ldhc Expression in Non-germ Cell Nuclei Is Repressed by NF-I Binding*

Received for publication, February 8, 2001, and in revised form, June 26, 2001
Published, JBC Papers in Press, July 10, 2001, DOI 10.1074/jbc.M101269200

Poonam Jethanandani‡ and Erwin Goldberg§
From the Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208-3500

Developmental and testis-specific expression of the mouse lactate dehydrogenase C (ldhc) gene requires mechanisms for activation in germ cells and repression in somatic cells. Promoter activity restricted to the testis has been demonstrated using in vitro transcription assays with a 60-base pair promoter sequence upstream of the transcription initiation site. This promoter fragment has a TATA box and an overlapping 31-base pair palindromic sequence. Here we have explored the role of the palindromic sequence as a silencer of the ldhc gene in somatic tissues. A gel retardation assay detected two sites within the palindromic sequence that were important for protein binding. A member of the NF-I/CTF family was identified as the protein binding to one of the sites. In transiently transfected mouse L cells, a promoter fragment in which the NF-I site was mutated showed a 4-fold greater activity as compared with the wild-type sequence. Overexpression of the four NF-I proteins, NF-IA, -B, -C, or -X, in mouse L cells transiently transfected with an ldhc reporter-constructor resulted in a 20–50% decrease in activity of the wild-type promoter but had no effect when the NF-I binding element in the palindrome was mutated. These results indicate a role for the NF-I proteins in regulation of the mldhc gene.

Mechanisms of negative regulation such as chromatin organization, methylation, and DNA-protein interaction increasingly are recognized as the basis in differential gene expression (1). We are interested in understanding regulation of the testis-specific member of the ldh gene family (ldhc), which encodes the C subunit of lactate dehydrogenase (LDH-C4) (2–4). The ldhc gene product is present only in developing and mature germ cells from the preleptotene-zygotene stage to the differentiated spermatozoan. The mechanism/s by which this gene is silenced in somatic tissues as well as during the early stages of spermatogenesis have not been resolved.

Transcription factors for activation or repression of the gene have yet to be identified. However, previous work demonstrating testis-specific expression with a 60-bp1 fragment of the mouse ldhc gene (2) implicated a 31-bp palindromic sequence that overlaps the TATA box and the transcription initiation site. This was confirmed with a transgene containing 100 bp of the promoter (3). The palindromic sequence binds proteins from testis and liver nuclear extracts in electrophoretic mobility shift assays (EMSA). Southwestern analysis reveals binding to a 105-kDa protein extracted from testis nuclei and a 65-kDa protein from liver nuclear extracts (2). Functional activity has been demonstrated by in vitro transcription assays using a promoter fragment with either the wild-type sequence or with random mutations introduced in the center or in the 5′-flanking region of the palindrome (4). Mutations in the center of the palindrome resulted in a loss of promoter activity with testis nuclear extracts, suggesting that an activator bound at this site. Random mutations in the 5′-flanking region of the mldhc promoter had no effect on activity with testis nuclear extracts but did result in a low but significant activity using liver nuclear extracts. This finding indicated that a repressor bound at this site in somatic tissues. The presence of a repressor was also suggested by the observation that liver nuclear extracts when added to testis nuclear extracts lowered the activity of the promoter (5). Additionally, competition with the palindromic oligonucleotide resulted in a low but significant activity by the otherwise silent mldhc promoter in liver nuclear extracts (5).

In this report we describe a mechanism by which the mldhc gene is silenced in somatic tissues. We demonstrate that the NF-I/CTF (CCAT box transcription factor) protein family binds to the palindrome and functions as a repressor of mouse ldhc gene expression. The NF-I proteins are encoded in mammals by four genes, Nf1a, Nf1b, Nf1c, and Nf1x (6), and are involved in transcriptional activation as well as viral replication. There is increasing evidence, however, that NF-I proteins also function in negative regulation (6).

EXPERIMENTAL PROCEDURES
Oligonucleotides Used for Gel Mobility Shift Assays and Competition Studies

The following oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA): Equimolar quantities of the sense and antisense strands were mixed in STE (TE with 100 mM NaCl) and annealed by boiling for 2 min and cooling gradually to room temperature.

Wild type palindrome (P) sequence was ATAACTGTTGGCTCCTG-GACCCCAACAGTTAT (31 bp); oligo I, ATAACTGTTGGCTCCTGOGCC (21 bp); oligo II, TCCTGGCTCGTGGACCCCAACA (21 bp); oligo III, GCTCCTGGACCCCAACAGTTAT (21 bp).

