Analysis of the rat lactate dehydrogenase A subunit gene promoter/regulatory region

Marc L. SHORT,*§ Delai HUANG,* Deborah M. MILKOWSKI,† Sabine SHORT,* Kevin KUNSTMAN,* Chu-Jing SOONG,‡ Kwang C. CHUNG* and Richard A. JUNGSMANN*||

*Department of Cellular, Molecular, and Structural Biology and Cancer Center and †Department of Medicine, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

The rat lactate dehydrogenase (LDH) A subunit gene promoter contains a putative AP-1 binding site at –295/–289 bp, two consensus Sp1 binding sites at –141/–136 bp and –103/–98 bp, and a single copy of a consensus cyclic AMP-responsive element (CRE) at –48 to –41 bp upstream of the transcription initiation site. Additionally, an as yet unidentified silencer element is located within the –1173/–830 bp 5'-flanking region. Transient transfection analyses of a –1173/+25 bp LDH A-chloramphenicol acetyltransferase fusion gene has indicated a complete inability of the promoter fragment to direct basal or forskolin-induced transcription. Deletion of the –1173/–830 bp sequence restored basal and cyclic AMP (cAMP)-inducible activity. Point mutations in the Sp1 binding sites of a –830/+25 bp promoter fragment reduced basal but not the relative degree of cAMP-inducible activity. cAMP-regulated transcriptional

activity was dependent upon an 8 bp CRE, –TGACGTCA–, located at the –48/–41 bp upstream region. Mutations in the CRE abolished cAMP-mediated induction and reduced basal activity by about 65%. The CRE binds a 47 kDa protein which has previously been identified as CRE binding protein (CREB)-327, an isoform of the activating transcription factor/CREB transcription factor gene family. Co-transfection of a vector that expresses the catalytic subunit of cAMP-dependent protein kinase stimulates LDH A subunit promoter activity suggesting that cAMP induces LDH A subunit gene expression through phosphorylative modification of CREB-327. This study emphasizes a fundamental role of several modules including Sp1 and CREB binding sites in regulating basal and cAMP-mediated transcriptional activity of the LDH A gene.

INTRODUCTION

The lactate dehydrogenase (LDH; EC 1.1.1.27) genes offer interesting and useful systems to study the developmental tissue-specific, as well as hormonal regulation of the metabolically important LDH isozymes. Analysis of LDH isozymes in various cell types shows widely differing ratios of LDH A and B subunit patterns indicating their selective tissue-specific expression under a variety of physiological conditions such as oogenesis [1], oestrogen stimulation [2], and neoplastic development [3]. The expression of an additional LDH isozyme, LDH C, is restricted to sperm cells and is not found in somatic cells [4]. A novel aspect of LDH A function is suggested by findings that LDH A may play a role in the regulation of gene transcription and/or DNA replication. For instance, LDH A subunit is present in the nucleus [5] and is identical with a previously studied single-stranded DNA-binding protein [6]. LDH-5 isozyme, consisting only of A subunits, can stimulate the activity of DNA polymerase *in vitro* [7] and thus may play a role in DNA replication.

In recent years, studies concerned with regulation of the LDH A subunit have shown that it is controlled by several effector agents. Epidermal growth factor [8], 17 β -oestradiol [9], catecholamines [10], and phorbol ester (D. Huang and R. A. Jungmann, unpublished work) are able to induce LDH A subunit expression by mostly undefined mechanisms, and it is likely that additional

effector agents may participate in LDH A regulation. Our laboratory has identified two basic mechanisms of LDH A subunit control in response to effector agents that activate the cyclic AMP (cAMP) signal transduction pathway [10]. Firstly, catecholamines, through activation of β -adrenergic receptors and generation of cAMP, achieve LDH A subunit regulation at the transcriptional level. Secondly, the half-life of LDH A subunit A mRNA is markedly increased after cAMP stimulation.

All of the above findings suggest that different modes of gene control are likely to operate between components of the LDH isozyme systems, thereby making it necessary for higher levels of control to operate at the gene level for the coordinate regulation of specific LDH isozyme patterns in different tissues. Based on our previous data, it is reasonable to postulate that the LDH A subunit gene is one of an increasing number of genes that are regulated by cAMP and share several common characteristics.

(a) Structural and functional analyses of the promoters of cAMP-inducible genes have led to the identification of a *cis*-regulatory sequence referred to as cAMP-regulatory element (CRE) with a consensus octamer motif 5'-TGACGTCA-3' [11]. As a general rule, the CRE appears to be required for maintaining the basal rate of transcription as well as attenuation of the level of induction by cAMP.

(b) CREs have the properties of enhancers, that is they regulate transcription from promoters in a manner independent

Abbreviations used: ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; cAMP, cyclic AMP; catalytic subunit, catalytic subunit of cAMP-dependent protein kinase; CRE, cAMP-responsive element; CREB, CRE binding protein; dibutyl cAMP, N⁶,O^{2'}-dibutyl cAMP; LDH, lactate dehydrogenase; LS, linker scanning; PCR, polymerase chain reaction; protein kinase A, cAMP-dependent protein kinase; Sp-8-CPT-cAMPS, Sp-8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphorothioate.

‡ Present address: VA Medical Center, 10701 East Boulevard, Cleveland, OH 44106, U.S.A.

§ Present address: Genetisches Institut der Justus-Liebig-Universität, W-6300 Giessen, Germany.

|| To whom correspondence should be addressed.

of orientation and available evidence supports a dual role for the CRE as both a basal and inducible enhancer.

(c) Mammalian CREs interact with a group of closely related transcription factor isoforms referred to as either activating transcription factor (ATF) or CRE binding protein (CREB) [12].

In this study, we have analysed the structure of the rat LDH A promoter and functionally characterized the 5'-flanking regulatory/promoter region by using chimaeric expression vectors transfected in cells which express the LDH A subunit. In addition to a promoter region with silencer activity, we identified discrete promoter elements including a CRE and two Sp1-binding sites capable of transcriptional regulation of the LDH A promoter.

