5-Aminolaevulinate synthase gene promoter contains two cAMP-response element (CRE)-like sites that confer positive and negative responsiveness to CRE-binding protein (CREB)

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The first and rate-controlling step of the haem biosynthetic pathway in mammals and fungi is catalysed by the mitochondrial-matrix enzyme 5-aminolaevulinate synthase (ALAS). The purpose of this work was to explore the molecular mechanisms involved in the cAMP regulation of rat housekeeping ALAS gene expression. Thus we have examined the ALAS promoter for putative transcription-factor-binding sites that may regulate transcription in a cAMP-dependent protein kinase (PKA)-induced context. Applying both transient transfection assays with a chloramphenicol acetyltransferase reporter gene driven by progressive ALAS promoter deletions in HepG2, and electrophoresis mobility-shift assays we have identified two putative cAMP-response elements (CREs) at positions –38 and –142. Functional analysis showed that both CRE-like sites were necessary for complete PKA induction, but only one for basal expression. Co-transfection with a CRE-binding protein (CREB) expression vector increased PKA-mediated induction of ALAS promoter transcriptional activity. However, in the absence

of co-transfected PKA, CREB worked as a specific repressor for ALAS promoter activity. A CREB mutant deficient in a PKA phosphorylation site was unable to induce expression of the ALAS gene but could inhibit non-stimulated promoter activity. Furthermore, a DNA-binding mutant of CREB did not interfere with ALAS promoter basal activity. Site-directed-mutagenesis studies showed that only the nearest element to the transcription start site was able to inhibit the activity of the promoter. Therefore, we conclude that CREB, through its binding to CRE-like sites, mediates the effect of cAMP on ALAS gene expression. Moreover, we propose that CREB could also act as a repressor of ALAS transcription, but is able to reverse its role after PKA activation. Dephosphorylated CREB would interfere in a spatial-disposition-dependent manner with the transcriptional machinery driving inhibition of gene expression.

Key words: gene expression, haem biosynthesis, hepatic cell, protein kinase A, transcription factor.

INTRODUCTION

Haem is an essential molecule for all eukaryotic cells because of its role as a prosthetic group for a number of proteins. It is synthesized in animal cells from succinyl-CoA and glycine by a sequence of reactions catalysed by eight enzymes. The first and rate-controlling step of this pathway is catalysed by the mitochondrial matrix enzyme 5-aminolaevulinate synthase (ALAS) [1–3]. There are two related isoenzymes of ALAS, which are encoded by two separate genes located on different chromosomes [4,5]. One of the genes encodes an isoenzyme that is expressed exclusively in erythroid cells; this gene is activated during erythropoiesis to provide the large amounts of haem needed during haemoglobin production [6,7]. The second ALAS gene encodes a housekeeping isoenzyme that is expressed in all tissues to provide haem for cytochromes and other haemoproteins [8].

Expression of housekeeping ALAS in the liver was found to be subject to feedback regulation by haem. Its rate of transcription is greatly increased in animals following administration of foreign chemicals such as phenobarbital [5,9]. This induction probably meets the increased demand for haem by induced hepatic cytochrome P450 apoprotein [10,11]. However, it has been suggested that these drugs would act directly by increasing the transcription rate of ALAS gene [12]. In addition to this major mechanism of regulation, we have demonstrated that other factors, like cAMP and phorbol esters, modulate the expression

of liver ALAS through cAMP-dependent protein kinase (PKA) and protein kinase C activation, respectively [13,14].

cAMP mediates the hormonal stimulation of a number of eukaryotic genes through a conserved cAMP-response element (CRE). CREs were identified in several gene promoters, and generally consist of small variations in the sequence TGACGTCA [15]. This element is recognized by the transcription factor CRE-binding protein (CREB) [16,17]. CREB activity, in turn, is regulated by PKA, which phosphorylates CREB at a single phosphoacceptor site Ser-133 [18]. As Ser-133 phosphorylation specifically stimulates the transcriptional activity of CREB without affecting its DNA-binding-protein properties, it has been proposed that such phosphorylation induces a structural change in the molecule that subsequently alters its interaction with CREB-binding protein (CBP) and other proteins in the RNA polymerase II complex [19,20]. In recent years, a whole family of transcription factors, the CREB/activating transcription factor (ATF) family, was reported to mediate cAMP responsiveness in different genes. These CREB/ATF proteins are members of the larger basic-leucine zipper (bZIP) family of transcription factors [15]. Although CREB is mostly described as a positive transcription factor, several reports show that it can also inhibit the transcriptional activity of diverse promoters. Ofir et al. [21] demonstrated that CREB inhibits transcription of the *c-fos* promoter, and that this negative regulation is relieved by phosphorylation. Also, dephosphorylated CREB inhibits

