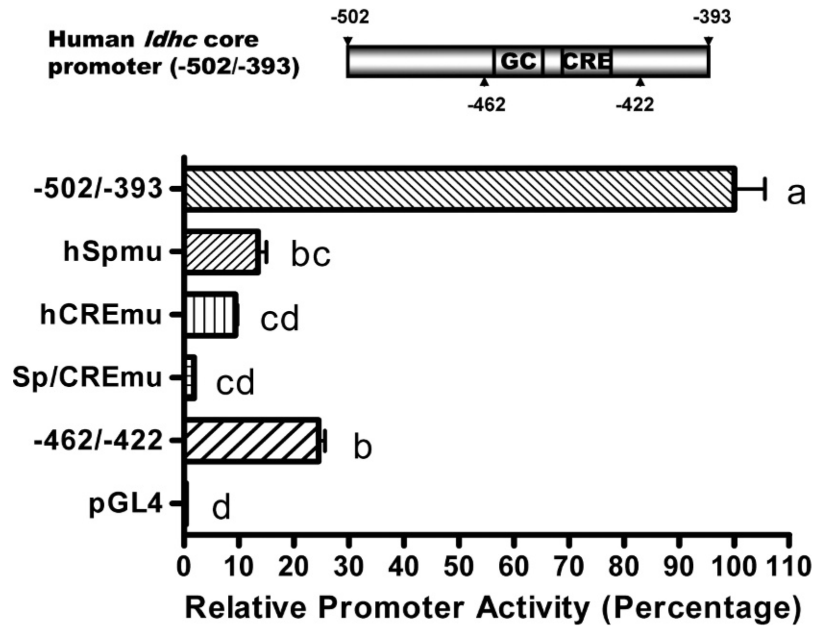

Figure 5. Effects of the GC box and cyclic adenosine monophosphate–responsive element (CRE) site mutation on *hLdhc* core promoter activity. Construct hSpmu contained a mutated GC box; hCREmu carried a mutated CRE site and Sp/CREmu contained both mutations in the construct. Constructs hSpmu, hCREmu, and Sp/CREmu contained the same promoter DNA fragment as the core promoter sequence (−502/−393) except with the corresponding mutations described above. A shorter 40-bp promoter construct (−462/−422) was also prepared to examine whether the pure GC box and CRE sites without franking sequence were sufficient for complete core promoter activity. pGL4 was an empty plasmid as a negative control. The promoter activities in hSpmu and hCREmu were reduced 87% and 91% respectively compared to the core promoter (−502/−393). Double mutations in Sp/CREmu abolished the promoter activity. Reporter activity for the core promoter was counted as 100% and other constructs were expressed as percentage of that in the core promoter. Different letters (a, b, c, d) indicate that the groups were significantly different ($P < .05$). Bars with shared letters indicate that the group did not differ significantly ($P > .05$). Thus, a is significantly different from all ($P < .05$); bc does not differ from cd; and cd does not differ from d ($P > .05$).

A 2-kb 5′ genomic DNA sequence of the *hLdhc* gene was analyzed for CpG dinucleotide distribution (Figure 7) using CpG Island Searcher software (Takai and Jones, 2002). A CGI was located in the core promoter region of the gene; other CpG dinucleotides were scattered upstream and downstream of these sequences. Noncore methylation is frequently observed for various genes, but it does not seem to block gene transcription (Gonzalgo et al, 1998; Deng et al, 1999; Miyakura et al, 2001). Therefore, we focused our attention on the island with 24 CpG dinucleotides sequestered in the 200-bp promoter region (Figure 2). The CGI overlaps with the core promoter region.