MATERIALS AND METHODS

Materials

Avian myeloblastosis virus reverse transcriptase, DNA-modifying enzymes, and acrylamide and deoxyribonucleoside triphosphates were from Boehringer Mannheim. Other reagents were of molecular biology grade and purchased from Sigma. Plasmid pMT-C α EV was a gift from Dr. G. S. McKnight.

Cell culture and preparation of nuclear extract

COS-1 cells, ATCC No. CRL 1650 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum. Rat C6 glioma cells (ATCC no. CCL 107) were maintained in Ham's F-10 nutrient medium (GIBCO) supplemented with 10% (v/v) dialysed foetal calf serum, 50 i.u./ml penicillin, and 50 μ g/ml streptomycin as described [13]. Nuclear extracts were prepared as described previously [14].

Synthesis of oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer using the phosphoramidite method [15]. The fragments were purified, annealed and labelled as described [16].

Construction of promoter-CAT fusion genes

The rat LDH A subunit promoter fragment (–1173/+25 bp) was synthesized by PCR using rat liver genomic DNA as template and the TA Cloning System with the pCRII vector (Invitrogen). Primers were constructed based on the published sequence of the mouse LDH A subunit gene [17]. The 5' and 3' primers were nucleotides –1173/–1152 and +25/+43, respectively, of the mouse sequence. Additional 5' primers were nucleotides –717/–705 and –295/–285 which were used to obtain additional partial fragments of the promoter for sequence analysis.

Plasmid pLDH(–1173/+25)chloramphenicol acetyltransferase (CAT) was constructed in the pCAT-Basic Vector (pSV0-CAT) (Promega). An *EcoRI*/*EcoRI* fragment from pCR II containing the LDH promoter was blunt-ended and ligated into a blunt-ended *HindIII*/*Sall*-restricted pSV0-CAT Basic vector. This construct contained 1173 bp of the 5'-flanking sequence of the rat LDH A gene, the transcription initiation site, and the first 25 bases of transcribed sequence of the LDH A gene. The deletion mutant pLDH(–830/+25)CAT was constructed by ligating an *EcoRI*/*PstI*-restricted promoter fragment into *PstI*-

restricted pSV0-CAT, followed by blunt end ligation. The deletion mutant pLDH(–637/+25)CAT was constructed similarly by ligating an *EcoRI*/*XbaI*-restricted promoter fragment into *XbaI*-restricted pSV0-CAT.

Plasmids pLDH(–159/+2)CAT, pLDH(–111/+2)CAT, pLDH(–80/+2)CAT, and pSV0-CAT with different 60 bp linker-scanning mutants (between –58/+2 bp) were constructed as follows. Synthetic LDH promoter fragments (from positions –159, –111 and –80, –58 to +2 bp) or 60 bp LDH linker-scanning mutants (from –58 to +2 bp) with 10-mer 5' and 3' *HindIII* linkers were inserted into the unique *HindIII* site of pSV0-CAT. pLDH(–159/+2)CAT with a mutated CRE (TAG ATC AT) or mutated Sp1 sites (CCACCC) (mutated bases are indicated in bold print) were constructed by oligonucleotide-directed mutagenesis. The sequence and correct orientation of all inserts were determined by restriction and sequence analyses. Sequencing was carried out in both directions with the dideoxynucleotide chain terminator method using specific synthetic oligonucleotides as primers.

DNA transfections and CAT assay

COS-1 cells (1×10^6) in 5 ml of culture medium were added to 60 mm² dishes. After reaching 80% confluence, the medium was replaced with 4 ml of serum-free medium containing 40 μ g of Lipofectin (GIBCO/BRL). After incubation for 1 h at 37 °C, varying amounts of CAT-constructs were added, and incubation was continued for 18 h. Because serum, at concentrations between 0.5 and 30%, was found to stimulate LDH A-CAT expression in a near linear fashion, the contribution of serum to CAT activity was minimized by reducing the serum concentration to 0.5%. Therefore, to stimulate cells medium was replaced with fresh medium containing 0.5% serum (0.25% foetal calf serum and 0.25% newborn calf serum) either without or with forskolin (10 μ M final concentration) and incubated for an additional 48 h prior to harvesting. Plasmid pCH110, which contains the *Escherichia coli* β -galactosidase gene driven by the simian virus 40 promoter, was used as internal control to determine transfection efficiency.

Enzyme assays

β -Galactosidase was assayed as described [18]. In initial experiments, CAT was assayed by incubating total cellular protein in a buffer containing 250 mM Tris, pH 7.8, 4 mM acetyl coenzyme A and 0.1 μ Ci of [¹⁴C]chloramphenicol (specific radioactivity 52 mCi/mol). All measurements were carried out within the linear range of the assays. CAT activity, expressed as the percentage of [¹⁴C]chloramphenicol converted to acetylated derivatives (% acetylation/ μ g of protein), was normalized to β -galactosidase activity (expressed as A_{420} / μ g of protein), to give a final value of % acetylation/units of β -galactosidase. In subsequent assays, the method of Neumann et al. [19] was used, substituting [1-¹⁴C]butyryl coenzyme A as acyl donor for radiolabelled acetyl coenzyme A as described in the Technical Information bulletin E94768 from Dupont/NEN. Accordingly, CAT activity was assayed by incubating cellular protein or CAT as standard in 100 mM Tris buffer, pH 7.8, 1.25 mM chloramphenicol/0.1 μ Ci of [1-¹⁴C]butyryl coenzyme A (specific radioactivity 4 mCi/mmol). The reaction mixture was overlaid with 5 ml of Econofluor and incubated at room temperature. At timed intervals, ¹⁴C radioactivity was determined in a liquid scintillation counter. CAT activity of each sample was plotted as a function of time and determined on the linear range of a standard curve. The extent of conversion of chloramphenicol to butyrylated

chloramphenicol was between 2.5 and 26%. Each transfection experiment was performed in triplicate.

