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Identification of a Silencer Module Which Selectively Represses Cyclic AMP-Responsive Element-Dependent Gene Expression

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The cyclic AMP (cAMP)-inducible promoter from the rat lactate dehydrogenase A subunit gene (LDH A) is associated with a distal negative regulatory element (LDH-NRE) that represses inherent basal and cAMP-inducible promoter activity. The element is of dyad symmetry, consisting of a palindromic sequence with two half-sites, 5′-TCTTG-3′. It represses the expression of an LDH A/chloramphenicol acetyltransferase (CAT) reporter gene in a dose-dependent, orientation- and position-independent fashion, suggesting that it is a true silencer element. Uniquely, it selectively represses cAMP-responsive element (CRE)-dependent transcription but has no effect on promoters lacking a CRE sequence. The repressing action of LDH-NRE could be overcome by cotransfection with LDH A/CAT vector oligonucleotides containing either the LDH-NRE or CRE sequence. This suggests that the reversal of repression was caused by the removal of functional active, limiting *trans*-acting factors which associate with LDH-NRE as well as with CRE. Gel mobility shift, footprinting, and Southwestern blotting assays demonstrated the presence of a 69-kDa protein with specific binding activity for LDH-NRE. Additionally, gel supershift assays with anti-CREB and anti-Fos antibodies indicate the presence of CREB and Fos or antigenically closely related proteins with the LDH-NRE/protein complex. We suggest that the LDH-NRE and CRE modules functionally interact to achieve negative modulation of cAMP-responsive LDH A transcriptional activity.

Regulation of eukaryotic gene expression can be viewed as an interplay between opposing positive and negative controlling influences. Several mechanisms for negative transcriptional regulation have been proposed (for reviews, see references 5, 7, 9, and 56) and include, for instance, competition for common DNA-binding sites (steric occlusion) between repressors and positively acting transcription factors; sequestering of limiting activating components away from the promoter of the affected gene (squelching); formation of an inactive activator-inhibitor complex; and binding of the activating and repressing proteins to adjacent, nonoverlapping DNA sequences, resulting in activator-repressor protein interaction and inhibition of transcription (quenching). The functionality of these inhibitory mechanisms appears to depend upon specific features of promoter elements and their interactive *trans* factors.

The eukaryotic genome encodes a number of genes whose expression is regulated by cyclic AMP (cAMP) at the transcriptional level (1, 26, 58). In these cases, the transcriptional response requires activation of an intracellular signaling pathway, ultimately converging and resulting in the activation of the cAMP response element (CRE). The molecular complexity of this response is evidenced by the family of transcription factors which interact with the CRE and consist of a group of very closely related isoforms referred to as either activating transcription factor (ATF) or cAMP-responsive element-binding protein (CREB) (26). The mechanism of the regulated transcriptional response by CRE involves intermediate molecular events, such as the cAMP-mediated dissociation and nuclear translocation of the catalytic subunit of protein kinase A (41, 42, 52), leading to the phosphorylation of CRE-binding proteins on specific serine residues by protein kinase A (for reviews, see references 1 and 26). Members of the ATF/CREB family of transcription factors activate or repress transcription through binding to highly related DNA sites and possess homologous leucine zipper (bZIP) domains responsible for dimerization. This property allows the formation of tissue-specific and functionally differentiated homo- and heterodimeric complexes that may interact, achieving cell-specific regulation (6, 25, 27).

During the course of our investigations into the structure and function of the cAMP-inducible lactate dehydrogenase A gene (LDH A) subunit promoter, we identified a negative regulatory element (NRE) within the bp −1173 to −830 promoter region (63). In this report, we present a detailed structural and functional analysis of the LDH-NRE. Our results show that the LDH-NRE module consists of an inverted palindromic sequence specifically binding a 69-kDa nuclear protein. The LDH-NRE selectively suppresses cAMP-inducible transcription from the LDH A subunit promoter in a position- and orientation-independent fashion but has no effect on promoters lacking the CRE.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes, acrylamide, and deoxyribonucleoside triphosphates were from Boehringer Mannheim Corp. Other reagents were of molecular biology grade and purchased from Sigma. Cell culture products were

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purchased from GIBCO. Antibody preparations were purchased from Santa Cruz Biotechnology, Inc. Plasmid p4×CRE-RSV(CAT), containing four tandemly linked vasointestinal CRE sequences just upstream of the Rous sarcoma virus (RSV) promoter, and pRSV(CAT), containing the RSV promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (13), were obtained from R. C. Scarpulla, and pBLCAT2, containing a bp −109 to +51 herpes simplex virus thymidine kinase (tk) promoter upstream of the CAT gene (47), was obtained from M. L. Short. Plasmid pCAT-Basic Vector [pSVO(CAT)], lacking promoter and enhancer sequences, and pCAT-Control Vector [pSV-(CAT)], containing simian virus 40 (SV40) promoter and enhancer sequences, were from Promega. Plasmid pMV7, containing a herpes simplex virus tk promoter linked to the coding sequence for the bacterial *neo* (neomycin phosphotransferase) gene (39), was obtained from M. D. Johnson.

Cell culture and preparation of nuclear extracts. COS-1 cells (ATCC CRL 1650) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. Rat C6 glioma cells (ATCC CCL 107) were maintained in Ham's F-10 nutrient medium (GIBCO) supplemented with 10% dialyzed fetal calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml as described before (11). Nuclear extracts were prepared as described by us (65).

Synthetic oligonucleotides. Synthetic complementary oligonucleotides were purified, annealed, and ligated into the respective plasmid vectors as described before (43).

Construction of CAT vectors containing unilateral 5′ deletions. The rat LDH A subunit promoter fragment (bp −1173 to +25) was synthesized, characterized, and ligated into plasmid pSVO(CAT) lacking promoter and enhancer elements to generate pLDH(−1173/+25)CAT as previously described (63). Plasmids with the 5′ promoter deletion fragments from bp −830 to +25 and bp −637 to +25 were generated by using conveniently located *Pst*I and *Xba*I restriction sites, respectively, in pLDH(−1173/+25)CAT (63).

Lacking convenient restriction sites, plasmids pLDH(−978/+20)CAT, pLDH(−862/+20)CAT, and pLDH(−843/+20)CAT were made by the PCR method with fragment bp −1173 to +25 as the template and the following oligonucleotide primers: 5′-GGGCATGCACAAAAGAGATCAC-3′, 5′-GGG CATGCGCTTGTTCAAATCT-3′, and 5′-GGGCATGCACTGTAATAGACC T-3′ as sense primers and 5′-GGGTAGGTGGCTTATAGCTCTA-3′ as the antisense primer. The blunt-ended PCR products were ligated into *Pst*I-cut and blunt-ended pSVO(CAT).

Plasmids containing either wild-type or site-mutated CRE sequences were constructed in pLDH(−159/+2)CAT by chemical synthesis of the bp −159 to +2 fragment containing 5′ *Hin*dIII and 3′ *Pst*I linkers and ligation into the *Hin*dIII and *Pst*I sites of pSVO(CAT) as described before (63). The CRE site at bp −48 to −41 was mutated from TGACGTCA to TAGATCAT.

Construction of pLDH(−159/+2)CAT vectors containing promoter LS mutations. Plasmids based on pLDH(−159/+2)CAT containing 5′ insertions of different 65-bp promoter fragments with linker-scanning (LS) mutations (between bp −890 and −826) (see Fig. 2B) were constructed by inserting synthetic wild-type fragments and LS mutations with *Hin*dIII linkers into the unique *Hin*dIII site of pLDH(−159/+2)CAT directly upstream of the bp −159 to +2 promoter fragment.

Construction of pLDH(−159/+2)CAT vectors containing 5′ and 3′ LDH-NRE insertions. Wild-type and mutated 29-bp LDH-NRE fragments (bp −875 to −847) were obtained by annealing the complementary oligonucleotide (wt, mut1, mut2, and mut3) fragments shown in Fig. 2D. Double-stranded fragments with *Hin*dIII linkers were inserted into the unique *Hin*dIII site of pLDH(−159/+2)CAT directly upstream of the bp −159 to +2 promoter fragment. To obtain 3′ insertions, double-stranded fragments with *Bam*HI linkers were cloned into the unique *Bam*HI site downstream from the CAT transcriptional unit in pLDH(−159/+2)CAT. An identical procedure was used for insertion of a wild-type LDH-NRE into pLDH(−159/+2)CAT with a mutated CRE site (63).