The methylation status of the 24 CpG dinucleotides of *hLdhc* was analyzed in the isogenic cell lines (Figure 8). Hela cells, which do not express *hLdhc*, were included as a generic control. Human testis genomic DNA was used for comparison with DNA from the cell lines. The CGI is hypermethylated in C8161, A375P, and Hela cells that do not express *hLdhc*; DNA from the testis, C81-61, and A375M cell lines that express *hLdhc* showed a hypomethylated CGI (Figure 8). However, methylation is not coincident in these cell lines; 4 out of 13 subclones showed a completely methylated CGI in melanoma C81-61. In the A375M cells, 50% of the subclones showed a scattered methylation pattern in CGI. The CGI in Hela cells was almost completely methylated in all clones assayed.

To confirm hypermethylation as the mechanism for silencing expression in the isogenic partners, C8161 and A375P, the demethylation agent 5′Aza-dC was used to treat these cell lines as well as Hela cells (Figure 9). CpG methylation usually promotes histone deacetylation; histone deacetylases (HDACs) are recruited to the promoter region and function as corepressors of transcription (Zlatanova et al, 2000; Jaenisch and Bird, 2003). Thus a HDAC inhibitor, TSA, was used with the 5′Aza-dC to treat the A375P and Hela cells. *Ldhc* expression was inducible in all 3 cell lines and transcription was enhanced by TSA treatment (Figure 9); TSA treatment alone did not induce *hLdhc* transcription (data not shown).

To rule out the possibility that aberrant methylation was a peculiarity of cell culture, the CGI of *hLdhc* was examined in 4 prostate tumor samples and 1 normal control sample (Figure 10). A differential methylation pattern was observed between these samples, but all the tumor samples (PC11, PC12, PC13, and PC14) had a higher methylation level compared to the normal tissue control section. Interestingly, sample PC14, with the

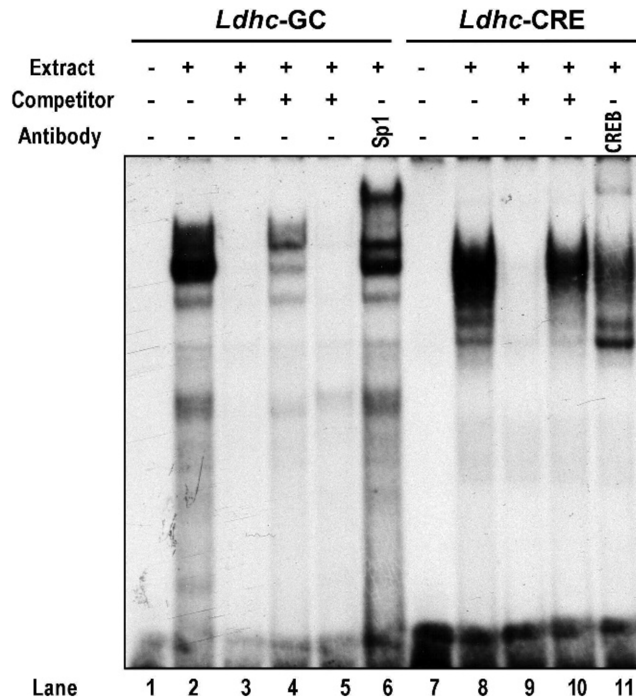

Figure 6. Electrophoretic mobility shift assay. There are specific DNA-protein interactions between the GC box or cyclic adenosine monophosphate–responsive element (CRE) in *hLdhc* promoter and nuclear protein extract (NE) from A375M melanoma cells (see text for description). Lanes 1–6, oligo Ldhc-GC used as a probe; lane 1, Ldhc-GC probe; lane 2, probe + NE; lanes 3–5, probe + NE + 100 × nonradioactive Ldhc-GC, Ldhc-GCmu1, and Ldhc-GCmu2, respectively; lane 6, probe + NE + antibody. Lanes 7–11, oligo Ldhc-CRE used as probe; lane 7, Ldhc-CRE probe; lane 8, probe + NE; lane 9, probe + NE + 100 × nonradioactive Ldhc-CRE; lane 10, probe + NE + 100 × Ldhc-CREmu; lane 11, probe + NE + antibody. Supershifts were observed for Sp1 antibody and CREB antibody.