Gel retardation and Southwestern blotting assays

The procedures were carried out as previously described by us [16].

DNAase I protection assay

A 120 bp *HindIII*/*NcoI* LDH promoter restriction fragment (prepared from pUC12_{LDH-159}) was end-labelled at the *HindIII* site with Klenow fragment and [α -³²P]dATP to obtain 3'-labelling, or [α -³²P]dCTP to obtain 5'-labelling. Various amounts of nuclear protein extracts were mixed with 2 μ g of poly(dI-dC), 10 mM Tris, pH 7.9, 60 mM NaCl/6.6 mM MgCl₂ and ~10000 c.p.m. of ³²P-labelled DNA in a 50 μ l reaction volume. Protein-DNA complex formation was carried out for 30 min at room temperature, after which 50 μ l of 5 mM CaCl₂/10 mM MgCl₂ was added. DNAase I [dissolved in 0.15 M NaCl/50% (v/v) glycerol] was diluted 1:200 (v/v) in water and added to the footprint reaction in 2–8 μ l aliquots containing 5–15 ng of DNAase I. The reaction was incubated for 60 s and terminated by adding 1 vol. of stop buffer (20 mM EDTA/200 mM NaCl/200 μ g/ml tRNA/1% SDS). DNA was isolated from the mixtures by phenol/chloroform and chloroform extractions followed by ethanol precipitation. The samples were dissolved in 99% formamide, heated for 3 min at 90 °C, and resolved on 12% (w/v) polyacrylamide sequencing gels.

Methylation protection assay

Methylation protection assays were carried out according to the guidelines given by Sambrook et al. [18]. Binding reactions were set up with either 5'- or 3'-labelled LDH promoter fragments (~50000 c.p.m.) as for DNAase footprinting. The labelled DNA was partially methylated at G residues using dimethyl sulphate. Free and protein-bound DNA were separated on a preparative gel. Equal Cherenkov counts from bound and free samples were run side-by-side on a 20% (w/v) acrylamide/urea sequencing gel. Radioactivity was determined by autoradiography.

RESULTS AND DISCUSSION

Sequence analysis of the 5'-flanking region of the LDH A subunit gene promoter

To study LDH A subunit gene regulation and to identify potential regulatory domains, a rat LDH A promoter sequence was constructed using PCR methodology and primers derived from a mouse LDH A genomic sequence [17]. Several PCR reactions were run and three independent clones were used for sequence determination. We find, with minor insignificant exceptions, that the rat promoter nucleotide sequence (–1173/+25 bp) is similar to the mouse LDH A subunit promoter sequence. Computer-assisted analysis of the 5'-flanking nucleotide sequence suggests the presence of several potential regulatory elements, e.g. an AP-1-like element at –295/–289 bp with a non-canonical AP-1 binding motif (–TGAGTCT–), two G-C boxes (Sp1-binding sites) at –141/–136 and –103/–98 bp (5'-CCGCCCC-3'), a CRE at –48/–41 bp (5'-TGACGTCA-3'), and a TATA box. The proximity of the CRE to the TATA box is similar to that found in several other cAMP-inducible promoters, including the soma-

tostatin, gonadotropin- α , tyrosine hydroxylase, proenkephalin, vasoactive intestinal polypeptide and several adenovirus promoters [20–23]. No other regulatory motifs could be identified by computer analysis. The rat LDH A promoter nucleotide sequence has been deposited in the GenBank/EMBL Data Bank with accession no. UO 5674.

Successive deletions of the LDH (–1173/+25 bp) promoter result in an increase of transcriptional activity

To define the sequences necessary for promoter activity, we initially subcloned the –1173/+25 bp fragment into the promoterless vector pSVO-CAT and generated the construct pLDH(–1173/+25)CAT to be analysed by transient transfection assay. Although our previous work on LDH gene regulation was done in rat C6 glioma cells [10], these cells turned out to be a very inefficient transfection host. Among other host cells examined, COS-1 cells, which also express cAMP-regulated LDH A, were chosen for the transient transfection experiments. These cells allowed for 11–15-fold higher transfection efficiencies than glioma cells. After transfection of the largest promoter fragment [pLDH(–1173/+25)CAT], we were unexpectedly unable to detect any CAT activity (see Figure 1). This finding was consistent in untreated cells and in cells treated with forskolin or various cAMP analogues such as 8-bromo-cAMP, *N*⁸,*O*²-dibutyrylcAMP (dibutyryl-cAMP), or Sp-8-(4-chlorophenylthio)-adenosine 3',5-cyclic monophosphorothioate (Sp-8-CPT-AMPS) (results not shown).

To elucidate the reason for the failure to demonstrate promoter activity with pLDH(–1173/+25)CAT, we constructed LDH promoter-CAT fusion genes containing progressive 5' promoter deletions whose CAT activities were determined after transfection into COS-1 cells. The results in Figure 1 show the deletion of the –1173 to –830 bp promoter region not only resulted in increased basal CAT expression but also in a significant stimulation of transcriptional activity by forskolin suggesting that the deleted nucleotide sequence interferes with both basal and inducible transcription regulation.

One explanation for the increase of basal and inducible CAT transcription may be the deletion of a silencer region within the

Figure 1 Expression of CAT gene under the control of different fragments from the LDH A subunit 5'-flanking region

Plasmid constructs with the CAT gene under the control of the LDH A subunit promoter fragments –1173/+25, –830/+25, –637/+25, –159/+2, –111/+2, –80/+2 and –58/+2 bp were tested for CAT activity by transfection into COS-1 cells in the presence of 0.5% serum (0.25% foetal calf serum and 0.25% newborn calf serum). Relative CAT activity values represent CAT/ β -gal activity ratios relative to the basal activity of construct –159/+2 bp set at a value of 100 (3.5% conversion of chloramphenicol to butyrate chloramphenicol). Data represent mean $\pm$ S.E. ($n = 3$) Open columns, assays carried out in the absence of forskolin; striped columns, assays carried out in the presence of forskolin.