Oligos A–K were 22 bp in length and had 2-bp mutations introduced successively in oligo II. Oligo I was taGGTGCTCGTGGACCCCAACA; oligo B, CTGGCCTCGTGGACCCCAACA; oligo C, CTGGCCTCGTGGACCCCAACA; oligo D, CTGGCCTCGTGGACCCCAACA; oligo E, CTGGCCTCGTGGACCCCAACA; oligo F, CTGGCCTCGTGGACCCCAACA; oligo G, CTGGCCTCGTGGACCCCAACA; oligo H, CTGGCCTCGTGGACCCCAACA; oligo I, CTGGCCTCGTGGACCCCAACA; oligo J, CTGGCCTCGTGGACCCCAACA; oligo K, CTGGCCTCGTGGACCCCAACA.
GACCCAACA; oligo D, CTGTTGtaTCCTGGACCCAACA; oligo E, CTGTTGTTGGCagCTGGACCCAACA; oligo F, CTGTTGGCTCacGGACCCAACA; oligo G, CTGTTGGCTCCTtaACCCAACA; oligo H, CTGTTGGCTCCTGGgaCCAACA; oligo I, CTGTTGGCTCCTGGACgaAACA; oligo J, CTGTTGGCTCCTGGACCCctCA; oligo K, CTGTTGGCTCCTGGA-CCAAac.

Other oligonucleotides used were adenoviral NF-I (NF-I), TTTTG-\textsubscript{GGATTGAAGCCAATATGATAA}; mutated NF-I (NFI\textsuperscript{*}), TTTTGGATT-\textsubscript{GAAGtaaATATGATAA}; mutated NF-I (NFI\textsuperscript{**}), TTTaatATTGAAGC-CAATATGATAA; and Sp1, ATTCGATCGGGGCGGGGCGAGC.

All oligonucleotides are sense oligonucleotides and are written in a 5\textsubscript{\textprime} to 3\textsubscript{\textprime} direction. Substitutions introduced in oligos A–K and mutated NF-I oligos are indicated by lowercase letters. The consensus binding elements for Sp1 and NF-I are based on the sequence from the Santa Cruz Biotechnology catalog.

Plasmids

\textit{β}-Galactosidase (β-gal) Reporter Constructs—A 430-bp mldhc 5\textsubscript{\textprime} flanking region fragment (−425 to +10) was amplified by PCR using the 5\textsubscript{\textprime} sense oligonucleotide Xhosens (5′-CCGCTCGAGGTTCTCA-

GAGTCCAGGACG-3′) (corresponding to −425 to −406 and including an XhoI site), and a 3′ antisense oligonucleotide, (5′-CCGAGGTTTATA-CTGTTGGTGCTCCAGGACG-3′) (corresponding to +10 to +12 and containing a HindIII site). The template was a pNAss (CLONTECH) vector, which contained the mldhc fragment (−425 to +10) cloned at the EcoRI/XhoI site. The amplified fragment was digested with XhoI/HindIII and cloned into the pβ-gal basic vector (CLONTECH) to give pWT. For generating mutations in the palindrome, PCR amplification was carried out using pWT as template and oligonucleotide Xhosens as 5′ sense primer and the 3′ antisense primers were MutBC (5′-CCGAGGTTTATA-CTGTTGGTGCTCCAGGACG-3′) corresponding to +10 to −26 and MutI (5′-CCGAGGTTTATA-CTGTTGGTGCTCCAGGACG-3′) corresponding to +10 to −17. The PCR product was digested with XhoI/HindIII and cloned into the β-gal basic vector to give pMutBC and pMutI.

Mutation at site GH was generated in two steps. In the first step, the mutation was introduced by using the 5′ sense primer Xhosens and the 3′ antisense oligonucleotide, Xhosens as 5′ sense primer and the 3′ antisense primer MutGH (5′-TGGCGCACTGTTGAGCCCAACGTTTAAACGG-3′) (corresponding to +1 to −26) containing a mutation at site GH. The template was pWT. The PCR product was used as a template for a

Fig. 1. A, sequence of the mouse mldhc (−430 to +58) promoter fragment. The common regulatory motifs YY1 and GC box, as well as the TATA element, are \textit{underlined}. The 31-bp palindrome is shown by the \textit{two inverted arrows}. The transcription initiation site is designated by an \textit{asterisk}. Restriction sites for AccI and KpnI are \textit{underlined}. B, the effect of deletion in the palindrome on protein binding. Panel shows EMSA using 10 µg of liver nuclear extract and the 31-bp palindrome (P; lane 2) or the palindrome constructs oligos I–III (lanes 3–5). Competition of protein binding to the palindrome P (lane 7) was at 200-fold excess of the unlabelled palindrome P (lane 8) or oligos I–III (lanes 9–11, respectively).
second PCR amplification to introduce a HindIII site using the 5’ XhoI primers and the antisense primer 5’-CTCGAGTCTATGACCCGAGCCAGCGAAGAAG-3’ (−10 to −11). The PCR product was digested with XhoI and HindIII and then cloned into pBluescript basic vector to give pMutGH.