highest Gleason grade, also had the highest methylation level, even though the tumor percentage (80%) in the tissue section for analysis was lower than in samples PC12 (91%–100%) and PC13 (90%) (Figure 10). Samples PC12 and PC15 were obtained from the same prostate gland, but PC12 was isolated from the region containing tumor and PC15 from the region that showed no evidence histologically of benign prostate hyperplasia, prostatic intraepithelial neoplasia, or atrophy. A distinctive higher methylation level in PC12 was observed compared to PC15.

Discussion

The availability of cancer cell lines that expressed the testis-specific *Ldhc* fortuitously allowed us to analyze transcriptional regulation and describe factors involved in activation and repression of this gene. As noted previously, reliable cultures of spermatogenic cells are not available for such assays. We report here that the GC box and CRE site are essential for the TATA-less human *Ldhc* promoter. It has been reported that either an Sp1 or a CRE binding site is indispensable for TATA-less promoters (Weis et al, 1997; Emami et al, 1998; Somboonthum et al, 2005); the Sp1 binding site is required for accurate positioning of the transcription initiation complex on the promoter. We speculate that the combination of a GC box and a CRE site in the core promoter is sufficient to drive a robust, tissue-specific gene expression in testis. These features are seen in both the human and murine *Ldhc* (Tang et al, 2008) core promoters. Enriched Sp1/Sp3 and testis-specific coactivator for CRE-site binding protein in testis is likely the key to fulfilling this task, because the consensus mutation of these transcription factor binding sites leads to a significant reduction/abrogation of reporter activity (Figure 5). In a 40-bp short *Ldhc* promoter (Figure 5) containing both GC box and CRE site, only 25% activity of the 110-bp core promoter was retained. It is likely that an oncogenic transcription factor may have a binding site in the 110-bp core promoter region. For example, in cancer cells c-myc is responsible for the up-regulation of *Ldha* gene expression (Shim et al, 1997). A stepwise deletion experiment may help to find the *cis* element for this potential transcription factor in the core promoter region.

In this study we demonstrated that the promoter CGI is hypomethylated only in the cancer cells that express *hLdhc*. The methylation inhibitor 5′Aza-dC induced

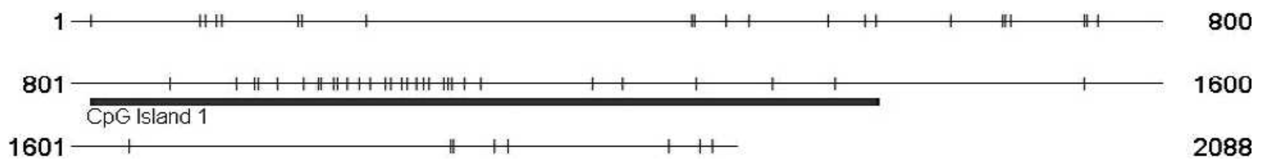

Figure 7. CpG dinucleotide distribution in 2-kb *hLdhc* 5′ genomic sequence. The CpG Island Searcher detected a single island in the core promoter region of *hLdhc*. Translation initiation codon is at 1454 nt. Criteria used for defining a CpG island: Select lower limits: %GC = 55; ObsCpG/ExpCpG = 0.65; length = 500; distance = 100. CpG island start = 814, end = 1393. %GC = 56.4; ObsCpg/ExpCpG = 0.651; length = 580.