–1773/–830 nucleotide sequence of the promoter fragment. Indeed, the data presented here and supported by detailed data to be reported elsewhere (K. C. Chung, and R. A. Jungmann, unpublished work) indicate the presence of an as yet unknown inhibitory *cis/trans*-regulatory module in the upstream LDH A promoter fragment. Inhibitory sequences have been identified in other hormonally regulated mammalian promoters, including human aromatase [24], rat prolactin [25], human parathyroid hormone [26], rat growth hormone [27], and others (for review see [28]).

A smaller LDH promoter fragment, the 159/+2 bp sequence, responded to forskolin stimulation with a maximal 3.5-fold increase of CAT activity. Its basal CAT activity, similar to the basal activities of the –830/+25 and –637/+25 bp fragments, was assigned a relative value of 100% (after correction for protein and β -galactosidase activity) to which all other CAT values were correlated. Deletion of the –637 to –159 bp fragment, containing the putative AP-1 element at –295/–289 bp, did not markedly affect basal or forskolin-inducible CAT activity of the resulting –159/+2 bp promoter fragment. In data not shown here we were able to identify the AP-1-binding site as a phorbol ester-responsive element (D. Huang and R. Jungmann, unpublished work). Deletion of the –159/–111 bp fragment resulted in an ~30% decrease of the basal activity as compared with the –159/+2 bp fragment, probably due to the elimination of one of the Sp 1 sites at –141/–136 bp (see also Table 3).

Hou and Li [29] have previously examined the basal and cAMP-responsive expression of a 2.4 kb mouse LDH A genomic fragment consisting of 1117 bases of 5'-upstream promoter and about 1200 bases transcribed downstream sequences. Although the authors did not list the absolute CAT conversion values for basal activity, they reported an approximate 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 may indicate the presence of regulatory regions in the downstream LDH A genomic sequences which could interact with 5'-flanking regions to suppress the inhibitory effect seen in our experiments.

The CRE is an essential positive regulatory element of the LDH A subunit gene

The –58/+2 bp region of the LDH A promoter contains no identifiable *cis*-acting motif other than the CRE (–48/–41 bp) and putative TATA box (–27/–22 bp). To test the functional integrity of the CRE, we systematically mutated the LDH –58/+2 bp promoter fragment to obtain linker scanning (LS) mutants in which the spatial and contextual surroundings of the promoter region were maintained. The ability of the LS mutants to express basal and forskolin-stimulated CAT activity was compared with that of the wild-type LDH A promoter fragment. As shown in Figure 2, LS mutant –46/–41 bp, containing a mutated CRE region, lost its ability to respond to forskolin but retained basal promoter activity [~30% of the basal activity of pLDH (–159/+2)CAT].

The retention of basal promoter activity of the LS CRE mutant appeared to be in contrast to previous findings indicating not only a loss of cAMP inducibility but also a reduction of basal promoter activity upon mutation of the CRE [30–36]. As the –58/+2 bp and LS mutants –46/–41 bp consisted of severely truncated promoter fragments and in consideration that CRE function is markedly influenced by the structural context of

Figure 2 Expression of CAT gene under the control of the wild-type –58/+2 bp LDH A subunit promoter fragment and promoter LS mutants

Plasmid constructs with the CAT gene under the control of the LDH A subunit wild-type (–58/+2 bp) and LS mutant promoter fragments were assayed for CAT activity in COS-1 cells as described in the legend of Figure 1. Relative CAT activity values represent CAT/ β -gal activity ratios relative to the basal activity of construct –58/+2 bp set a value of 100. Data represent means $\pm$ S.E. ($n = 3$). Open columns, assays carried out in the absence of forskolin; striped columns, assays carried out in the presence of forskolin. The double-stranded sequences (from –58 to +2 bp) were constructed from complementary strands which had been synthesized by the phosphoramidite method [15]. Each sequence (shown below) possessed a 3' *Hind*III site and was blunt-ended at the 5' position. The CRE consensus is underlined. Mutated nucleotides are bold-faced. Only partial sequences (–53 to –20 bp) indicating the mutations are shown.

–50	–40	–30	–20	
↓	↓	↓	↓	
CACTCTGACGTCAGCGCGGAGCTTCCATTTAAGG				
CACTCTGACGTCAGCGCGGAGCTT TAG CTTAAGG				
CACTCTGACGTCAGCG GACT TCGTCATTTAAGG				
CACTCT TAACACA AGCGCGGAGCTTCCATTTAAGG				
CAGACT TAACGTC AGCGCGGAGCTTCCATTTAAGG				
				Wild-type
				LS –29/–26
				LS –37/–33
				LS –46/–41
				LS –51/–48

Table 1 Effect of CRE site mutation on CAT expression

Plasmid constructs with the CAT gene under the control of the wild-type –159/+2 bp (wt LDH –159/+2) and CRE-site mutated LDH A promoter fragments (CRE_m LDH –159/+2) were tested for transcriptional activity by transient transfection into COS-1 cells. The CRE site (at –48/–41 bp) was mutated from –TGACGTCA– to –TAGATCAT–. Relative CAT activity values represent CAT/ β -gal activity ratios relative to the basal activity of the wild-type construct –159/+2 bp set at a value of 100. Data represent means $\pm$ S.E. ($n = 3$).

Transfected plasmid	CAT activity		
	– Forskolin	+ Forskolin	-Fold
wt pLDH (–159/+2)CAT	100	310 $\pm$ 35	3.1
CRE _m pLDH(–159/+2)CAT	35 $\pm$ 2.7	41 $\pm$ 6.9	1.2

neighbouring promoter sequences [37], we tested the effect of CRE mutation using the larger –159/+2 bp fragment containing both Sp 1 sites and which expressed maximal basal and forskolin-inducible transcriptional activity. Indeed, mutation of the CRE in pLDH (–159/+2) CAT resulted in a 65% decrease of basal CAT activity and a loss of forskolin inducibility (Table 1).