All plasmids were sequenced to confirm ligation ends, the generation of the mutation, and the PCR amplification sequence.

Expression Vectors—Mammalian expression vectors for NF-IA (pCHNFIA), NF-B (pCHNFIB), NF-C (pCHNFIC), and NF-D (pCHNFD) as well as the empty vector pCH were a generous gift from Prof. R. Gronostajski (Lerner Research Institute, Cleveland, OH). The expression vector for CCAAT displacement protein, pMT2CDP, and the control pMT2 vector were obtained from Prof. E. J. Neufeld (Harvard Medical School, Boston, MA).

Gel Mobility Shift Assays—Nuclear extracts were prepared from adult CD-1 mouse livers. The livers were dissected from 15 mice, minced, and homogenized in buffer as described previously (2). Testes from 50 mice were decapsulated, minced, and homogenized. Freshly prepared proteinase inhibitors (leupeptin, 10 μg/ml; aprotinin, 10 μg/ml; pepstatin, 1 μg/ml; benzamidine, 1 mM; dithiothreitol, 0.5 mM; and phenylmethylsulfonyl fluoride, 0.5 mM) were added to all buffers. The denaturing 5% polyacrylamide gel in 0.5 M NaOH/0.5% SDS was run on ice for 30 min. Protein-DNA complexes were resolved on a non-denaturing 5% polyacrylamide gel in 0.5 M NaOH/0.5% SDS and autoradiographed at −70 °C. The protein concentration of each extract was determined using an assay based on Bradford. Protein concentrations were between 5 and 10 μg/ml. Binding reactions were set on ice in buffer containing 10% glycerol, 25 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 0.05 mM phenylmethylsulfonyl fluoride. Each reaction contained 1 μl of poly[d(I-dC)] (Sigma-Aldrich), 5.0 μg of denatured salmon sperm DNA, and 10 μg of liver nuclear proteins. Cold competitors when needed were added at 200-fold molar excess unless otherwise indicated. Complementary oligonucleotides were annealed and labeled at the 5’ end using [γ-32P]ATP. The probes were purified through a 50-μl Sephadex spin column. The labeled probe (50,000 cpm) was added, and the binding reaction was incubated on ice for 30 min. Protein-DNA complexes were resolved on a non-denaturing 5% polyacrylamide gel in 0.5× TBE at 4 °C. Gels were dried and autoradiographed at −70 °C using intensifying screens. For supershift analysis, 3 μl of pre-immune or polyclonal antisera raised against NF-ICTP (gift from Prof. N. Tanese, New York University, New York, NY) was added to the binding reaction and kept overnight at 4 °C. The probe was then added, and the reaction mixture was kept on ice for an additional 30 min before resolving it on a polyacrylamide gel as above.

Transient Transfection Assay—Mouse L cells (a gift from Prof. D. Linzer, Northwestern University, Evanston, IL) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cultured cells were grown in six-well plates and transfected with 5 μg of the reporter vector at ~50% confluence using Superfect (Qiagen, Chatsworth, CA). The constructs tested were the empty β-gal basic vector, phasic, and β-gal basic vector with 430 bp of wild-type mouse ldhc promoter, pWT. Other constructs included those with mutations in the 430-bp promoter, pMutBC, pMutGH, and pMutI. Cells were harvested 48 h after transfection and assayed for β-galactosidase activity using the Galacto-Plus kit from Tropix (Foster City, CA) and a luminometer. The plasmid pRLTK (Promega, Madison, WI) was used as an internal control to normalize transfection efficiencies. β-Galactosidase activities are expressed as -fold differences compared with those obtained with the wild-type promoter, pWT.

For co-transfection assays, mouse L cells were cultured as above. Cells were transfected at ~50% confluence in 12-well plates with 2 μg of the reporter plasmids pWT or pMutBC and the expression vectors (1 μg) for NF-I or for CCAAT displacement protein, CDP. The empty vectors, pCH and pMT2, for NF-I and CDP, respectively, were also transfected (1 μg each) in parallel. The internal control pRLTK was used to normalize transfection efficiencies. Cells were harvested 48 h later and lysed. β-Galactosidase activities were determined as above. The β-gal activity of pWT or pMutBC co-transfected with either pCH or pMT2 was arbitrarily set at 100. All other activities are expressed as a percentage of this activity.