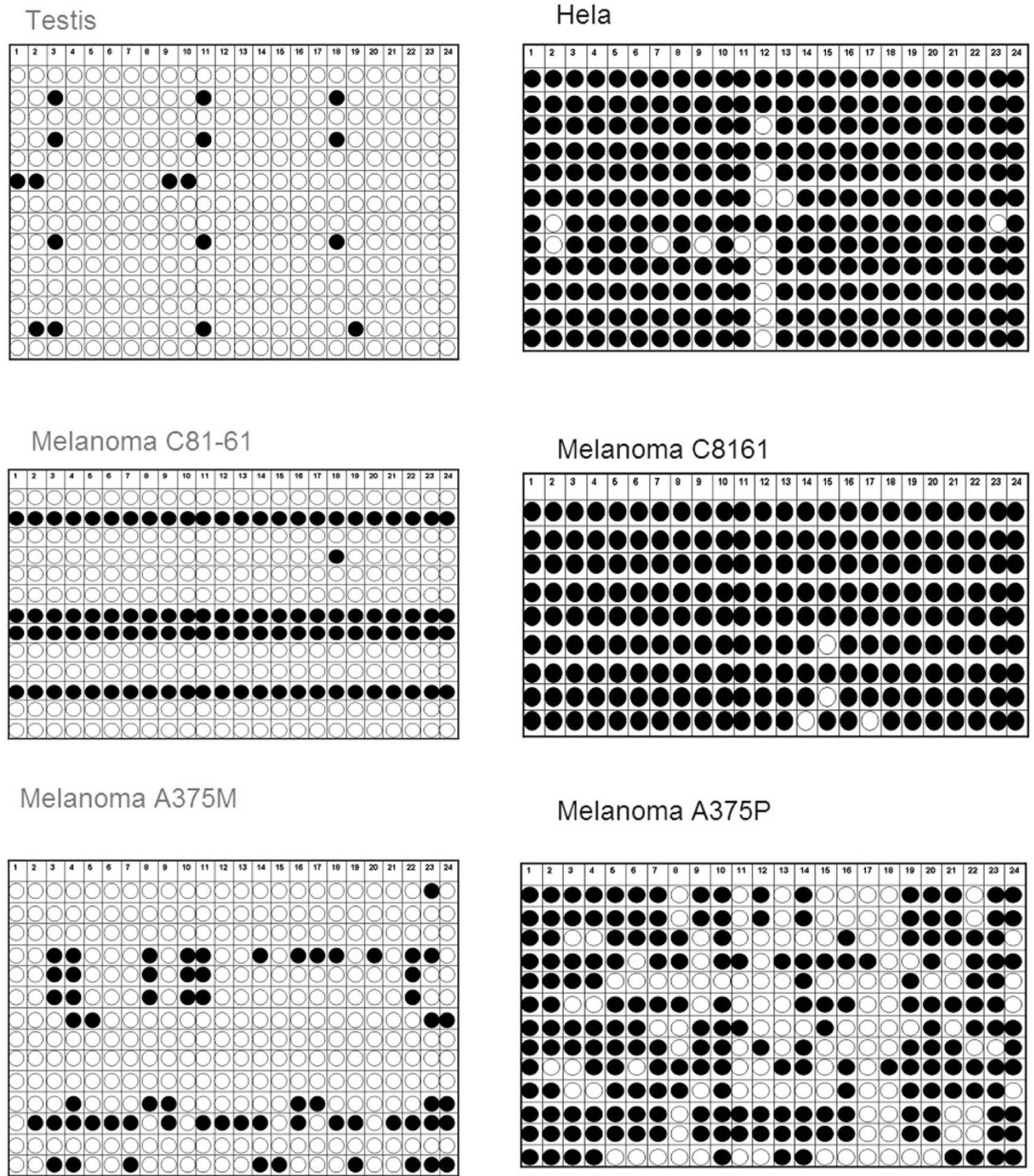

Figure 8. Bisulfite sequencing analysis of *hLdhc* CpG island (CGI) methylation. CGI is hypomethylated in *hLdhc*-expressing cells (left panel) and hypermethylated in cells that do not express *hLdhc* (right panel). Left column, 3 *hLdhc*-expressing samples: testis, melanoma C81-61, and A375M cell lines; right column, 3 cancer cell lines that do not express *hLdhc*: Hela cell, melanoma C8161, and A375P; melanoma cell lines C81-61 and C8161 are isogenic; melanoma cell lines A375M and A375P are isogenic. The columns numbered 1–24 in the table indicate each CpG dinucleotide in the island. Each row in the table contains subclones of amplified PCR products. Each black circle indicates a methylated CpG.