Overexpression of the catalytic subunit of cAMP-dependent protein kinase (catalytic subunit) A stimulates LDH A subunit promoter activity

It has previously been demonstrated that cAMP-dependent protein kinase A (protein kinase A) appears to be the major

Table 2 Transcription of the LDH A subunit promoter is induced by the catalytic subunit of protein kinase A

Plasmid construct pLDH(−159/+2)CAT (5 µg) was transfected without and with 10 µg of pMT-CαEV, an expression vector that produces the catalytic subunit of protein kinase A [39]. Forskolin (10 µM final concentration) was added 30 h before harvest to increase cAMP levels. Zinc (50 µM) was added 48 h before harvest to activate the metallothioneine promoter and increase expression of the catalytic subunit. Relative CAT activity values represent CAT/β-gal activity ratios relative to the basal activity of pLDH(−159/+2)CAT in the presence of zinc or forskolin set at a value of 100. Data represent means ± S.E. (*n* = 3).

Transfected plasmid	Zn ²⁺	CAT activity	
		− Forskolin	+ Forskolin
pLDH(−159/+2)CAT	(−)	100	410 ± 39
	(+)	106 ± 16	425 ± 29
pLDH(−159/+2)CAT + pMT-CαEV	(−)	210 ± 18	515 ± 38
	(+)	477 ± 29	522 ± 41

mediator of cAMP responses on expression of CRE-regulated mammalian genes [38]. To investigate the effect of overexpression of the catalytic subunit of protein kinase A on LDH A promoter activity, we co-transfected a metallothioneine-regulated promoter expression vector (pMT-CαEV) that produces the α-isoform of the catalytic subunit [39]. As Table 2 illustrates, co-transfection of the catalytic subunit expression vector in the absence of zinc and forskolin induced expression of the CAT reporter gene by 2.1-fold, an effect that was increased to 4.8-fold when catalytic subunit expression was induced via the metallothioneine promoter with zinc. The induction of CAT activity by the catalytic subunit was similar to that produced by forskolin in cells that had not been co-transfected with pMT-CαEV. Furthermore, the addition of forskolin to Zn²⁺-induced cells did not lead to a further significant increase of CAT activity. Therefore, it can be concluded that the effect of overexpressed catalytic subunit was not additive with that produced by forskolin which raises intracellular cAMP levels, indicating that the same signal pathway was operative for both mechanisms of stimulation.

Sp1 sites are essential for maintaining maximal LDH A promoter activity

The -GGGCGG- sequence (or, as in the LDH promoter, in its inverted form -CCGCCC-) is present in the Sp1-responsive promoters and appears to play a critical role in directing efficient transcription from a select group of promoters [40]. Although Sp1 is universally expressed and has often been implicated in the transcription of 'housekeeping' gene promoter, recent evidence suggests that Sp1 expression may be modulated to confer tissue-specific and developmental regulation on target genes [41,42]. As the LDH −58/+2 bp sequence consists of a severely truncated promoter fragment, it is possible that its diminished ability to direct basal CAT expression is due to the deletion of sequences required for maintaining maximal basal activity. Sequence analysis identifies two identical structural motifs, 5'-CCGCCC-3' at −141/−136 and −103/−98 bp, which constitute consensus binding sites for the transcription factor Sp1 [40]. The 70% drop of basal promoter activity of the −58/+2 bp sequence as compared with the larger −159/+2 bp sequence correlates with the deletion of Sp1 sites which are important for promoter activity [43]. Since repeated DNase I protection and methylation

Table 3 Effect of Sp1 site mutation on CAT expression

Plasmid constructs with the CAT gene under the control of the wild-type −159/+2 bp (wt LDH −159/+2) and Sp1-site mutated LDH A promoter fragments (Sp1_m LDH −159/+2) were tested for transcriptional activity by transient transfection into COS-1 cells. Both Sp1 sites (at −141/−136 bp and −103/−98 bp) were mutated from -CCGCCC- to -CCACCC-. Relative CAT activity values represent CAT/β-gal activity ratios relative to the basal activity of construct −159/+2 bp set at a value of 100. Data represent means ± S.E. (*n* = 3).

Transfected plasmid	CAT activity		
	− Forskolin	+ Forskolin	-Fold
wt pLDH(−159/+2)CAT	100	380 ± 25	3.8
Sp1 _m pLDH(−159/+2)CAT	16 ± 2.7	58 ± 6.9	3.6

Figure 3 Electrophoretic band-shift analysis of nuclear protein extracts

(a) The 60 bp wild-type LDH promoter fragment and LS mutants shown in the legend of Figure 2 were used as ³²P-labelled probes. The lanes show the complex formation of the wild-type LDH (lane 1) and LS DNA fragments LS −29/−26 (lane 5); LS −37/−31 (lane 6); LS −45/−41 (lane 7) in the absence (−) and presence of 15 µg of rat C6 glioma cell nuclear protein. A 10-fold (lane 2), 20-fold (lane 3), and 50-fold (lane 4) molar excess of unlabeled LDH wild-type DNA was added to the reaction mixtures containing 0.15 ng of labelled LDH wild-type probe. Two DNA-protein complexes (a and b) are marked with arrows. (b) The 60 bp wild-type LDH promoter fragment was used as ³²P-labelled probe. The following synthetic double-stranded sequences (from −53 to −34 bp) were used as competitors:

−50   −40   −34
 | | |
 CACTCTGACGTCAGCGCGGA   Wild-type
 CACTCTAACACAAGCGCGGA   LS −46/−41

The wild-type and mutated CRE sites are underlined. Mutated nucleotides are bold-faced. The lanes show the complex formation of the 60 bp wild-type LDH fragment in the absence (−) and presence of 15 µg of glioma cell nuclear protein (lane 1). Two DNA-protein complexes (a and b) are marked with arrows. A 10-, 20- and 50-fold molar excess of unlabeled LDH wild-type DNA (lanes 2–4), respectively, and a 50-, 100-, and 200-fold molar excess of LS −46/−41 DNA (lanes 5–7), respectively, was added to the reaction mixtures as competitors.