Construction of pLDH(−80/+2)CAT vectors containing 5′ LDH-NRE insertions. A double-stranded wild-type 29-bp LDH-NRE fragment (see above) with appropriate restriction sites was inserted into *Hin*dIII- and *Afl*III-cut pLDH(−159/+2)CAT, resulting in pLDH-NRE(−80/+2)CAT. Similarly, the 29-bp LDH-NRE fragment was inserted into pLDH(−159/+2)CAT in which the CRE site had been mutated (see above), resulting in pLDH-NRE(−80/+2)CAT with a mutated CRE.

Construction of p4×CRE-RSV(CAT) and pRSV(CAT) vectors containing the LDH-NRE. Plasmids p4×CRE-RSV(CAT) (13) and pRSV(CAT) (lacking the CRE) were digested with *Nde*I and *Bst*XI to delete a 223-bp fragment just 5′ of the 4×CRE-RSV or RSV promoter, respectively. Double-stranded LDH-NRE oligonucleotides with appropriate linkers were then cloned into the *Nde*I and *Bst*XI sites.

Construction of pSV40(CAT) containing 5′ and 3′ LDH-NRE insertions. The Promega pCAT-Control Vector [pSV(CAT)], which contains the SV40 promoter and enhancer, was digested 5′ of the SV40 promoter with *Bgl*II or 3′ of the SV40 enhancer with *Sal*I, respectively. The double-stranded wild-type LDH-NRE oligonucleotide wt with appropriate linkers was cloned into the *Bgl*II or *Sal*I site, respectively.

Construction of ptk(CAT) containing a wild-type 5′ LDH-NRE insertion. pBLCAT2 (47) was digested with *Sal*I and *Xba*I just 5′ of the herpes simplex virus tk promoter. Double-stranded wild-type 29-bp LDH-NRE oligonucleotide wt with appropriate linkers was cloned into the *Sal*I and *Xba*I sites.

Construction of p2×CRE$_{wt}$-tk(CAT) and p2×CRE$_{mut}$-tk(CAT). pBLCAT2 was digested with *Sal*I and *Xba*I just 5′ of the tk promoter. A 39-bp oligonucleotide with two tandemly linked CREs with appropriate linkers was cloned into the restricted sites. The sequence of the inserted oligonucleotide (CRE sites shown in boldface) was 5′-CTG**TGACGTCA**GCTCGAATTCGTCATACTG**T GACGTCA**G-3′.

A vector, p2×CRE$_{mut}$-tk(CAT), in which the two CRE sites were mutated to TAGATCAT, was similarly constructed by insertion of the above fragment containing two mutated CREs.

Construction of p2×CRE$_{wt}$-tk(CAT) and p2×CRE$_{mut}$-tk(CAT) containing 5′ LDH-NRE$_{wt}$ and LDH-NRE$_{mut}$ insertions. pBLCAT2 was digested with *Sal*I and *Xba*I just 5′ of the tk promoter. A 62-bp oligonucleotide containing either the wild-type or mutated (mut1, mut2, or mut3) NRE sequence with two tandemly linked CREs was cloned into the restricted sites. The sequence of the inserted oligonucleotide (wild-type NRE and wild-type CRE sites shown in boldface) was 5′-AAA**TCTTGCTCAAGA**CTGTTACTG**TGACGTCA**GCTCGAATTCGTC ATACTG**TGACGTCA**GCT-3′.

A vector in which the CRE sites were mutated to TAGATCAT was similarly constructed by insertion of the above fragment containing two mutated CREs.

The sequence and correct orientation of all inserts were determined by restriction and DNA sequence analyses. Sequencing was carried out in both directions by the dideoxynucleotide chain terminator method with specific synthetic oligonucleotides as primers.

DNA transfections and CAT assay. Transfections of COS-1 cells (10^6 cells) by the Lipofectin (GIBCO/BRL) procedure and assay of cellular extracts for CAT activity were performed as previously described (33, 63).

Gel mobility shift, DNase I protection assay, and Southwestern (DNA-protein) blotting analysis. Assays were carried out as previously described by us (33, 43, 63). For gel supershift assays, 1 μg of antibody was added to the DNA-protein binding reactions, after which the mixture was incubated at 4°C for 2 h before gel electrophoresis.

UV DNA-protein cross-linking. UV cross-linking of proteins to DNA was performed as described by Ausubel et al. (2).

In vitro transcription assays and primer extension analysis. The procedures for in vitro transcription and primer extension were described in detail in a previous publication (65).

Nucleotide sequence accession number. The sequence of the LDH promoter fragment has been assigned GenBank/EMBL accession number U05674.

RESULTS

Transcriptional regulation by the LDH A subunit gene upstream region and identification of a negative regulatory element. We have previously characterized and identified several functional binding sites for known transcription factors within the promoter region of the LDH A subunit gene, including two Sp1 binding sites, a phorbol ester-inducible AP-1-binding site (TRE), and a cAMP-inducible, CREB-responsive CRE (33, 63). Additionally, functional testing of the upstream LDH A region suggested the presence of a negative transcription regulatory element between bases −1173 and −830. To delineate the borders of the negative element, our strategy consisted of testing the transcriptional activity of several LDH A 5′ promoter deletions followed by detailed mutational analysis with LS as well as point mutations. Promoter fragments were inserted in front of the CAT coding sequence in a pSVO(CAT) basic vector, which uses SV40 splicing and polyadenylation signals for eukaryotic expression but lacks any eukaryotic promoter and enhancer sequence. Several of the promoter deletions were selected because of the location of convenient restriction sites (Fig. 1A). In order to maintain the physiological tissue specificity, the analyses were carried out with cells expressing cAMP-inducible LDH A, either the COS-1 or rat C6 glioma cell line. Both cell lines express high levels of cAMP-inducible LDH A subunit endogenously (11, 37). For transient-transfection assays, we chose the COS-1 cell line, because transfections of COS-1 cells turned out to be highly efficient (63). To determine the transcriptional efficacy of different reporter genes, several precautionary controls were carried out as part of the experiments. CAT assays were carried out in the linear range of the acyltransferase activity, and transfection efficiencies were normalized by cotransfection of the β-galactoside expression vector pCH110. Furthermore, pSVO(CAT)