hLdhc promoter activity in the cell lines with a hypermethylated CGI. The C81-61/C8161 cells were isolated from a patient with metastatic disease and differ only in passage numbers (14 and 59, respectively). Apparently ectopic expression of *hLdhc* present in the parental cells was lost with increasing passages by hypermethylation of the promoter; a likely mechanism that silences most nonessential genes in cultured cells (Antequera et al, 1990). Therefore, it is not unusual for a cell line to lose expression of tissue-specific markers as passage number increases. Methylation could be a major reason that directly or indirectly silences gene expression, indirectly by silencing the essential transcription regulators of the target gene or directly by limiting access to transcriptionally important binding sites on the promoter.

The A375P/A375M cell lines were derived from the heterogeneous cell line A375 by sequential IV injection via the tail vein in a nude mouse model. A375P is parentallike and A375M was selected for increased lung metastasis characteristics. The passage numbers were 29

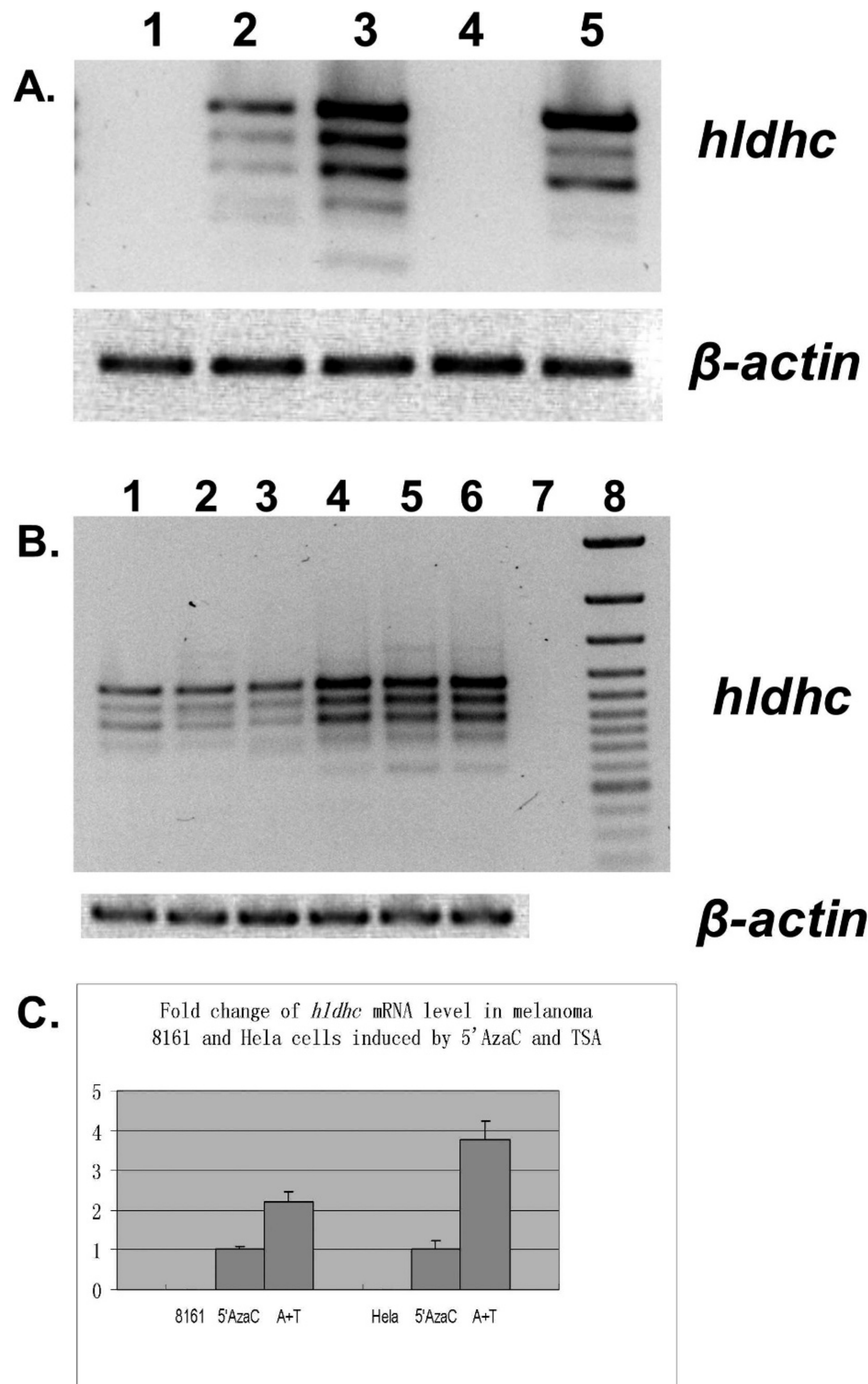