Figure 4 DNAase I footprinting analysis of nuclear protein-binding sites in the LDH A subunit 5'-flanking region

The LDH $-118/+2$ bp fragment (flanked by *Nco*I and *Hind*III sites) was labelled at the 5' or 3'-site and the DNAase protection assay was carried out as described in the Methods section. Lanes A+G, sequencing reactions. (a) Upper strand: lanes 1, 2 and 3, reactions containing 0, 20, and 40 μ g respectively of rat C6 glioma cell nuclear protein extract; lane 4, binding assay with 40 μ g of nuclear protein carried out in the presence of a 50 molar excess of LS mutant $-46/-41$ (see legend to Figure 2); lane 5, binding assay with 40 μ g of nuclear protein carried out in the presence of a 50-fold molar excess of unlabelled LDH wild-type fragment. (b) lower strand: lanes 1, 2 and 3, reactions containing 0, 20, and 40 μ g respectively of nuclear protein extract. The DNAase I protected regions and nucleotide sequences are indicated.

interference assays using glioma and COS cell nuclear extracts fail to give consistent and unequivocal protection patterns over the putative Sp1 sites, probably due to low concentrations of Sp1, we analysed Sp1 site mutants for their transcriptional activity. Comparison of the CAT activities of wild-type and mutant pLDH($-159/+2$)CAT constructs showed an 84% reduction of basal CAT activity in the Sp1 site mutants (Table 3). Despite the marked reduction of basal transcriptional activity, the mutant promoter retained its ability to respond to forskolin. The strong effect of the mutation in the context of the entire $-159/+2$ bp promoter indicated that the Sp1 sites were essential for maintaining a relatively high level of promoter activity but that active Sp1 sites were not a requirement to achieve cAMP regulation of the LDH A subunit promoter.

Nuclear protein(s) bind specifically to the LDH A subunit CRE

Identification of factor(s) exhibiting binding affinity for and functional interaction with the LDH A subunit CRE would strengthen the results of the transfection assays and help to establish the -48 to -41 bp promoter sequence as a functional cAMP-responsive module. The 60 bp wild-type and LS mutant promoter fragments used above for the *in vivo* functional studies afforded us a set of DNA fragments to determine binding sites of CRE-regulatory protein(s) *in vitro*. As analysed by the gel mobility shift, the ^{32} P-labelled LDH wild-type promoter fragment formed two DNA-protein complexes (a) and (b) (see Figure 3, panel A, lane 1). Radiolabelled LS mutants $-29/-26$ bp (lane 5) and $-37/-31$ bp (lane 6) were also able to form DNA-protein complexes, whereas the ability of LS mutant $-45/-41$ bp to form a protein complex was markedly reduced (lane 7). Competition assays using the 60 bp wild-type promoter fragment as competitor indicated the specificity of complex formation (panel

A, lanes 2–4). Based on the competition experiments it appears that the two DNA/protein complexes (bands a and b) appear to have slightly different binding affinities for the LDH $-58/+2$ bp fragment, with complex (b) showing a higher binding affinity than complex (a).

Because we used relatively large DNA fragments (60 bp), we needed to allow for the possibility that additional binding sites other than the CRE may have formed DNA/protein complexes. To ascertain that protein factor binding was solely due to the CRE site, the competitive ability of two smaller (20 bp) promoter fragments ($-53/-34$ bp), one fragment carrying the wild-type and the other fragment a mutated CRE site, were evaluated. The results are shown in Figure 3, panel B. The wild-type fragment effectively competed for protein factor binding of the two DNA/protein complexes (lanes 2–4), whereas the fragment with a mutated CRE site was unable to compete even at a 200-fold molar excess (lanes 5–7).

DNAse I footprint analyses of the proximal LDH A 5'-flanking region shows protection of the CRE

The promoter/reporter gene expression experiments described above indicate that a functional CRE is located downstream of position 58. DNAase I protection analysis was used to identify more precisely the site of CRE/protein interaction. A 120 bp ($-118/+2$ bp) fragment of the LDH A promoter was labelled on either the sense strand (-118 bp) or the antisense strand ($+2$ bp) and incubated with varying amounts of nuclear protein extract before partial DNAase I digestion. One major region containing the CRE is protected on the sense strand (from -53 to -33 bp) by rat C6 glioma cell extracts (see Figure 4a). Protection on the antisense strand corresponds very closely (from -55 to -35 bp) to the protected area on the sense strand

(Figure 4b). A 28 bp promoter fragment containing the wt CRE (−58/−12 bp) successfully competes for binding (Fig. 4a, lane 5), whereas the fragment with a mutated CRE (LS −46/−41 bp) has no competitive ability (lane 4). Similar results are obtained with COS-1 cell nuclear extracts (results not shown).

Methylation interference pattern shows sites of contact of protein with the CRE

The above findings are supported by methylation-interference experiments. In these experiments a 120 bp DNA fragment containing the promoter sequences from positions −119 to +1 bp was labelled at the 3' end and methylated randomly by dimethyl sulphate. Dimethyl sulphate methylates N-7 of guanines in the major groove of a DNA double helix, interfering with protein binding at this site. The methylated probe was used in the DNA binding assay. The bound and unbound fragments were extracted from the gel and further processed to produce the sequence ladders. If methylation of guanosine or adenosine residues interfered in factor binding, the bound fraction in the DNA binding assay was enriched only with the molecules that were not methylated within the target sequence. Results of such an experiment are shown in Figure 5. In DNA sequence ladders, those guanosine residues from the DNA fragment isolated from the bound fraction (Figure 5, lane B) and that are involved in factor binding will be absent or much reduced when compared with those from the unbound fraction (Figure 5, lane F). Lane B, corresponding to the bound fraction, shows the guanosine residues at positions −47 and −44 with a much reduced intensity compared with that of the unbound (free) fraction, while the

Figure 5 Methylation interference analysis of nuclear protein binding in the LDH A subunit 5'-flanking region

3'-End-labelled LDH promoter fragment (see legend to Figure 4) was methylated by treatment with dimethyl sulphate as described in the Methods section. Specific glioma cell nuclear protein-DNA complexes were excised from a preparative mobility shift gel, and DNA from free (lane F) and protein-bound (lane B) bands was purified and separated on a denaturing gel following piperidine cleavage of the modified DNA. Solid circles indicate the positions of guanine nucleotides which inhibit protein binding when methylated. Lane A + G, sequencing reaction.

remaining guanosine residues in the sequence ladder remain unaffected.