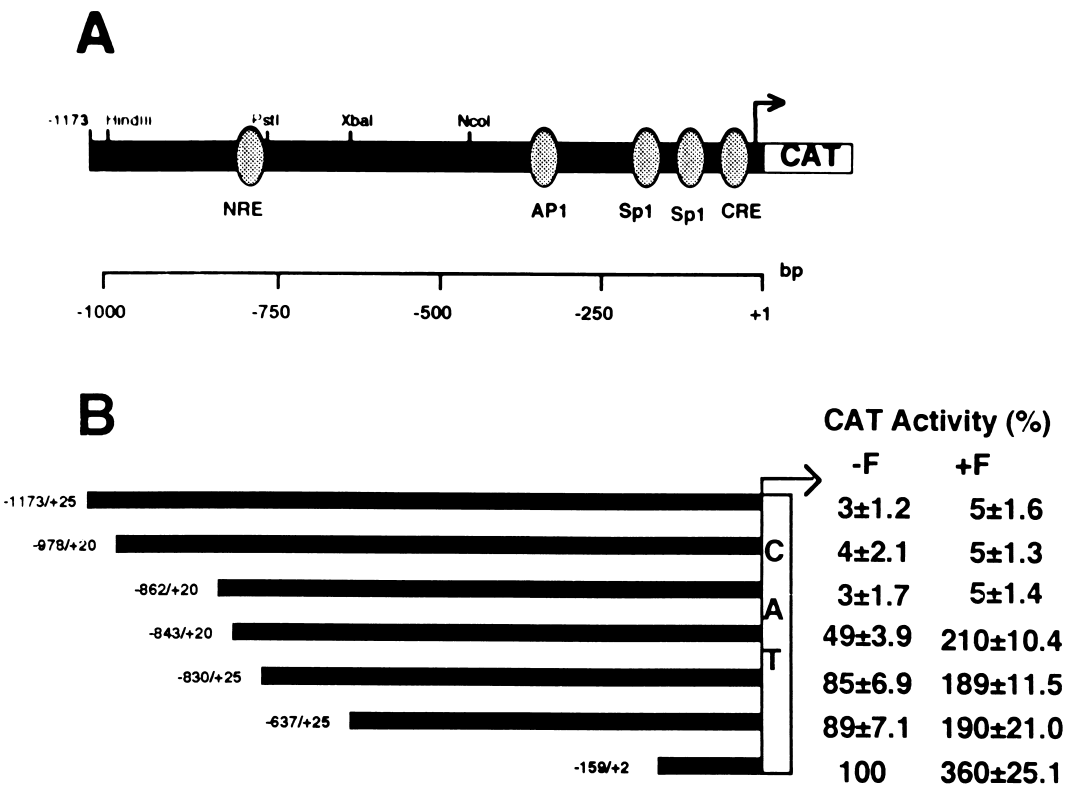

FIG. 1. Basal expression and forskolin induction of 5′ deletion LDH A/CAT fusion genes. (A) Diagram of LDH A subunit promoter structure with functionally identified regulatory regions. Several relevant restriction sites are indicated. The arrow indicates the transcription initiation site. (B) Basal (−F) and forskolin-induced (+F) expression of LDH A subunit/CAT constructs. Plasmid constructs with the CAT gene under the control of the indicated LDH A subunit promoter fragments were tested for CAT activity by transfection (5 μg) into COS-1 cells in the presence of 0.5% serum (0.25% fetal calf serum and 0.25% newborn calf serum). CAT activity values represent CAT/β-galactosidase activity ratios relative to the basal activity of the construct carrying bp −159 to +2, which was set at 100% (3.5% conversion of chloramphenicol to butyrated chloramphenicol). CAT expression values for each plasmid are the means ± standard error (SE) for three separate transfections with three separate plasmid preparations.

basic vector (lacking a promoter and enhancer) and pSV-(CAT) control vector (containing an SV40 promoter and enhancer) were included as negative and positive controls, respectively.

The results of transient-transfection experiments with seven LDH A promoter fragments of various lengths are summarized in Fig. 1B. Because the data are pooled from several experiments, they are presented in arbitrary units of CAT activity, taking the mean basal activity level of pLDH(−159/+2)CAT, exhibiting the highest activity of the CAT vectors, as 100%. The longest fragment of the LDH A subunit upstream region tested (bp −1173 to +25) as well as deletions to bp −978 and −862 exhibited very low, if any, basal and forskolin-inducible CAT activities. As the deletions progressed from bp −862 to −843, basal as well as cAMP-inducible activities increased markedly. Thus, this initial series of transient-transfection assays revealed the existence of a core fragment with strong negative regulatory activity. Considering the gradual increase in basal promoter strength as the deletions progressed from bp −843 to −159, the existence of an additional negative regulatory sequence(s) may be indicated. However, at this time we have not tested this possibility.

To further delineate the borders of LDH-NRE, we generated and tested a series of LDH promoter fragments with LS mutations generated between bp −884 and −829 (sequences shown in Fig. 2B). In the experiments summarized in Fig. 2A, the wild-type bp −890 to −826 promoter fragment was isolated and inserted in the sense and antisense orientations 5′ of the −159 to +2 promoter fragment in pLDH(−159/+2)CAT. Analyses of these constructs showed that the bp −890 to −826 fragment in either orientation strongly repressed basal and cAMP-inducible transcriptional activities. On the basis of their ability to reverse repression, transcriptional analyses of the various LS mutation-CAT vectors allowed us to locate the LDH-NRE in a core region ranging from approximately bp −873 through bp −850.

A palindromic LDH-NRE acts independently of orientation and position. Visual inspection of the bp −873 to −850 fragment reveals the presence of a 12-base region of dyad symmetry (see Fig. 2D) in which the inverted repeats TCTTGCTC AAGA are separated by the two bases C and T. On the assumption that the palindromic sequence is the region conveying negative regulatory activity, we analyzed its transcriptional effect in more detail. Short 29-bp promoter fragments were constructed in which either one (mut1 and mut2) or both (mut3) palindromic half-sites were mutated (see Fig. 2D). After insertion of these fragments either upstream or downstream of the LDH-CAT transcription unit in pLDH(−159/+2)CAT, the transcriptional activities of the wild-type and mutated hybrid promoter fragments were determined. The results are shown in Fig. 2C. While insertion of the wild-type palindromic sequence in either orientation markedly repressed basal and forskolin-inducible CAT activities, mutation of either or both palindromic half-sites fully restored the transcription regulatory activity of the bp −159 to +2 fragment. These

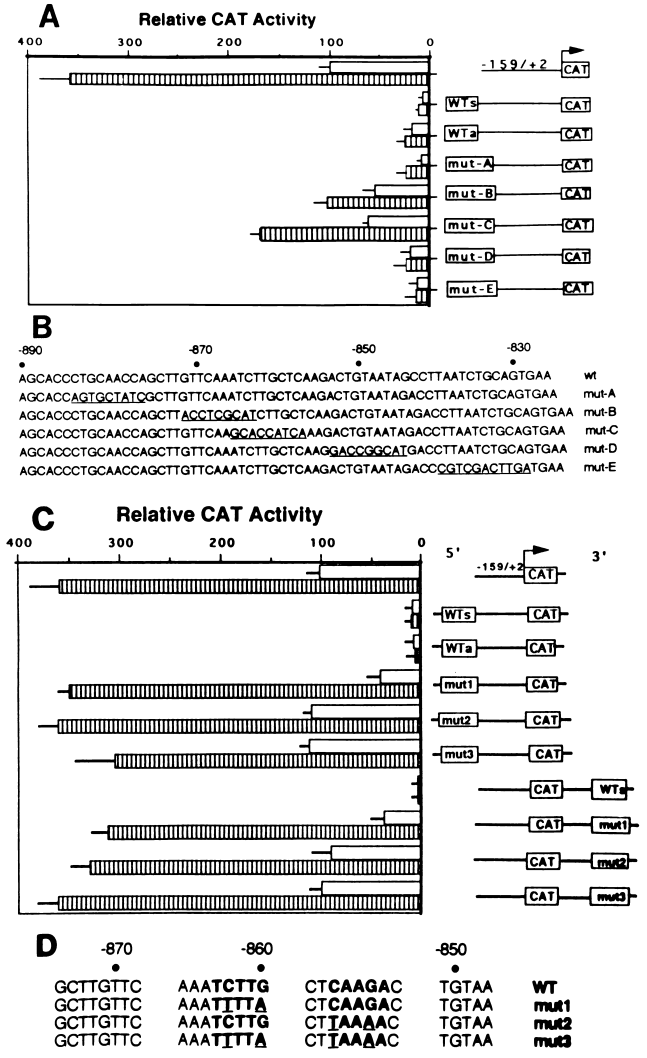

FIG. 2. Control of LDH-CAT fusion gene expression by wild-type and mutated bp −890 to −826 LDH A subunit promoter fragments. (A) LDH A promoter repression by wild-type and LS-mutated NRE sequences. CAT expression in cells transiently transfected with plasmids containing wild-type and mutated LDH-NRE sequences is shown relative to that obtained from pLDH(−159/+2)CAT, which contains no LDH-NRE. Assay conditions are as described in legend to Fig. 1. Error bars indicate SEs (three determinations per sample). Assays were carried out in the absence of forskolin (open columns) and in the presence of forskolin (hatched columns). WT_s, wild-type LDH-NRE inserted in the sense direction; WT_a, wild-type LDH-NRE inserted in the antisense direction. (B) Sequences of wild-type (wt) and LS-mutated LDH-NRE fragments (bp −890 to −826) that were inserted upstream of LDH A fragment bp −159 to +2. The regions containing the LS mutations are underlined. (C) LDH A promoter-CAT expression directed by wild-type and point-mutated LDH-NRE sequences inserted in the upstream and downstream positions. For experimental conditions, see the legend to panel A. (D) Sequences of wild-type and mutated LDH-NRE fragments (bp −875 to −847) that were inserted upstream and downstream of LDH A fragment bp −159 to +2. Mutated bases are underlined. The bases constituting the palindromic sequence are in boldface.

results indicate a requirement for two intact palindromic half-sites for LDH-NRE activity.

Negative regulation mediated via specific DNA sequences may be envisaged to involve either local position-dependent interference with positive regulatory factors or a position-independent effect analogous to that of silencers (7, 12). To test the potential position-independent effect of LDH-NRE, wild-type and mutated LDH-NRE sequences were inserted into the unique *Bam*HI site downstream from the CAT gene in pLDH(−159/+2)CAT. The results of the CAT assays, displayed in Fig. 2C, show that wild-type LDH-NRE in the downstream position retains its transcription-repressing effect, on both basal and cAMP-inducible activities, while mutation of the LDH-NRE restored promoter activity.