Figure 9. Induction of *hLdhc* transcription. **(A)** Reverse transcriptase polymerase chain reaction (RT-PCR) results showing that *hLdhc* transcription is induced in nonexpressing cells A375P and C8161 by methylation inhibitor 5′Aza-dC treatment and enhanced by coupled histone deacetylase inhibitor trichostatin A (TSA) treatment. Total RNA was extracted from cells and subjected to RT-PCR to examine the transcription levels in different treatment groups. Multiple transcripts are of splicing variants of the gene. Lane 1: Melanoma C8161 cells with no addition; lanes 2 and 3: C8161 cells treated with 5 μM 5′Aza-dC and with 5 μM 5′Aza-dC + 300 nM TSA, respectively; lane 4: melanoma A375P cells with no addition; lane 5: A375P cells treated with 5 μM 5′Aza-dC + 300 nM TSA. The lower panel shows the corresponding β-actin control. **(B)** RT-PCR showing effects on *hLdhc* transcription by acetylation inhibitor TSA treatment in melanoma C8161 cells. Lanes 1 to 3: Triplicate assays of cells treated with 5 μM 5′Aza-dC; lanes 4 to 6: triplicate assays of cells treated with 5 μM 5′Aza-dC + 300 nM TSA respectively; lane 7 is PCR negative control. The lower panel shows the corresponding β-actin control. **(C)** Comparison of the transcription level of full-length *hLdhc* in C8161 and Hela cells treated with 5′Aza-dC or 5′Aza-dC + TSA (A + T).