UV Cross-linking—Binding reactions were carried out as described for EMSA except that the reaction was scaled up 5 times. The probes used were the consensus NF-I element, oligo II, oligo A, and oligo C. After a 30-min incubation on ice, the tubes were exposed to short wavelength UV light for 1 h. The reactions were then boiled in 2× Laemmli buffer and electrophoresed on a 10% SDS-PAGE gel. The gel was dried and exposed overnight to x-ray film at −70 °C.

Western Blot—Liver nuclear extracts from adult mice (150 μg of protein) and testis nuclear extracts from adult and 10-day mice (150 μg of protein each) were run on a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane. After blocking with 5% bovine serum albumin, the proteins were incubated with anti-NF-I serum for 3 h at room temperature. The blot was washed with Tris-buffered saline and incubated with goat anti-rabbit secondary antibody. Proteins were detected using the chemiluminescent ECL system from Amersham Pharmacia Biotech.

Mouse L cells transfected with expression vectors for NF-IA, -B, -C, or -X were pelleted, lysed directly in 1× Laemmli buffer, boiled for 10 min, separated on a 10% SDS-PAGE gel, and blotted onto a nitrocellulose membrane. After blocking with 5% bovine serum albumin, the blot was hybridized with 2 μg/ml of C125A antibody to HA (Roche Diagnostics, Indianapolis, IN). Antibody binding was detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).
RESULTS

Identification of Nucleotides within the Palindrome Necessary for Protein Binding in Somatic Tissue Extracts—EMSA was used to demonstrate protein binding to specific domains of the mldhc gene. Fig. 1A shows the sequence of the 430-bp mouse ldhc promoter and the location of the palindrome (inverted arrows), which overlaps the TATA box and includes the transcription initiation site (indicated by an asterisk). Previous studies revealed that the palindrome bound a 65-kDa protein from liver nuclear extracts (2). In vitro transcription assays demonstrated that mutations in the 5’ region of the palindrome resulted in low activity with liver nuclear extract in what otherwise would be a silent mldhc promoter (4). We decided to determine first if the entire 31-bp palindrome was necessary for protein binding. Three deletion constructs, each 21 bp in length (oligo I (−21 to −1), oligo II (−16 to +5), and oligo III (−11 to +10)) were generated. EMSA results are seen in Fig. 1B. The wild-type 31-bp palindrome (P) showed multiple protein complexes (lane 2). Oligo I (lane 3) and oligo III (lane 5) bound very little protein, but oligo II retained protein binding capacity similar to the wild-type 31-bp palindrome (lane 4). These results were confirmed by cross-competition (Fig. 1B). Oligo II competed for protein binding to the palindrome (lane 10), but oligos I and III did not (lanes 9 and 11). Oligo II binds protein even though it lacks the first four nucleotides (ATAA) and the last four nucleotides (TTAT) of the 31-bp palindrome. Oligo I, which lacks the CAACA nucleotides present in oligo II, does not bind protein. Similarly, oligo III lacks CTGTTG present in oligo II and loses protein binding capacity. These results indicate that the nucleotides CAACA and CTGTTG within the palindrome are essential for an electrophoretic mobility shift.

Since a gel shift was observed with the 22-bp oligo II, it was used instead of the 31-bp palindrome (P) in subsequent experiments. In order to identify the nucleotides within oligo II required for somatic tissue protein binding, a series of oligos designated oligo A through oligo K were generated by introducing successive 2-bp mutations in oligo II. These oligonucleotides were then used in EMSA with liver nuclear extracts. As seen in Fig. 2A, oligo A shows the same multiple protein complexes (lane 3) that were observed when oligo II was used (lane 2).
2). Oligo A competed for protein binding to oligo II (Fig. 2B, lane 3). Interestingly, mutation of nucleotides corresponding to sites B, C, and D resulted in a selective loss of the lower mobility bands (Fig. 2A, lanes 4–6) and oligos B, C, and D could not compete with oligo II for the proteins present in these bands (Fig. 2B, lanes 4, 5, and 6, respectively). When site E was mutated, only the lowest band was lost (Fig. 2A, lane 7) and oligo E could not compete for the lowest band (Fig. 2B, lane 7).

The results of EMSA using oligos F–K are shown in Fig. 2 (C and D). Mutations at sites F, G, and H resulted in a selective loss of the lowest band (Fig. 2C, lanes 3, 4, and 5, respectively). Oligos G and H could not compete for the lower band (Fig. 2D, lanes 3 and 4). Oligo F competed weakly for both bands even though, when used as a probe, oligo F did not bind the protein in the lower band (Fig. 2D, lane 2). This suggests that sites G and H rather than F may be more critical for protein binding. Mutation at the I site led to a loss of both upper bands as well as the lowest band (Fig. 2C, lane 6). Oligo I competed only weakly for the upper bands (Fig. 2D, lane 5). Mutations in sites J and K did not affect binding of proteins in the lowest band (Fig. 2C, lanes 7 and 8). This was confirmed by competition (Fig. 2D, lanes 6 and 7).