LDH-NRE represses transcription of heterologous cAMP-inducible promoters. Since the functional properties of the LDH-NRE described so far were obtained by testing its effect as part of the homologous LDH A promoter, we wanted to know whether the silencer acted only within the context of the LDH A promoter or exerted a more general action on CRE-containing heterologous promoters. To test this notion, we chose two different vectors, p4×CRE-RSV(CAT) and p2×CRE-tk(CAT), in which the CAT marker gene was under the control of a heterologous promoter, either the RSV (13) or tk (47) promoter, which, through insertion of four and two CREs, respectively, linked in tandem, had acquired a high degree of transcriptional inducibility by cAMP.

Wild-type and mutated LDH-NREs were cloned just upstream of the CRE hybrid promoters. This placed the LDH-NRE 223 bases upstream of the 4×CRE-RSV promoter and 18 bases upstream of the 2×CRE-tk promoter. Transient transfections showed that p4×CRE-RSV(CAT) and p2×CRE-tk(CAT) exhibited relatively high basal and cAMP-inducible CAT activities, whereas CRE-CAT fusion genes with wild-type LDH-NRE inserts in either the forward or reverse orientation expressed little or no detectable transcriptional activities (Fig. 3A). In contrast, insertion of mutated LDH-NRE fragments, mut1, mut2, and mut3, in place of the wild-type LDH-NRE partially restored cAMP inducibility. These results demonstrate that (i) LDH-NRE is not promoter specific and can repress cAMP-inducible heterologous cellular promoters and (ii) the LDH-NRE represses transcription to the same extent when present in either orientation [at least in p4×CRE-RSV(CAT)].

Silencing activity of the LDH-NRE is dependent on the presence of a CRE module. To shed some light on the silencing mechanism, it is important to know whether the silencer acts in concert with other positive transcription modules. We first assessed whether the LDH-NRE could confer negative regulation on heterologous promoters lacking a CRE. To this end, we tested its effect on three heterologous promoters that do not contain CREs and are not cAMP inducible, such as the RSV promoter and also the SV40 and tk promoters, which both contain SP1 sites. In each experiment, the LDH-NRE was cloned upstream of the promoters and, in the case of the pSV(CAT) control, containing the SV40 promoter and enhancer, also downstream of the CAT marker gene. As shown in Fig. 3B, insertion of the silencer element had no effect on the activity of either the RSV or the SP1-containing SV40 and tk promoters.

To investigate the functional effect of the NRE on promoters which contain CRE as well as SP1 elements in more detail, we prepared several additional vectors in which the CRE sites had been mutated. Figure 4 shows the inability of the NRE to modulate the transcriptional activity of the homologous promoter pLDH(−159/+2)CAT, containing a mutated CRE site, as well as the heterologous promoter construct p2×CRE_{mut}-tk(CAT). Similarly, NRE was unable to repress the activity of CRE-mutated pLDH(−80/+2)CAT, which lacks the two SP1 sites located at bp −136 and −102 in the LDH bp −159 to +2 fragment. We conclude from these experiments that the presence of a CRE is absolutely required for functional LDH-NRE activity and that deletion of the SP1 sites does not modulate the silencer activity of NRE.

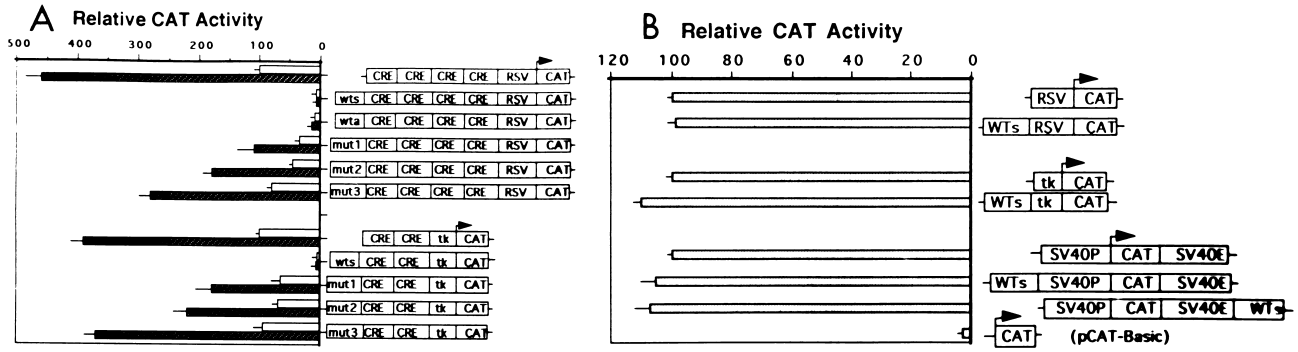

FIG. 3. Repression of heterologous cAMP-inducible promoters by LDH-NRE. (A) Expression of the CAT gene under the control of hybrid CRE-CAT promoter constructs. Plasmid constructs under the control of the 4×CRE-RSV or 2×CRE/tk hybrid promoters without and with inserts of wild-type and mutated LDH-NRE fragments were assayed for CAT activity without (open columns) and with (shaded columns) forskolin. For experimental conditions, see the legend to Fig. 1. The basal CAT activities of p4×CRE-RSV(CAT) and 2×CRE-tk(CAT) were arbitrarily set at 100%. The basal activity of p4×CRE-RSV(CAT) was 4.5-fold higher and that of 2×CRE-tk(CAT) was 0.8-fold lower than that of pLDH(−159/+2)CAT. (B) Expression of the CAT gene under the control of the RSV, tk, and SV40 promoters without and with 5′-inserted wild-type LDH-NRE in the sense orientation (WT_s). The SV40-CAT plasmid was also tested with the LDH-NRE in the downstream position of the CAT gene. pCAT-Basic represents a promoterless control plasmid.

Competition analysis demonstrates a functional interaction of LDH-NRE with a cellular *trans*-acting factor(s). To test whether a saturable nuclear *trans*-acting factor(s) specifically interacts with the LDH-NRE to repress transcription, competitive analyses were carried out by cotransfecting excess 29-bp LDH-NRE fragment with pLDH(−978/+20)CAT. If the cellular concentration of the *trans*-acting factor is limiting, its transcription regulatory effect will be partially titrated out by cotransfection of the LDH-NRE fragment. In the experiments shown in Fig. 5A, competition for the putative LDH-NRE binding factor(s) was evaluated by cotransfecting increasing quantities of either wild-type or mutated 29-bp LDH-NRE fragments with pLDH(−978/+20)CAT. Whereas the three mutated LDH-NRE fragments had little or no effect on transcriptional activity, cotransfection of wild-type LDH-NRE relieved the repression in a dose-dependent fashion. These results allow the conclusion that the palindromic LDH-NRE exerts its transcription-repressing activity by binding directly or indirectly to one or more *trans*-acting protein factors present in only limiting concentrations.

To expand and confirm the above results, we examined the effects of the wild-type and mutated LDH-NRE fragments in an in vitro transcription system previously developed by us to functionally analyze *cis*- and *trans*-regulatory elements of the LDH A promoter (65). Cell-free transcription analysis is a powerful technique for elucidation of transcriptional mechanisms under a variety of carefully controlled experimental conditions, and it complements transcriptional studies by transient-transfection techniques. We have previously shown (65) that transcription from the LDH A promoter in pLDH(−159/+2)CAT occurs accurately from the transcription initiation site, responds to cAMP addition with an upregulation of in vitro transcription, and allows analysis of the competitive effects of promoter mutations. While HeLa cell nuclear extracts

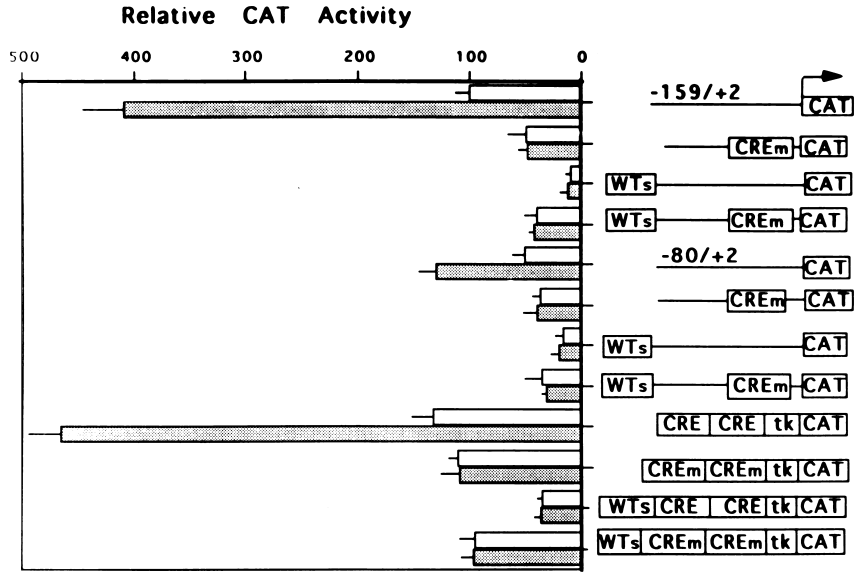

FIG. 4. Requirement for a functional CRE for NRE silencer activity. The activities of plasmid constructs with the CAT gene under the control of the LDH bp −159 to +2, the LDH bp −80 to +2, and the 2×CRE-tk promoter containing either a wild-type (CRE) or site-mutated (CRE_{mut}) CRE were compared with those of identical vectors that had an insert of wild-type NRE fragment (WT_s) just upstream of the promoter fragments. Assay conditions are as described in legend to Fig. 1. Data represent means ± SE (n = 3). Open columns, without forskolin; shaded columns, with forskolin.