The above data indicate that G residues at positions −47 and −44 on the coding and −45 on the non-coding strand respectively are involved within the LDH A subunit CRE in binding to a CRE-binding protein. These contact sites are similar to the CRE contact sites in other CRE-regulated promoters [44–46] where binding of CREB/ATF to the site 5'-GACG-3' protects two guanosines on one strand and a third guanosine on the opposite strand from methylation. The finding that this prevalent binding pattern is also found in the LDH A CRE suggests a similar protein/DNA binding mechanism probably involving interaction of protein with guanosine residues in the major groove of the CRE DNA.

Southwestern blotting assays identify a 47 kDa LDH A subunit CRE-binding protein

To characterize nuclear protein(s) that interact with the LDH A subunit CRE, glioma cell nuclear proteins were separated by SDS/PAGE, renatured, blotted onto nitrocellulose, and hybridized with a ^{32}P -labelled oligonucleotide consisting either of the wild-type CRE from the LDH A gene (Figure 6, lane 1) or a sequence in which the CRE octamer was mutant (LS mutant −46/−41) (lane 2). As shown in Figure 6, the ^{32}P -labelled wild-type CRE sequence recognized a limited number of nuclear proteins which among others included a 47 kDa and an approx. 105 kDa protein that lacked binding affinity to the mutated LS −46/−41 probe (see lane 2). After addition of a 100-fold molar excess of LDH A wild-type fragment (−58/+2 bp) as competitor, binding of the radiolabelled probe to the 47 kDa protein and the slowest-migrating 105 kDa protein was effectively competed (lane 3). LS mutant −46/−41 DNA and an AP-1 binding

Figure 6 Southwestern blot analysis of the LDH A subunit DNA binding activity in nuclear protein extracts

Glioma cell nuclear protein extracts (150 $\mu\text{g}/\text{lane}$) were separated electrophoretically on 7.5% SDS/polyacrylamide gels, renatured, and transferred by passive diffusion to nitrocellulose. The filter was probed with either ^{32}P -labelled wild-type LDH (lane 1) or LDH LS mutant −46/−41 (lane 2). For DNA fragments see legend to Figure 2. Competition of protein binding to the ^{32}P -labelled LDH fragment was carried out with a 100-fold molar excess of either wild-type LDH DNA (lane 3), LS mutant −46/−41 DNA (lane 4), or AP1 binding DNA fragment (5'-CTGACTCAGCG-3') (lane 5). The positions of protein molecular-mass markers are shown on the left-hand side of the Figure.

DNA fragment as competitors did not markedly interfere with binding of the wild-type LDH probe to the 47 kDa protein. However, the binding and competition properties of the 105 kDa protein indicated that it had slightly different binding site requirements than the 47 kDa protein. Based on these data, the 47 kDa protein can be identified as possessing specific binding activity for the LDH CRE fragment. Indeed, we were recently able to identify the binding protein as the CREB-327 isoform [47].

Assay of nuclear extracts from various cell types for the 47 kDa CRE-binding protein showed its presence in rat ovary [16], rat liver, rat H4-IIE, COS-1 and HeLa cells (J. Kwast-Welfeld and R. A. Jungmann, unpublished work) which all express the LDH A subunit. We were unable to demonstrate the presence of the 47 kDa LDH CRE-binding protein in nuclear extracts from rat heart (J. Kwast-Welfeld and R. A. Jungmann, unpublished work). Rat heart expresses little or no LDH A subunit. However, failure to demonstrate LDH CRE-binding activity does not necessarily indicate an absence of the 47 kDa protein in rat heart. It may result from a lack of protein(s) which is specifically required for functional complex formation of the 47 kDa binding protein with the CRE of the LDH A promoter.

In conclusion, while attempting to elucidate the molecular mechanisms that determine cell-specific expression and regulation of LDH isozyme patterns, the present studies have identified functional elements within the LDH A promoter, the CRE and Sp1 binding site modules, that are involved in transcriptional regulation of the LDH A gene. It appears that the CRE module is part of a cell and isozyme-specific regulatory element present in the A subunit gene which is not involved, at least not directly, in LDH B subunit regulation. Based on our previous studies [10,13], the LDH B subunit gene is not subject to regulation by cAMP, which agrees with our preliminary structural analysis of the LDH B subunit promoter (R. A. Jungmann, unpublished work) indicating that the promoter lacks a CRE-consensus sequence.

This work was supported in part by grants from the National Institutes of Health (to R.A.J.), by a Nellie Fox Fellowship from the Chicago Baseball Charities (to M.L.S. and D.M.M.), by a Fellowship from the Neuroscience Training Grant T23NS07140 (to K.C.C.), and by the Research and Education Fund, Northwestern University. We thank Dr. G. S. McKnight for providing the catalytic subunit expression plasmid pMT-CαEV.