The above results indicate that there are probably two different proteins that bind the palindrome. One of these binds at sites B, C, and D and also requires an intact site I. The binding of this protein results in multiple protein complexes. The second protein binds at sites E, F, G, H, and I, resulting in a single band, which runs with the highest mobility in EMSA. The requirement of site I for binding both proteins indicates a possible cooperation between the two, whereby the binding of each is enhanced in the presence of the other.

Identification and Characterization of Protein Binding to the BCD Site of the Palindrome—After delineation of the two protein binding sites in the palindrome, we wanted to identify the protein binding to the BCD and EFGH sites. A search of the transcription factor data base, Transfac (GBH), resulted in identification of the BCD site, the nucleotide sequence GTTGGC, as a putative NF-L/CTF protein binding site. Competition gel-shift assays tested NF-I binding to this site. As seen in Fig. 3A, the wild-type palindrome oligo II forms multiple protein complexes with liver nuclear extract (lane 1). A 200-fold excess of the same oligo competed for all the bands (lane 2). The adenoviral NF-I element competes only for the upper bands at 200-fold molar excess (lane 3). The fastest migrating band was not competed, even with an 800-fold excess of the adenoviral NF-I element (data not shown). The mutated NF-I oligonucleotides, NF-I* (CCA to TAA, which disrupts the 3′-half of the NF-I binding site) did not compete with oligo II (Fig. 3A, lane 4) and NF-I** (TGG to ATT, which disrupts the 5′-half of the NF-I binding site) competed slightly (lane 6). An unrelated consensus sequence for the transcription factor Sp1 did not compete (lane 5). We also assayed a variety of other transcription factor consensus elements such as those for the closely related CCAAT/enhancer-binding protein C/EBP, NF-Y (another CAATT-binding protein), glucocorticoid receptor, and androgen receptor for their ability to compete with oligo II (data not shown). Since none of these elements competed, the specificity of protein binding to the palindrome was confirmed.

Additional evidence for the NF-I binding was obtained using the adenoviral NF-I oligonucleotide as probe and either the wild-type or mutated palindrome as competitor. As seen in Fig. 3A (lane 7), the adenoviral NF-I element bound the same broad band of proteins with similar mobility as the upper bands obtained with oligo II and liver nuclear extract. Oligo II as well as oligo A (mutated palindrome, which retains protein binding capacity; see Fig. 2A, lane 3) could compete out the proteins from the NF-I element (Fig. 3A, lanes 8 and 9 at 200- and 400-fold molar excess and lanes 12 and 13 at 200- and 400-fold molar excess). However, oligo C (mutated palindrome, which was unable to bind the upper bands; see Fig. 2A, lane 4) did not compete (Fig. 3A, lanes 14 and 15 at 200- and 400-fold molar excess). The NF-I self oligo competed efficiently at 200- and 400-fold excess (Fig. 3A, lanes 10 and 11). These results further demonstrate the similarity of proteins binding to the adenoviral NF-I element and the palindromic oligo II. Further verification of NF-I protein binding to the palindrome was provided by a supershift analysis using anti-NF-I serum. As seen in Fig. 3B, only the immune serum (lane 2) but not the pre-immune serum (lane 1) could shift protein binding.

If the proteins binding to the adenoviral NF-I oligo and the mldhc palindrome are the same, then their size should be similar. This was examined by UV cross-linking. As seen in Fig. 3C, the adenoviral NF-I element showed multiple bands (lane 2). This was expected, as there are multiple NF-I isoforms.
ranging from 35 to 66 kDa in somatic tissues (6). However, the size of the proteins cross-linked to the adenoviral NF-I element ranged from 60 to 80 kDa (lane 2). The larger size observed by us could be due to the oligo protein complex, which would increase the size by ~15–20 kDa. As seen in lane 1, the size of the proteins binding to the palindrome was approximately the same as that for the NF-I consensus oligo. The negative controls were oligo C (lane 3) and oligo III (lane 4) (see Figs. 2A (lane 4) and 1B (lane 5)). As noted above, Southwestern analysis (2) revealed binding of a 65-kDa protein present in liver nuclear extracts. This finding was confirmed by the UV cross-linking results.

A Transfac database search conducted to identify potential transcription factors that might recognize the nucleotide sequence TCTTGACCC (corresponding to the EFGHI site) was not informative.