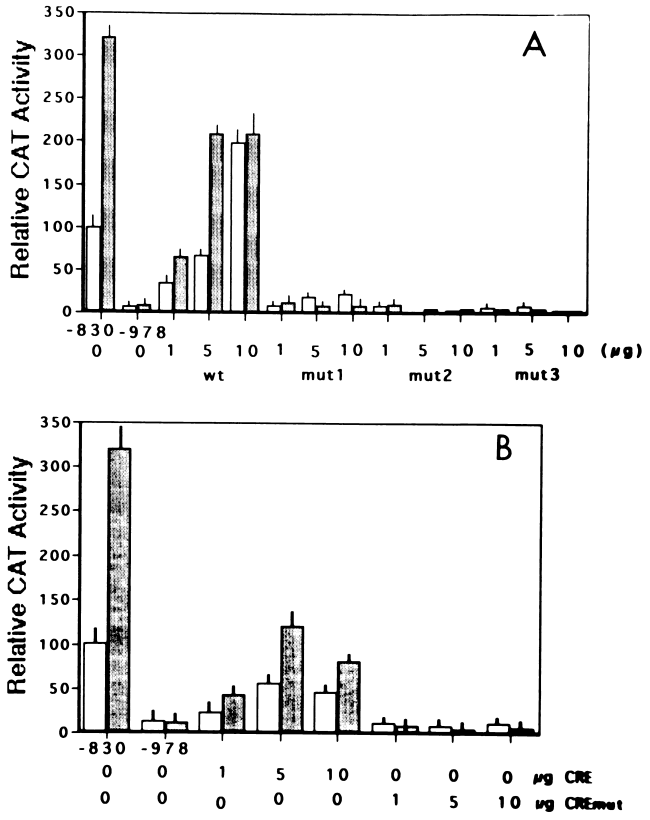

FIG. 5. Competition CAT assays with isolated LDH-NRE or CRE fragments. All transfections were carried out with 5 μg of CAT vectors without (open bars) and with (shaded bars) forskolin. Results are expressed as CAT activity relative to the basal CAT activity of plasmid pLDH(−830/+25)CAT, which was set at 100%. (A) Plasmid pLDH(−978/+20)CAT was either transfected alone or cotransfected with the indicated micrograms of isolated 27-bp wild-type (wt) and mutated LDH-NRE fragments (for sequences, see Fig. 3B). Transfection conditions were as described in the legend to Fig. 1. Bars show the means + SE for at least three separate transfections with three separate plasmid preparations. (B) Experimental conditions are identical to those described in the legend to panel A except that the indicated micrograms of isolated 20-bp wild-type (CRE) and mutated (CRE_{mut}) CRE fragments were cotransfected as competitors. The following synthetic double-stranded sequences (from bp −53 to −34) containing the CRE (underlined) and mutated CRE (mutations in boldface type) were used as competitors: wild-type CRE, 5′-CACTCTGACGTCAGCGCGGA-3′; mutated CRE, 5′-CACTCTAACACAAGCGCGGA-3′.

apparently do not respond to cAMP addition by increasing the transcriptional activity of CRE-containing promoters (51), nuclear extracts from several other cell types, such as rat liver (53), porcine kidney LLC-PK_1 cells (51), and rat C6 glioma cells (65), increase transcription of cAMP-inducible promoters in response to cAMP addition.

The templates used in the in vitro transcription assays were the test plasmid LDH(−159/+2)CAT without (Fig. 6D, line a) and with (Fig. 6D, line b) NRE insertions and the tk-neo plasmid pMV7 (39), which served as the internal control. Using the template shown in Fig. 6D, line a, we demonstrated that cAMP increased transcription from the LDH A bp −159 to +2 promoter template about 3.5-fold (Fig. 6A, lanes −159/+2). Insertion of the wild-type LDH-NRE immediately before the bp −159 to +2 promoter in either orientation (see template b in Fig. 6D) reduced cAMP-induced transcription to slightly below basal levels (Fig. 6A, lanes WT_s and WT_a). Figure 6B shows that transcription from the tk promoter was not affected under these experimental conditions. In additional control experiments, we were able to show that cloning of an unrelated 29-bp DNA fragment in place of the LDH-NREs had no inhibitory effect on transcription (data not shown). Inclusion in the assays of increasing amounts of competitor wild-type LDH-NRE fragment (lanes WT [Fig. 6C]) relieved repression of basal activity and cAMP inducibility. In contrast, the mutated LDH-NRE mut3, within the concentration range used, restored neither basal activity nor cAMP sensitivity. The addition of an unrelated 29-bp DNA fragment at a 4:1 molar excess did not relieve repression (data not shown).

Identification of specific LDH-NRE/protein complexes. Since the competition CAT assays described above indicated that a cellular component(s) is necessary for negative regulatory activity, we used competition gel retardation and footprinting experiments to identify the nuclear LDH-NRE binding protein(s). Rat C6 glioma cell nuclear extracts were probed with a 3′-end-labeled wild-type LDH-NRE fragment. Figure 7A shows that gel shift assays gave three bands of different intensities, designated a, b, and c (lane 2). All three bands were eliminated by increasing molar concentrations of unlabeled wild-type LDH-NRE (Fig. 7A, lanes 3 to 5). Mutated LDH-NRE fragment mut3 appeared to eliminate only band a, indicating the specificity of complex formation with bands b and c.

We also performed footprinting experiments to identify the nuclear protein binding to LDH-NRE. We found that one major region containing the palindromic LDH-NRE is protected on the sense strand from bp −876 through −853 [Fig. 7B(a), lanes 1 through 6] and on the antisense strand from bp −870 through −851 [Fig. 7B(b), lanes 1 through 6]. Similar footprinting patterns were obtained with nuclear extracts from rat liver and COS-1 cells. Each footprint was eliminated by a 29-bp oligonucleotide containing the wild-type LDH-NRE sequence [lane 6 in Fig. 7B(a) and lanes 1 and 2 in Fig. 7B(b)].

Identification of LDH-NRE binding protein by Southwestern blotting and UV cross-linking. Information concerning the molecular size of the LDH-NRE binding protein was obtained by two independent methods, a Southwestern blotting assay and UV cross-linking. For the Southwestern assay, nuclear proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, renatured, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled wild-type LDH-NRE probe. As shown in Fig. 8A, lane 1, the LDH-NRE sequence recognized a nuclear protein with a molecular mass of approximately 69 kDa. Competition experiments were carried out to further ascertain the specificity of interaction between the 69-kDa protein and the LDH-NRE. Protein blots were again probed with ^{32}P-LDH-NRE but in the presence of competitor LDH-NRE, mutated LDH-NRE (mut3), and poly(dI-dC). While addition of nonspecific poly(dI-dC) (lane 2) and mutated LDH-NRE (lane 4) had no effect on binding, addition of wild-type LDH-NRE fragment blocked binding of ^{32}P-LDH-NRE to the 69-kDa protein (lane 3). The experiments demonstrating the UV cross-linking of ^{32}P-LDH-NRE to glioma cell protein are shown in Fig. 8B; lane 1 identifies a protein of approximately 69 kDa with binding affinity for wild-type LDH-NRE. Mutated (mut3) LDH-NRE did not cross-link (Fig. 8B, lane 2). From these data, the 69-kDa protein can be identified as possessing selective binding affinity for the LDH-NRE.