Abbreviations used: ALAS, 5-aminolaevulinate synthase; CRE, cAMP-response element; CREB, CRE-binding protein; PKA, cAMP-dependent protein kinase; CAT, chloramphenicol acetyltransferase; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; ATF, activating transcription factor.

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expression of somatostatin gene by competing with C/EBP (CCAAT-enhancer-binding protein) for binding to CRE [22]. On the other hand, Lemaigre et al. [23] showed that CREB inhibits transcription of multiple activators, whose DNA-binding domains and activation regions are unrelated to one another. Inhibition requires CREB dimerization and DNA-binding domains to be intact.

The structure of the 5'-upstream region of housekeeping ALAS gene from rat liver has been reported by Braidotti et al. [24] and Yomogida et al. [25]. Computer-aided analysis of the ALAS promoter [26] revealed three highly homologous consensus CREs located at positions -261 to -254, -142 to -149 and -38 to -45 as putative *cis*-acting elements in the promoter. In a recent work, we have demonstrated that PKA increases ALAS gene expression [27]. The aim of the present study was to establish the main regulatory elements and transcription factors that determine cAMP responsiveness in rat ALAS gene. Our results show the presence of two functional binding sites for the transcription factor CREB in the proximal ALAS promoter. Co-transfection with a CREB expression vector increases PKA-mediated induction of transcriptional activity of the ALAS promoter in HepG2 cells. However, in the absence of co-transfected PKA, CREB works as a specific repressor of ALAS promoter basal activity. A CREB mutant deficient in PKA phosphorylation site is unable to induce expression of ALAS gene but can inhibit unstimulated promoter activity. Furthermore, a DNA-binding mutant of CREB does not interfere with ALAS promoter basal activity. Both CRE-like elements, located at -142 and -38, are necessary for complete PKA induction, although the upstream site seems to be the more effective. However, only the nearest element to the transcription start site is able to inhibit promoter activity. In view of the agreement between physical and functional analysis, we conclude that CREB, through CRE-like sites, mediates the effects of cAMP on ALAS gene expression. We propose that CREB could also act as a repressor of ALAS transcription, but that it is able to reverse its role after PKA activation.

EXPERIMENTAL

Materials

Minimum essential medium, Dulbecco's modified Eagle's medium (high glucose), 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP), forskolin, agarose, chloramphenicol, butyryl-CoA and *o*-nitrophenyl β -D-galactopyranoside were purchased from Sigma (St. Louis, MO, U.S.A.). [ring-3,5- 3 H]Chloramphenicol (specific radioactivity 1.1–2.2 GBq/mmol), [γ - 32 P]ATP (specific radioactivity 222 TBq/mmol) and [α - 32 P]dCTP (specific radioactivity 111 TBq/mmol) were purchased from New England Nuclear-Dupont (Wilmington, DE, U.S.A.). Random primers kit, restriction endonucleases and DNA-modifying enzymes were from New England Biolabs. All other chemicals were of analytical grade. The oligodeoxynucleotides were synthesized chemically by Bio-Synthesis (Lewisville, TX, U.S.A.).

Plasmids

The plasmid p-833ALAS/CAT contains the ALAS -833 to +42 sequence cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene [27]. The deletion-mutant plasmids p-459ALAS/CAT, p-354ALAS/CAT, p-156ALAS/CAT, p-75ALAS/CAT, p-38ALAS/CAT and p-110/-38ALAS/CAT were generated by digestion of pACAT with *Pst*I, *Afl*II/*Bam*HI, *Stu*I/*Bam*HI, *Bss*HII/*Bam*HI, *Bst*EII/*Bam*HI and *Bst*EII/*Ppu*MI, respectively. If necessary, gel-eluted plasmids were treated

with T_4 DNA polymerase to generate blunt ends, prior to ligation. Mutated plasmids were generated by site-directed mutagenesis using Gene Editor *in vitro* Site-directed Mutagenesis System from Promega (Madison, WI, U.S.A.). The CRE-like site at -142 was mutated from wild-type TGACTATG (non-coding) to TGTGTATG (non-coding). The CRE-like site at -38 was mutated from wild-type TGACCGCT (non-coding) to TGT-GCGCT (non-coding).