Functional Relevance of the BC, GH, and I Sites—The demonstration of protein binding elements in a promoter has meaning only if substantiated in a functional assay. The relevance of the BC, GH, and I protein binding sites in the palindrome was examined by analysis of promoter-reporter constructs including the 430-bp wild-type mouse ldhc promoter, pWT, as well as reporter vectors carrying mutations in the BC (pMutBC), GH (pMutGH), or the I (pMutI) sites in the palindrome. Each of these fragments was cloned upstream of the β-galactosidase reporter in the pb-gal basic vector, and tested for promoter activity in transiently transfected mouse L cells.

As seen in Fig. 4 mutation of the BC site led to a 3–4-fold increase in activity compared with the wild-type promoter. Mutation at the GH site, however, had no effect, whereas mutation at the I site resulted in a 2-fold increase in promoter activity over the control.

These results indicate that the BC as well as the I sites but not the GH site in the palindromic element of the mldhc promoter function as negative regulatory regions for mouse ldhc gene expression.

Overexpression of the NF-I Proteins Represses the Native Mouse ldhc Promoter—We have identified an NF-I protein binding site in the palindrome and demonstrated that mutation of this site leads to an increase in promoter activity. In order to investigate whether NF-I functions in silencing this gene, we examined the effects of overexpression of NF-I on mldhc promoter activity. Expression vectors for NF-IA, -B, -C, or -X were co-transfected with the 430-bp mldhc promoter-reporter plasmid into mouse L cells. Overexpression of NF-IA resulted in a slight decrease in promoter activity (Fig. 5A) compared with control cells transfected with the empty vector, pCH. A Western blot probed with antibody to HA confirmed transfection of these cells with each of the NF-I isoforms (Fig. 5B). Overexpression of NF-I B, -C, and -X resulted in a statistically significant 40–50% reduction in transcriptional activity (p < 0.001, Student’s t test). In order to determine whether repression due to NF-I involves the BC site in the palindrome, we tested overexpression of NF-IB and -C on the activity of pMutBC (430-bp promoter with a mutated BC site in the palindrome). Neither NF-IB nor NF-IC repressed the activity of pMutBC. These results suggest that the NF-I binding site BC is important for repression and that NF-I acts through this site to negatively regulate mldhc transcription.

The specificity of NF-I repression was confirmed by overexpression CDP. We chose this protein because CDP binds close to CAAT boxes and negatively regulates testis-specific genes (7, 8). Since the NFI protein is a CCAAT box protein, we wanted to eliminate the possibility that CDP was involved in mldhc regulation. Neither the empty vector pMT2 nor the CDP expression vector pMT2CDP repressed the wild-type mouse ldhc promoter. In fact there was an increase in activity with the CDP expression vector. The binding element for CDP is not well defined, and a Transfac search did not reveal any sites for CDP in the mldhc promoter sequence. Although increased transcriptional activity with CDP is interesting, the important point from these results is that the repression of the mldhc promoter by NF-I is specific.

Presence of NF-I Proteins in Liver and Testis—With a role demonstrated for NF-I proteins in silencing the mldhc gene in somatic tissues, it became of interest to compare NF-I protein levels between testicular and somatic tissues. A Western blot using equal amounts of liver and testis nuclear extracts was resolved on an SDS-PAGE gel and probed with polyclonal anti-NF-I serum. As seen in Fig. 6 liver nuclear extracts from adult mice had abundant NF-I proteins ranging from 50 to 60 kDa. Testis nuclear extracts from adult mice had extremely low levels of NF-I proteins of ~70 kDa. The level of NF-I appeared to be slightly higher in day 10 mouse testis as compared with adult testis.

![Fig. 5. A](image-url) The effect of overexpression of NF-I on the transcriptional activity of mldhc. The promoter-reporter plasmids were pWT and pMutBC (2 μg each). Co-transfection was done with 1 μg of expression vectors for NF-IA, -B, -C, -X, pMT2CDP, or the blank vectors pCH or pMT2. β-Galactosidase assays were performed, and the activities were expressed relative to the control activity of pWT or pMutBC co-transfected with the blank vector pCH or pMT2 set at 100%. Bars indicate standard deviations of four independent experiments. B, Western blot demonstrating the expression of the NFIA, -B, -C, and -X proteins. Whole cell lysates of mouse L cells transfected with expression vectors for HA-tagged NFIA, -B, -C, or -X (see legend for A) were analyzed on a 10% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane and probed with the C125A anti-HA antibody (Roche). The numbers on the left are the size markers. The arrowheads indicate expression and size of the NF-IA (~65 kDa), -B (~55 kDa), -C (~55 kDa), and -X (~50 kDa) proteins. Differences in blot signal intensity are due only to their being run at different times, and are not meaningful in terms of level of protein detected.