REFERENCES

- Markert, C. L. and Ursprung, H. (1962) *Dev. Biol.* **5**, 363–366
- Richards, A. H. and Hilf, R. (1972) *Endocrinology* **91**, 287–295
- Lee, C., Oliver, L., Coe, E. L. and Oyasu, R. (1979) *Cancer Res.* **62**, 193–199
- Goldberg, E. (1977) in *Isozymes: Isozymes in Testes and Spermatozoa* (Rattazi, M. C., Scandalios, J. G. and Whitt, G. S., eds.), Vol. 1, pp. 79–124, Alan R. Liss, New York
- Cattaneo, A., Biocca, S., Corvaja, N. and Calissano, P. (1985) *Exp. Cell Res.* **161**, 130–140
- Williams, K. R., Reddigari, S. and Patel, G. L. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5260–5264
- Grosse, F., Nasheuer, H., Scholtissek, S. and Schomburg, U. (1986) *Eur. J. Biochem.* **160**, 459–467
- Matrisian, L. M., Rautmann, G., Magun, B. E. and Breathnach, R. (1985) *Nucleic Acids Res.* **13**, 711–726
- Li, S. S. and Hou, E. W. (1989) *Cell Biol. Intl. Rep.* **13**, 619–624
- Jungmann, R. A., Kelley, D. C., Miles, M. F. and Milkowski, D. M. (1983) *J. Biol. Chem.* **258**, 5312–5318
- Roesler, W. J., Vandenbark, G. R. and Hanson, R. W. (1988) *J. Biol. Chem.* **263**, 9063–9066
- Habener, J. F. (1990) *Mol. Endocrinol.* **4**, 1087–1094
- Derda, D. F., Miles, M. F., Schweppe, J. S. and Jungmann, R. A. (1980) *J. Biol. Chem.* **255**, 11112–11121
- Short, S., Short, M. L., Milkowski, D. M. and Jungmann, R. A. (1991) *J. Biol. Chem.* **266**, 22164–22172
- Matthes, H. W. D., Staub, A. and Chambon, P. (1987) *Methods Enzymol.* **154**, 250–287
- Kwast-Welfeld, J., Soong, C.-J., Short, M. L. and Jungmann, R. A. (1989) *J. Biol. Chem.* **264**, 6941–6947
- Fukasawa, K. M. and Li, S. S.-L. (1986) *Biochem. J.* **235**, 435–439
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Neumann, J. R., Morency, C. A. and Russian, K. O. (1987) *BioTechniques* **5**, 444–448
- Jameson, J. L., Jaffe, R. C., Deutsch, P. J., Albanese, C. and Habener, J. F. (1988) *J. Biol. Chem.* **263**, 9879–9886
- Deutsch, P. J., Hoeffler, J. P., Jameson, J. L., Lin, J. C. and Habener, J. F. (1988) *J. Biol. Chem.* **263**, 18466–18472
- Leza, M. A. and Hearing, P. (1989) *J. Virol.* **63**, 3057–3064
- Fink, J. S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G. and Goodman, R. H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6662–6666
- Wang, J. and Chen, S. (1992) *Mol. Endocrinol.* **6**, 1479–1488
- Zhang, Z.-X., Kumar, V., Rivera, R. T., Chisholm, J. and Biswas, D. K. (1990) *J. Biol. Chem.* **265**, 4785–4788
- Okazaki, T., Zajac, J. D., Igarashi, T., Ogata, E. and Kronenberg, H. M. (1991) *J. Biol. Chem.* **266**, 21903–21910
- Pan, W. T., Liu, Q. and Bancroft, C. (1990) *J. Biol. Chem.* **265**, 7022–7028
- Clark, A. R. and Docherty, K. (1993) *Biochem. J.* **296**, 521–541
- Hou, E. W. and Li, S. S.-L. (1987) *Biochem. Biophys. Res. Commun.* **147**, 501–505
- Deleage, A. M., Ferland, L. H. and Mellon, P. L. (1987) *Mol. Cell. Biol.* **7**, 3994–4002
- Deutsch, P. J., Jameson, J. L. and Habener, J. F. (1987) *J. Biol. Chem.* **262**, 12169–12174
- Andrisani, O. M., Zhu, Z. N., Pot, D. A. and Dixon, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2181–2185
- Lafyatis, R., Lechleider, R., Kim, S.-J., Jakowlew, S., Roberts, A. B. and Sporn, M. B. (1990) *J. Biol. Chem.* **265**, 19128–19136
- Quinn, P. G., Wong, T., Magnuson, M. A., Shabb, J. B. and Granner, D. K. (1988) *Mol. Cell. Biol.* **8**, 3467–3475
- Kim, K.-S., Lee, M. K., Carroll, J. and Joh, T. H. (1993) *J. Biol. Chem.* **268**, 15689–15695
- Abrahamsen, M. S., Li, R.-S., Dietrich-Goetz, W. and Morris, D. R. (1992) *J. Biol. Chem.* **267**, 18866–18873
- Jameson, J. L., Powers, A. C., Gallagher, G. D. and Habener, J. F. (1989) *Mol. Endocrinol.* **3**, 763–772
- Mellon, P. L., Clegg, C. H., Correll, L. A. and McKnight, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4887–4891
- Uhler, M. and McKnight, G. S. (1987) *J. Biol. Chem.* **262**, 15202–15207
- Kadonaga, J. T., Jones, K. A. and Tijan, R. (1986) *Trends Biol. Sci.* **11**, 20–23
- Dynan, W. S. and Tijan, R. (1983) *Cell* **35**, 79–87
- Chen, H.-M., Pahl, H. L., Scheibe, R. J., Zhang, D.-E. and Tene, D. G. (1993) *J. Biol. Chem.* **268**, 8230–8239
- Araki, E., Murakami, T., Shirotani, T., Kanai, F., Shinohara, Y., Shimada, F., Mori, M., Shichiri, M. and Ebina, Y. (1991) *J. Biol. Chem.* **266**, 3944–3948
- Lin, Y.-S. and Green, M. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3396–3400
- SivaRaman, L., Subramanian, S. and Thimmappaya, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5914–5918
- Gilman, M. Z., Wilson, R. N. and Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 4305–4316
- Short, M. L., Manohar, C. F., Furtado, M. R., Ghadge, G. D., Wolinski, S. M., Thimmappaya, B. and Jungmann, R. A. (1991) *Nucleic Acids Res.* **19**, 4290