Plasmid pMtC α is a 5.4 kb expression vector plasmid containing the mouse metallothionein-1 promoter cloned on to the cDNA for the α isoform of the mouse PKA catalytic subunit [28]. Expression vector for the wild-type CREB (pCREB3) [18] and reporter plasmids p4xCRE/CAT and p4xCRELUC were provided kindly by Dr P. Sassone-Corsi (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Expression vectors for negative mutants of CREB, pCREB M1 [18] and pCREB K [29] were a generous gift of Dr M. E. Greenberg (Harvard Medical School, Boston, MA, U.S.A.). Plasmid pRSV β gal contains the β -galactosidase gene under the control of the Roux sarcoma virus promoter. To perform transfection assays, plasmids were purified with the Maxipreps Wizard kit (Promega), and the DNA concentration was estimated spectrophotometrically.

HepG2 and F9 cell cultures and transfections

The human hepatoma cell line HepG2 was grown in minimum essential medium supplemented with 10% (v/v) fetal calf serum. The rat teratocarcinoma cell line F9 was grown in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) fetal calf serum in 0.1% (w/v) gelatin-coated dishes. Both cell lines were grown as monolayer cultures and maintained at 37 °C with 5% CO $_2$. Cell transient transfection was performed according to the standard calcium phosphate precipitation method as described previously [27].

Each well was transfected with a mix containing 4 μ g of pACAT or its derivatives or reporter plasmid p4xCRE/CAT and 6.5 μ g of pRSV β gal. In the experiments designed to over-express catalytic subunit of PKA, cells were co-transfected with 3 μ g of pMtC α . Overexpression of CREB, CREB M1 and CREB K was achieved by co-transfecting cells with 6 μ g of the respective plasmids. Competition experiments with p4xCRELUC were carried out by co-transfecting the cells with 8 and 16 μ g of this plasmid. The final DNA concentration was adjusted to 30 μ g/35 mm dish with non-specific DNA carrier. Control transfections with carrier alone and carrier plus vector pBLCAT6 were done in parallel. After 16 h, the medium was replaced with 3 ml of serum-free medium, and incubated for 24 h. As indicated in some of the experiments, transfected cells were incubated for 24 h in serum-free medium with 100 μ M 8-CPT-cAMP, 10 μ M forskolin or 10 μ M CREB antisense oligodeoxynucleotide (25 nmol/well). A 20-mer antisense oligodeoxynucleotide, 5'-GCGGAGTGTGTTGGTGTGAGTGAC-3', complementary to bases 9–28 of human CREB mRNA, was designed to block synthesis of the protein.

Analysis of CAT and β -galactosidase activity assays were performed in cell extracts as described previously [27], according to the Seed and Sheen phase-extraction assay for CAT activity [30]. β -Galactosidase activities were expressed as A_{420} $\cdot$ μ g of protein $^{-1}$ $\cdot$ h $^{-1}$. CAT activity was expressed as the amount of radiolabelled chloramphenicol acetylated by 1 mg of protein in 1 min, normalized for equal transfection efficiency with β -galactosidase activity. β -Galactosidase activity was not modified by any of the treatments used. Each series of experiments was

performed at least three times and each transfection was performed in duplicate.