Repression of Mouse ldhc by NF-I/CTF Protein
Repression of Mouse ldhc by NF-I/CTF Protein

DISCUSSION

Previous studies from our laboratory implicated a palindrome sequence between the TATA and transcription start site in repression of the mouse ldhc gene. We have investigated further the role of this palindrome in transcriptional regulation. We first defined the nucleotides important for protein binding. Two sites (GTTGGC, site BCD; and TCCTGGAC, site EFGH) bound protein in gel shift assays. An intact CC dinucleotide (site I), which is 8 nucleotides downstream of GTTGGC, was also needed for protein binding. The protein binding to GTTGGC was identified as the NF-I/CCAAT transcription factor based on competition in EMSA, supershift with anti-NF-I antibody, and UV cross-linking. The consensus binding site for NF-I proteins is TTGG(N7)CCAA. However, in the mldhc palindrome, the TTGG is separated by 8 nucleotides from CCAA. The spacing between TGG and CCA in the NF-I binding element is thought to be important (9). However, it is possible that there are subtle differences in protein binding site requirements not yet defined for the different NF-I isoforms. Our results clearly indicate the ability of NF-I protein to bind the palindrome.

We next asked whether these sites function as negative regulatory elements by testing the effect of mutations introduced at the BC, GH, and I sites on promoter activity in transient transfections of mouse L cells. Mutation of the BC site led to a 4-fold increase in promoter activity compared with wild-type promoter, indicating a functional role in repression. Mutation of the GH site, however, did not change activity significantly. Functional activity was greatest with NF-IC followed by NF-IB and -X, whereas NF-IA was least effective. NF-I transcription factors are encoded in mammals by four genes Nf1a, Nf1b, Nf1c, and Nf1x (6). There are more than 20 isoforms of the multiple genes and their alternatively spliced products. These proteins form homo- and heterodimers, giving rise to further variants. All the proteins share a highly homologous N-terminal DNA-binding domain, but their C-terminal activation domains differ significantly. Both activator and repressor functions for these proteins have been reported. NF-I suppresses transcription of genes encoding the L-type pyruvate kinase (10), phosphoenol pyruvate carboxykinase (11), the glucocorticoid-inducible mouse mammary tumor virus promoter (12), androgen receptor (13), cartilage matrix protein (14), and the GLUT4 (15) genes. The mechanism by which it represses these genes is not clearly understood. One model suggests direct competition with transactivators for binding at adjacent sites. An example is the repression of the mouse a1(I) collagen promoter by competition with Sp1 for overlapping binding sites (16). Similarly, competition between NF-I and HNF4 for overlapping binding sites on the rat pyruvate kinase promoter is proposed to play a role in cell-type specific repression by NF-I proteins (17). In addition, the C-terminal regions of NF-I proteins can function as repressors when attached to heterologous DNA binding domains (18), indicating that direct repression can occur either through recruitment of corepressors or interaction with the basal transcription apparatus.

The NF-I site in the palindrome is adjacent to the TATA box. One model to explain the mechanism by which NF-I silences the mldhc gene is prevention through steric hindrance, of assembly of the basal transcriptional complex. This is consistent with the demonstration by Chaudhry et al. (19) that NF-I mRNA levels for all four NF-I isoforms are high in somatic tissues and extremely low in the testis. In fact, NF-I may not be expressed at all in germ cells, with the low NF-I mRNA level accounted for by somatic cells in the testis. This is supported by our Western blot analysis, which shows higher NF-I levels in 10-day testis (when mostly somatic cells are present) as compared with the adult with a preponderance of germ cells. Testicular tissues have much higher levels of TATA-binding protein (TBP) than somatic tissues (20). A low ratio of NF-I/TBP or
a total lack of NF-I in germ cells may favor assembly of the transcriptional complex in testis but not in somatic tissues, which have a high NF-I/TBP ratio. Alternatively, NF-I proteins may negatively regulate the mldhc gene through differences in the NF-I isoforms found in somatic and germ cells. NF-IA mRNA predominates in the testis (19) and has the lowest repressor activity in transient transfection assays. NF-IB3, was isolated and characterized from a human fibroblast cell line. This isoform is generated by the use of a premature polyadenylation site located in an intron. NF-IB3 lacks an activation domain may predominate in somatic tissues. Such isoforms would not be detected by the anti-NF-I serum, which was raised against the C-terminal portion of NF-I. A third mechanism for differential gene expression could involve differences in tissue cofactors such that the testis may lack co-repressors needed by NF-I. With the results presented here, the mechanism by which NF-I represses the mldhc gene in somatic tissues as well as during the early stages of spermatogenesis can now be elucidated.