Functional interaction between the LDH-NRE and CRE modules. In a number of cases, affinities of otherwise positive transcription factors for negative regulatory elements have been identified (19, 23, 29, 59, 61, 66). However, when the DNA-binding sites for several transcription factors (Sp-1, CAAT box-binding protein, AP-1, AP-3, GRE, NF-κB, and OCT1) were used in gel shift assays as competitors, no effects

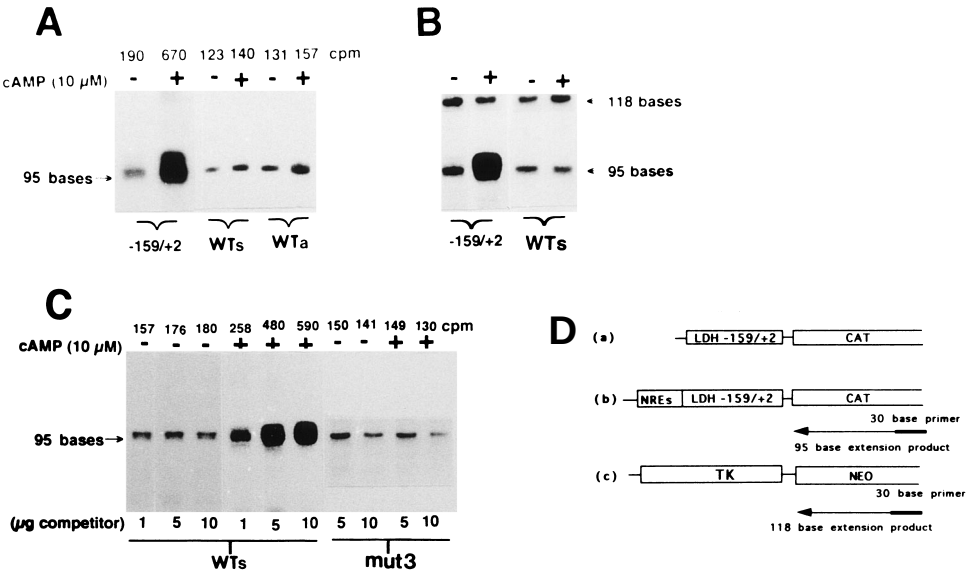

FIG. 6. Effect of LDH-NRE on in vitro transcription directed from the LDH A subunit promoter fragment bp −159 to +2. (A) In vitro transcription assay of the LDH bp −159 to +2 promoter without and with upstream insertions of LDH-NRE. Two micrograms of closed circular plasmid pLDH(−159/+2)CAT, either without (lanes −159/+2) or with insertions of wild-type 27-bp LDH-NRE fragments in the sense (lanes WTs) or antisense (lanes WTa) orientation were used in reaction mixtures containing 160 μg of glioma cell nuclear protein. The reactions were carried out in the absence (−) or presence (+) of 10 μM cAMP. The primer extension products of 95 bases are marked with an arrow. After autoradiography of the gels, ^{32}P radioactivity in corresponding gel slices was determined by scintillation counting. The counts per minute (cpm) are listed above each lane. (B) Effect of cAMP on the transcriptional activities directed by a control tk promoter and the LDH bp −159 to +2 promoter without and with a sense insertion (WTs) of LDH-NRE. Transcription reactions were carried out as described in the legend to panel A but with the addition of 1 μg of pMV7 template. The primer extension products of 118 bases (pMV7) and 95 bases [pLDH(−159/+2)CAT] are marked with arrows. (C) Analysis of the competitive effects of isolated wild-type and mutated LDH-NRE fragments on in vitro transcription of the LDH bp −59 to +2 promoter with a sense insertion (WTs) of LDH-NRE (see template b in panel D). In vitro transcription was carried out as described for panel A with 2 μg of DNA template and the indicated micrograms of 27-bp wild-type LDH-NRE (WTs) or mut3 competitor DNA in the presence (+) or absence (−) of forskolin. The counts per minute (cpm) per band are listed above the lanes. (D) Diagrammatic representations of the templates used for transcription.

on LDH-NRE/nuclear protein binding were found. Nevertheless, our data demonstrated an absolute requirement for CRE to achieve repression, suggesting an interaction between LDH-NRE-binding protein and CRE (and/or CRE-binding protein) and conversely between CRE-binding protein and LDH-NRE [and/or an LDH-NRE-binding protein(s)]. This idea was tested in several ways. First, we determined the competitive effect, if any, of CRE on LDH-NRE/nuclear protein binding and, conversely, the effect of LDH-NRE on CRE/nuclear protein binding by gel shift analysis. Second, we evaluated the competitive effect of CRE in a functional CAT assay with pLDH(−978/+20)CAT, a vector with minimal transcriptional activity. We reasoned that if CRE does indeed interact with the LDH-NRE binding protein complex, CRE may have a competitive effect in the gel shift and, in addition, could relieve repression of cAMP inducibility through titration of the LDH-NRE binding protein.

Accordingly, a 20-bp CRE-containing LDH A promoter fragment (bp −53 to −34) was tested for its ability to compete for binding in a ^{32}P-LDH-NRE/protein binding assay. The results of these experiments are shown in Fig. 9A. The CRE fragment (lanes 7 through 9) competed as efficiently as the LDH-NRE fragment (lanes 3 through 5) in band b complex formation. A slight degree of competition by the CRE of complex a was also observed but only at a higher concentration of competitor CRE (lane 9), suggesting a nonspecific effect. Interestingly, whereas LDH-NRE competed efficiently for complex c formation (Fig. 9A, lanes 3 through 5), the CRE fragment was unable to reproduce this effect (lanes 7 through 9) even at a relatively high concentration. Neither site-mutated CRE (5′-CACTCT**AA**C**AC**AAGCGCGGA-3′; mutations shown in boldface) nor NRE mut3 was able to complete (Fig. 9A, lanes 10 to 13). Figure 9B shows the efficient competition by LDH-NRE on CRE-nuclear protein binding. The upper band was completely eliminated by a 50-fold molar excess of LDH-NRE (Fig. 9B, lane 3), whereas higher concentrations were needed to eliminate the lower band (lanes 3 to 5). Mutated CRE (Fig. 9B, lanes 6 and 7) and mutated LDH-NRE (lanes 8 and 9) fragments were ineffective as competitors even at relatively high molar concentrations.

As an extension of these gel shift data, we proceeded to test the functional effect of CRE on LDH-NRE-mediated silencing activity by cotransfecting wild-type and mutated 20-bp CRE oligonucleotides with pLDH(−978/+20)CAT and assessing their effect on CAT activity. Cotransfection of CRE partially restored basal and forskolin-stimulated CAT activity of the otherwise inactive LDH A promoter (Fig. 5B). Interestingly, cotransfection of CRE caused a concentration-dependent reversal up to 5 μg of CRE fragment. Further increases in CRE concentrations led again to a lower degree of inhibition reversal, probably due to an onset of cellular CREB titration by higher levels of CRE. A mutated CRE fragment showed no effect on CAT activity.

LDH-NRE/protein complexes include CREB and c-Fos proteins. On the premise that the action of LDH-NRE and CRE is mediated through the interaction and affinity of their respective binding proteins, we proceeded to gather information about the nature of the binding proteins. Since the action of CRE could conceivably involve an interaction between LDH-NRE binding protein and CREB, the composition of LDH-NRE/protein complexes was investigated by gel supershift assays with antibody to CREB. The LDH-NRE oligonucleotide-protein complexes b and c (see Fig. 10, lane 2) were supershifted by the anti-CREB antibody (Fig. 10, lane 8), suggesting