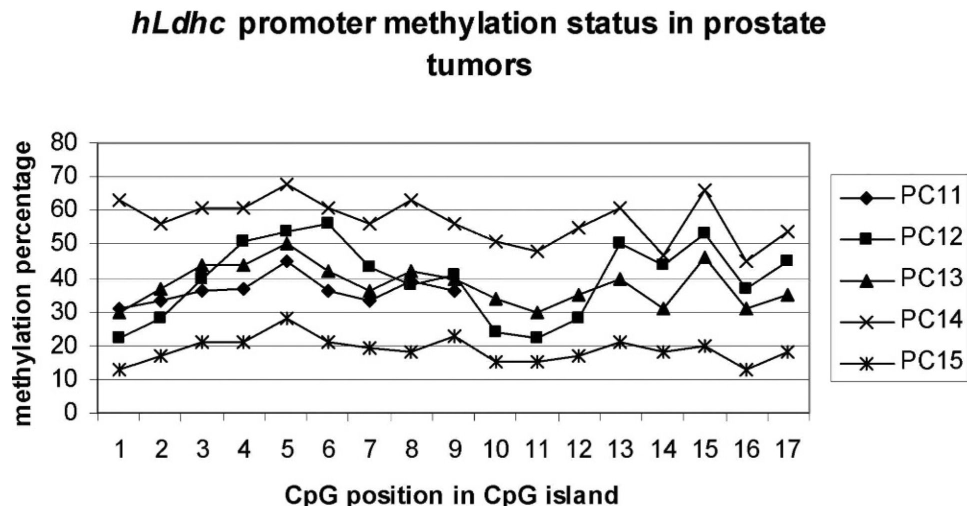

Figure 10. Methylation status of *hLdhc* promoter CpG islands in prostate tumors (PC11, PC12, PC13, and PC14) and normal tissues (PC15). Methylation level was analyzed quantitatively by pyrosequencing. The diagram indicates a differential methylation pattern in which all 4 prostate tumor samples with various Gleason scores had a higher methylation level compared to the normal tissue control. Tumor sample PC11: Gleason grade 4 + 3, score 7, with 51%–60% tumor in the section for genomic DNA methylation analysis and no biochemical relapse. Sample PC12: Gleason grade 3 + 3, score 6, 91%–100% tumor with relapse. Sample PC13: Gleason grade 3 + 3, score 6, 90% tumor, unknown relapse. Sample PC14: Gleason grade 3 + 5, score 8, 80% tumor with relapse.

for A375M and 32 for A375P when used for methylation analysis of the *hLdhc* promoter. Szyf et al (2005) proposed that global hypomethylation in cancer cells is associated with activation by demethylation of metastasis-associated genes. In other words, selection for high metastasis likely favors demethylation. Even though direct evidence is required for such a proposal, a selection toward higher metastasis potential in A375 cells indeed led to a hypomethylated CGI and therefore *hLdhc* expression in A375M cells.

HLdhc transcription was greatly enhanced by combining 5′Aza-dC and TSA compared with methylation inhibitor treatment alone. Previously, Cameron et al (1999) reported a similar synergistic effect of demethylation and HDAC inhibition in the re-expression of genes silenced in cancer. In a transcriptionally silenced status, the methyl-CpG–binding proteins MeCP1 and MeCP2 bind methylated DNA tightly and associate with a complex composed of transcription repressor mSin3A and HDAC (Jones et al, 1998; Nan et al, 1998); the histones in the chromatin are extensively deacetylated through the action of HDAC. The deacetylated histone leads to chromatin condensation and transcription repression (Jones et al, 2002). Even though both demethylation and acetylation contribute to the reactivation of transcription, methylation seems to be dominant because demethylation is prerequisite for reactivation of gene expression rather than acetylation induced by the HDAC inhibitor. The dominant role of methylation is supported both by *hLdhc* gene activation data and by results from Cameron et al (1999). However, the general molecular mechanism for the interaction between DNA methylation and histone deacetylation will require further clarification (Jones and Baylin, 2002). Overall, the experimental data suggest that both DNA methylation and histone acetylation of the *hLdhc* promoter are involved in regulating transcription of this gene in these cancer cells.

The methylation status of the *hLdhc* promoter was examined in 4 prostate tumor biopsy samples by pyrosequencing analysis (Figure 10). Pyrosequencing offers the most sensitive quantitative analysis of methylation in multiple CpG sites of a CGI (Tost et al, 2003; Mirmohammads et al, 2006). Our data indicated that differential methylation patterns indeed exist in these prostate tumor tissue samples with different degrees of malignancy. A very interesting observation was that the specimens with relatively higher methylation levels (PC14 and PC12) were obtained from patients who had suffered a biochemical relapse. Although many more specimens need to be examined to formally prove a correlation, these results may provide a lead to developing a biomarker based on the methylation status of the *hLdhc* CGI.

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