In the mldhc gene, the palindrome sequence is flanked by a TATA box and the transcription initiation site. Sequences in this region have been described previously as negative regulators of testis-specific genes including the rat sperm H2B gene and the rat H1t genes (22, 23). In these cases the protein binding these sites was not identified. The rat sperm H2B gene is expressed in meiotic pachytene spermatocytes. Repression in pre-meiotic cells appears to be due to proteins binding at an E element, which lies between the TATA box and the transcription initiation site (22). On analyzing the E element using the Transfac program, we found that it did contain an NF-I binding site. However, NF-I may not be involved in the repression of this gene in spermatogonia, since the protein binding to the E element is found in testis of 7-day-old rats but not in somatic tissue extracts. The testis-specific histone H1t gene is also expressed only in pachytene spermatocytes. A negative regulatory region designated GC box 2 lies just downstream of the TATA box and plays a major role in gene repression (23). The Sp1 proteins bind weakly to this element but are not involved in repression of this gene. Observations from these two studies and mldhc regulation suggest that different mechanisms and transcription factors seem to be involved in determining testis-specific gene expression in different species at the same developmental stages of spermatogenesis.

Acknowledgments—We thank Chongwen Duan for constructing the vector, pMutGH. We also thank Drs. T. L. Kroft and Siming Li for comments on the manuscript.

REFERENCES
1. Kornberg, R. D. (1999) Trends Cell Biol. 9, M46–M49
2. Zhou, W., Xu, J., and Goldberg, E. (1994) Biol. Reprod. 51, 425–432
3. Li, S., Zhou, W., Doglio, L., and Goldberg, E. (1998) J. Biol. Chem. 273, 31191–31194
4. Zhou, W., and Goldberg, E. (1996) Biol. Reprod. 54, 84–90
5. Zhou, W. (1994) Transcriptional Regulation of the Murine Testis-specific Lac-tate Dehydrogenase c Gene. Ph.D. thesis, Northwestern University, Evanston, IL
6. Gronostajski, R. M. (2000) Gene (Amst.) 249, 31–45
7. Barberi, A., Superti-Furga, G., and Busslinger, M. (1997) Cell 50, 347–359
8. Higgy, N. A., Tarnasky, H. A., Valarche, I., Nepveu, A., and van der Hoorn, F. A. (1997) Biochem. Biophys. Acta 1351, 313–324
9. Gronostajski, R. M. (1987) Nucleic Acids Res. 15, 5545–5559
10. Cuif, M.-H., Poteu, A., Kahn, A., and Vaulouet, S. (1993) J. Biol. Chem. 268, 13769–13772
11. Crawford, D. R., Leahy, P., Hu C. Y., Chaudhry, A., Gronostajski, R., Grossman, G., Woods, J., Hakimi, P., Roesler, W. J., and Hanson, R. W. (1998) J. Biol. Chem. 273, 13387–13390
12. Chaudhry, A. Z., Vitullo, A. D., and Gronostajski, R. M. (1999) J. Biol. Chem. 274, 7072–7081
13. Song, C. S., Jung, M. H., Supakkar, P. C., Chatterji, B., and Roy, A. K. (1999) Mol. Endoerocrinol. 13, 1487–1496
14. Szabo, P., Moitra, J., Rencendorj, A., Rakhehly, G., Rauch, T., and Kiss, I. (1995) J. Biol. Chem. 270, 10212–10221
15. Cooke, D. W., and Lane, M. D. (1999) J. Biol. Chem. 274, 12917–12924
16. Nehls, M. C., Grapilon, M. L., and Brenner, D. A. (1992) DNA Cell Biol. 11, 443–452
17. Yamada, K., Tanaka, T., and Noguchi, T. (1997) Biochem. J. 324, 917
18. Osada, S., Ikeda, T., Xu, M., Nishihara, T., and Imagawa, M. (1997) Biochem. Biophys. Res. Commun. 238, 744–747
19. Chaudhry, A. Z., Lyons, G. E., and Gronostajski, R. M. (1997) Dev. Dynamics 208, 313–325
20. Schmidt, E. E., Ohbayashi, T., Makino, Y., Tamura, T., and Schibler, U. (1997) J. Biol. Chem. 272, 5326–5334
21. Liu, Y., Bernard, H.-U., and Apt, D. (1997) J. Biol. Chem. 272, 10739–10745
22. Kim, K., and Chae, C.-B. (1992) J. Biol. Chem. 267, 15271–15273
23. Clare, S. E., Fantz, F. A., Kistler, S., and Kistler, M. K. (1997) J. Biol. Chem. 272, 33036–33040