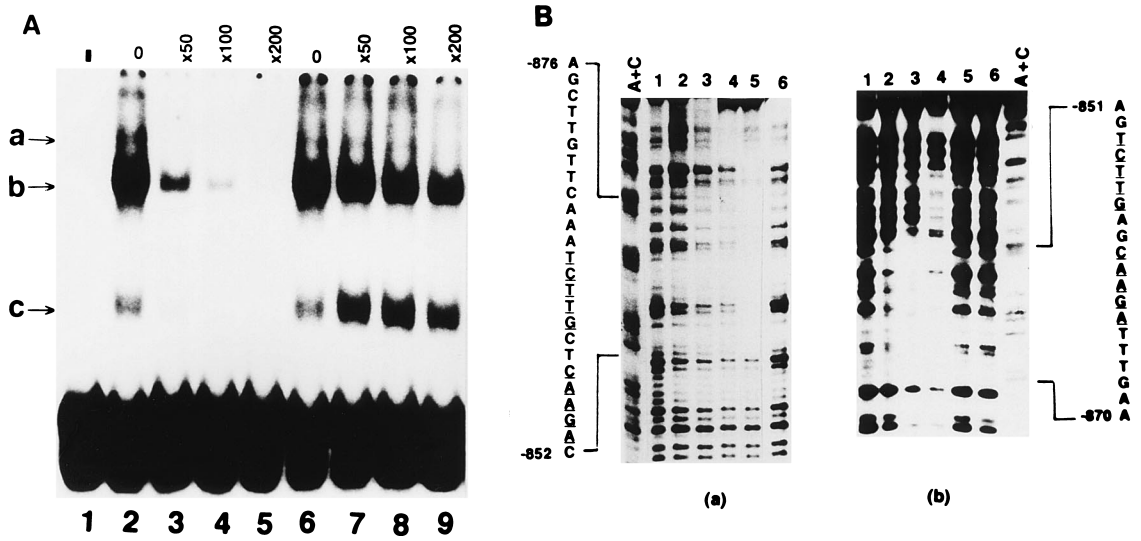

FIG. 7. Nuclear protein binding to LDH-NRE. (A) Electrophoretic band shift analysis of nuclear protein extracts. The 32-bp wild-type LDH-NRE fragment shown in Fig. 2D was used as the ^{32}P-labeled probe. The lanes show the complex formation of the wild-type LDH-NRE in the absence (lane 1) and presence of 15 μg of rat C6 glioma cell nuclear protein (lanes 2 through 9). Competition with wild-type LDH-NRE is shown in lanes 3 to 5 (50- to 200-fold molar excess of competitor), and competition with mutated LDH-NRE mut3 is shown in lanes 7 to 9. The DNA-protein complexes a, b, and c are marked with arrows. (B) DNase I footprinting analysis of nuclear protein-binding sites in the upstream region of the LDH A promoter. The LDH bp −906 to −788 fragment was labeled at the 5′ or 3′ site, and DNase protection assays were carried out as described in the text. Lanes A and C, sequencing reactions. (a) Sense strand. Lanes 1 to 5, 0, 20, 40, 60, and 80 μg of glioma cell nuclear protein extract, respectively; lane 6, competition binding assay with 80 μg of nuclear protein and a 100-fold molar excess of a 29-bp LDH promoter fragment (bp −875 to −848) containing the LDH-NRE (see Fig. 2D for sequence). (b) Antisense strand. Lanes 3 to 6, 60, 40, 20, and 0 μg of nuclear protein extract, respectively; lanes 1 and 2, competition binding assay with 60 μg of nuclear protein and a 200- and 50-fold molar excess of the 29-bp LDH promoter fragment, respectively. The DNase I-protected regions and nucleotide sequences are indicated.

the presence of CREB or a protein of similar antigenicity. We also tested the ability of several other transcription factors involved in LDH A transcription regulation to interact with the LDH-NRE binding protein. Of these, only anti-c-Fos antibody supershifted the LDH-NRE/protein complex (Fig. 10, lane 5).

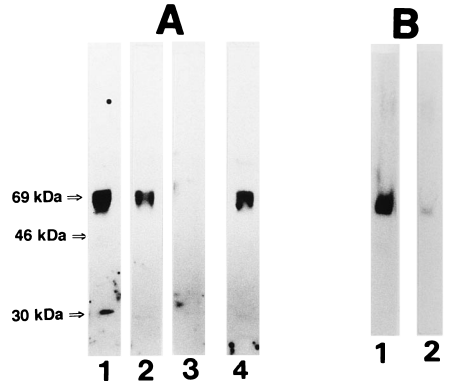

FIG. 8. Southwestern blotting and UV cross-linking analyses of LDH-NRE DNA-binding activity in nuclear protein extracts. (A) Southwestern blotting assay. Glioma cell nuclear protein extracts (180 μg per lane) were separated electrophoretically on denaturing gels. After renaturation and transfer to nitrocellulose, filters were probed with ^{32}P-labeled wild-type LDH-NRE fragment (see Fig. 2D for sequence) without competitor (lane 1), in the presence of 3 μg of poly(dI-dC) (lane 2), a 50-fold molar excess of wild-type LDH-NRE (lane 3), and a 200-fold molar excess of LDH-NRE mut3 (lane 4). The positions of protein molecular mass markers are shown on the left-hand side of the figure. (B) UV cross-linking assay. Nuclear protein extracts containing ^{32}P-labeled wild-type and mutant (mut3) LDH-NRE fragments (see Fig. 2C) were irradiated (254 nm) for 15 min at 4°C at an intensity of 7,000 μW/cm^2. After micrococcal nuclease digestion, proteins were separated electrophoretically on denaturing gels. Radioactively labeled protein was identified by autoradiography. Lane 1, wild-type LDH-NRE with non-specific poly(dI-dC) (0.25 μg/μl) as the competitor; lane 2, mutant LDH-NRE (mut3) as the probe.

Anti-SP1, anti-c-Jun, and anti-c-Myc antibodies were unable to supershift the complex.

DISCUSSION

During previous studies of several positive regulatory promoter modules in the rat LDH A subunit gene, we obtained evidence suggesting a negative regulation of the gene (63). Our findings identified a region within the subunit promoter that abolished cAMP-inducible as well as basal transcriptional activities. Here, we present a detailed analysis of this negative regulatory region, LDH-NRE. We have determined its palindromic structure and demonstrated that it has the properties of a typical silencer module (12) inasmuch as it mediates a position- and orientation-independent repression of the LDH A gene. The relative orientation and position independence of LDH-NRE are properties shared with a number of negative regulatory elements named transcriptional silencers (for reviews, see references 7, 30, and 45).

In vivo as well as in vitro transcriptional competition studies indicated the involvement of a limiting cellular component(s) in LDH-NRE silencing activity. This observation was further explored by gel mobility shift assays. We found that three distinct complexes form between cellular proteins and LDH-NRE. Interestingly, the competition of one of the LDH-NRE/protein complexes by CRE and vice versa (see Fig. 9) suggests that both LDH-NRE and CRE bind a similar factor(s). This appears to be confirmed by the presence of CREB, or a protein with similar antigenicity, in the LDH-NRE/protein complex and is consistent with the functional data obtained with in vivo and in vitro transcriptional assays (see Fig. 5 and 6). Indeed, sequence comparison of LDH-NRE and CRE does show some similarity (e.g., TG-C-TCA versus TGACGTCA), and some ambiguity of factor binding to *cis* elements is not unprece-

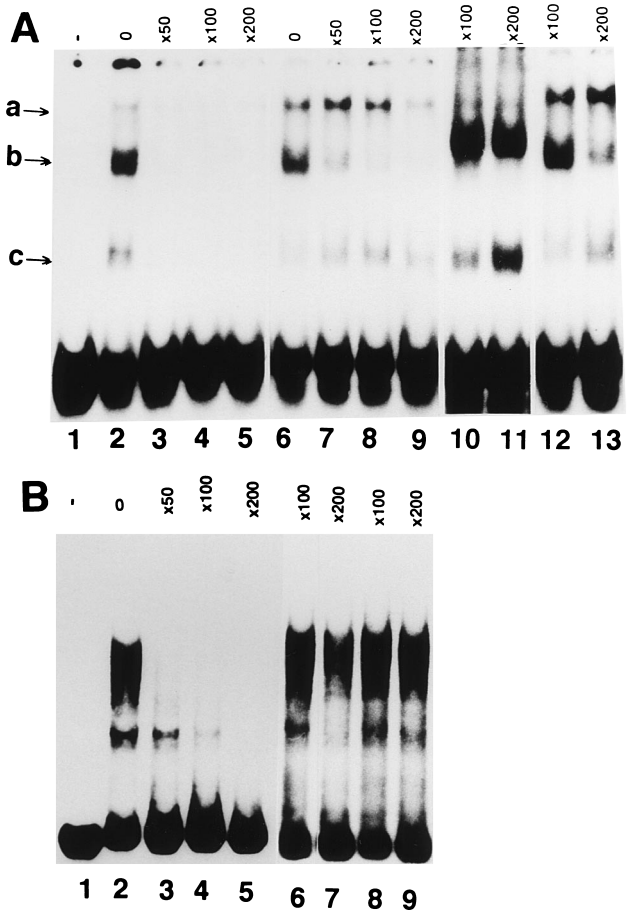

FIG. 9. Gel retardation analysis of the competitive effects of CRE and LDH-NRE on CRE and LDH-NRE DNA-protein complex formation. The assay conditions are the same as described in legend to Fig. 6A. (A) The 32-bp wild-type LDH-NRE (see Fig. 2D) was used as the ^{32}P-labeled probe in the absence (lane 1) and presence (lanes 2 through 13) of 15 μg of nuclear protein. Competition was carried out by the addition of a 50- to 200-fold molar excess of LDH-NRE fragment (lanes 3 to 5), a 50- to 200-fold excess of CRE fragment (lanes 7 to 9), a 100- to 200-fold excess of site-mutated NRE (mut3) (lanes 10 and 11), and a 100- to 200-fold excess of site-mutated CRE_{mut} (see the legend to Fig. 5 for sequence) (lanes 12 and 13). Note: band a is not consistently observed in different nuclear protein preparations. (B) A 20-bp wild-type LDH promoter fragment (bp −53 to −34) containing the CRE (see the legend to Fig. 5B) was used as the ^{32}P-labeled CRE probe. The lanes show the complex formation of ^{32}P-CRE in the absence (lane 1) and presence of 15 μg of glioma cell nuclear protein (lanes 2 through 9). Competition was carried out by the addition of a 50- to 200-fold excess of wild-type LDH-NRE fragment (lanes 3 to 5), a 100- to 200-fold excess of CRE_{mut} (lanes 6 and 7), and a 100- to 200-fold excess of mutated NRE (mut3) (lanes 8 and 9).

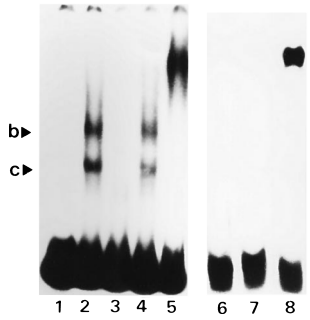

FIG. 10. Gel supershift analysis of LDH-NRE/protein complexes. The assay conditions are the same as described in the legend to Fig. 6A. ^{32}P-labeled LDH-NRE was incubated with no nuclear extract (lane 1), 10 μg of glioma cell nuclear extract (lanes 2 through 5 and 8), nuclear extract plus a 50-fold molar excess of LDH-NRE as competitor (lane 3), nuclear extract plus 1 μg each of anti-c-Jun (lane 4), anti-c-Fos (lane 5), and anti-CREB (lane 8) antibody. Lanes 6 and 7, ^{32}P-LDH-NRE with no nuclear extract but with 1 μg of anti-c-Fos or anti-CREB antibody, respectively. Arrows on the left side of the figure indicate LDH-NRE/protein complexes b and c.

dented, particularly among members of the bZIP family of transcription factors (26, 44, 48, 60).

Negative regulation may involve many different mechanisms of transcriptional repression (7, 30, 45). However, the unique functional feature of LDH-NRE consists of the fact that its silencing activity is strictly dependent upon the presence of a functional CRE, specifically acts via repression of CRE enhancer activity, and does not function as a general negative regulator. Thus, it is very likely that the molecular mechanism of transcription repression involves an interaction between the CRE/LDH-NRE modules. Evidence supporting this notion is based on several key findings: (i) CRE was able to compete in LDH-NRE/protein binding assays and, conversely, LDH-NRE competed in CRE/protein binding assays (see Fig. 9); (ii) CRE counteracted LDH-NRE silencing activity in functional CAT assays (Fig. 5B); and (iii) gel supershift assays indicate the presence of CREB (or an antigenically related protein) in LDH-NRE/protein complexes. Considering the overall implications of these data together with the fact that both inducible as well as basal transcription of several cAMP-regulated genes depends on the presence of CRE (10, 36, 38, 53), it is conceivable that the CRE module is the target for LDH-NRE and that LDH-NRE/CRE interaction is part of the mechanism by which the expression of the LDH A subunit and conceivably other cAMP-responsive genes is regulated. Differential interaction of these modules would be of marked physiologic significance, inasmuch as it increases the versatility and fine-tuning ability of a transcription regulatory system.

What is the molecular basis of the CRE/LDH-NRE interaction? An important clue comes from the fact that LDH-NRE has a palindromic structure with two identical half-sites. Therefore, it is a reasonable assumption that the putative LDH-NRE-binding protein may belong to the bZIP family of transcription factors, which bind DNA as dimers and activate or repress transcription (4). As a leucine zipper protein, LDH-NRE binding protein could possess the potential for heterodimer formation with other members of the bZIP family. Indeed, some of our data are compatible with a potential heterodimer formation between CREB and/or c-Fos and LDH-NRE binding protein. Whereas several of the leucine zipper proteins heterodimerize with various other members of their class (3, 27), CREB has been reported to act selectively in that it heterodimerizes only with ATF-1 (34) and CREM (17). However, interaction between members of the CREB-ATF and Fos-Jun families, for example, can occur through direct protein-protein interactions (3, 27) which do not involve the leucine zipper (15). Thus, as judged from the selective binding affinity between CREB and LDH-NRE binding protein, complex formation which does not involve the mechanism of leucine zipper formation may take place. This scenario may be likely in our system, since neither Southwestern blotting nor UV cross-linking assays identified a protein similar in molecular size to CREB (65) or c-Fos (see Fig. 8). However, a relatively low affinity of CREB for LDH-NRE or other unknown experimental factors may have played a role in our failure to identify LDH-NRE/CREB binding.

A second clue is based on the recent finding that CREB interacts with a component of the transcription factor TFIID

complex and may thus function as a potent constitutive transcriptional activator (15). It may be speculated that LDH-NRE binding protein, through its affinity to CREB, may transmit its inhibitory function by somehow interfering with transcription initiation through a quenching type of mechanism (7, 30, 45).

c-Fos itself cannot form homodimers (28, 40, 54). Instead, Fos-DNA binding is mediated through heterodimer formation with c-Jun (54, 55). However, we failed to identify Jun protein as a component of the LDH-NRE/protein complex. The presence of c-Fos in LDH-NRE/protein complexes could be explained through heterodimer formation with LDH-NRE binding protein. LDH-NRE binding protein might, by replacing c-Jun, function as an alternative partner for c-Fos, thus switching the target of c-Fos action and contributing to the negative regulatory action of LDH-NRE. Indeed, c-Fos can form complexes with proteins other than c-Jun and act through sequences other than the AP-1 element (21, 46). Through the Fos/LDH-NRE binding protein interaction, downregulation of cAMP-induced transcription could be achieved. This is consistent with data demonstrating that transfection of a *fos* expression vector into HepG2 cells caused a marked inhibition of protein kinase A-mediated phosphoenolpyruvate carboxykinase gene expression (25).

In this context, it is appropriate to consider the potential physiological significance of LDH-NRE function in relation to LDH isozyme expression. Mammalian tissues express widely differing ratios of LDH A and B subunits under different physiological conditions. Tissue-specific LDH isozyme shifts are seen in different developmental patterns (18, 49), during neoplastic development (14, 22), and in response to phorbol ester (33), epidermal growth factor (50), 17β-estradiol (57), and catecholamine (11, 37) stimulation, indicating that the selective tissue-specific regulation of the expression of the LDH isozyme genes is complex. Our previous work has established that the LDH B subunit gene is not subject to regulation by cAMP and lacks the CRE as well as LDH-NRE modules (11, 63). Thus, hormonal fine tuning of the cellular LDH A/B subunit ratios may, at least in part, be achieved by the interplay between the CRE and LDH-NRE modules, thus representing an underlying mechanism for tissue-specific regulation of the LDH A gene. In similar studies, Ishiguro et al. (35), examining the neuron-specific expression of the human dopamine β-hydroxylase gene, have proposed an important role for the interaction between the CRE and a neuron-specific silencer.

It is important to point out that the silencer element apparently does not affect to any degree the cAMP-responsive expression of the endogenous LDH A gene in rat C6 glioma cells. At present, the reason for this is not clear. Using Chinese hamster ovary cells as the transfection host, Hou and Li (32) examined the cAMP-responsive expression of a 2.4-kb mouse LDH A genomic fragment, including 1,117 bases of 5′ upstream promoter and about 1,200 bases of transcribed downstream sequences. The authors reported an approximately 3.8-fold stimulation of CAT activity by 8-bromo-cAMP. Since the 5′-flanking region of the mouse LDH-CAT chimeric construct used by Hou and Li is similar to the rat sequence used in our experiments, the apparent absence of inhibitory activity in the 2.4-kb mouse LDH-CAT construct indicates the presence of as yet unidentified regulatory elements in the downstream LDH A genomic sequences which could functionally interact with the NRE. It is clear that further studies are needed to resolve this issue.

ACKNOWLEDGMENTS

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