RNA isolation and Northern-blot analysis

Total cellular RNA was isolated from cultured HepG2 cells according to Chomczynski and Sacchi [31]. The yield and purity of RNA samples were assessed by the ratio of absorbance at 260 and 280 nm. For Northern-blot analysis, 20 µg of total RNA were denatured, electrophoresed on 1% glyoxal/agarose gels, and transferred to nylon membranes (Hybond N, Amersham). The membranes were hybridized sequentially with ³²P-labelled probes to human liver ALAS and β-tubulin. To detect ALAS mRNA, a 26-mer oligodeoxynucleotide was synthesized complementary to bases +328 to +353 of human housekeeping ALAS mRNA [32]. The oligodeoxynucleotide was purified and 5'-end-labelled using [γ-³²P]ATP and T₄ polynucleotide kinase (Promega). The resulting probe had a specific activity of about (4–5) × 10⁸ c.p.m./fmol. Hybridization was carried out overnight at 70 °C in the same prehybridization solution by adding the ³²P-labelled oligodeoxynucleotide (3.0 × 10⁵ c.p.m./cm²) as described previously [14]. To detect β-tubulin mRNA, chicken β-tubulin cDNA (a generous gift of Dr J. Messina, University of Birmingham, Birmingham, AL, U.S.A.) was labelled by random priming using [α-³²P]dCTP and Klenow to a specific activity of about 6 × 10⁸ c.p.m./µg. The membranes were prehybridized, hybridized and washed in standard conditions described by Sambrook et al. [33]. Autoradiographs were obtained by exposing these blots to Kodak XAR-5 films with an intensifying screen for 5–7 days at –70 °C. Blot autoradiograms were quantified with a Bio-Rad densitometric scanner model GS-670.

Electrophoretic mobility-shift assay

Nuclear extracts were prepared from confluent HepG2 cell cultures as described by Andrews and Faller [34]. The double-stranded DNA probes and unlabelled competitors used were C1 (putative CRE at –38), 5'-AGCGCATGCGCAGCGGTCA-CCCCGGCTC-3', and C2 (putative CRE at –142), 5'-GCCTAAGACATAGTCACAAGAGTTGGAGC-3', and mutants of C1 and C2, 5'-AGCGCATGCGCAGCGCACACCCCCGGCTC-3' and 5'-GCCTAAGACATACACACAAGAGTTGGAGC-3', called C1m and C2m respectively, in which underlined bases have been mutated to disrupt the specific binding of CREBs. A positive control oligodeoxynucleotide containing a consensus CRE site corresponding to –58 to –31 bp of the rat somatostatin gene promoter, and a negative one containing a Sp1-binding site, were also used. To generate radioactive probes, the sense and antisense oligodeoxynucleotides were heated to 75 °C for 5 min and then cooled slowly over 2 h at room temperature in annealing buffer (10 mM Tris/HCl, pH 7.5/100 mM NaCl/1 mM EDTA). The oligodeoxynucleotides were 5'-end-labelled using T₄ polynucleotide kinase and [γ-³²P]ATP (222 TBq/mmol) at 37 °C for 45 min.

Binding reactions were performed mixing 5 µg of nuclear extract or 1 µg of bacterially produced CREB protein (a generous gift of Dr P. Sassone-Corsi) with 3 µg of poly(dI:dC) and 50000 c.p.m. of the labelled probe in TM buffer (50 mM Tris/HCl, pH 7.9/12.5 mM MgCl₂/1 mM EDTA/1 mM dithiothreitol/20%, v/v, glycerol) in a 20 µl final volume. The reaction was incubated for 20 min at room temperature. When noted, the protein was first incubated for 20 min at room temperature in a binding cocktail with a 100-fold molar excess of unlabelled competitor DNA before the addition of labelled DNA. After the binding reaction, electrophoresis was carried out through a 4% non-denaturing polyacrylamide gel containing 0.25 × TBE

(1 × TBE = 50 mM Tris/borate/1 mM EDTA, pH 8.3). The gel was then dried, and autoradiography was performed.

RESULTS

Elements required for PKA-mediated induction of ALAS promoter activity are located within 156 bp of the transcription-initiation site

To confirm previously observed results from rat hepatocytes demonstrating that cAMP produced transcriptional activation of the ALAS gene [13], we tested the effect of cAMP on the expression of the ALAS gene in HepG2 cells. These cells were incubated with 100 µM 8-CPT-cAMP for up to 16 h or transiently transfected with a vector encoding the catalytic subunit of PKA for different periods up to 24 h. Northern-blot analysis showed that in both cases there was a time-dependent increase in the mRNA levels for ALAS, suggesting that cAMP induced the ALAS gene expression in HepG2 cells (results not shown).

In order to determine whether the ALAS 5'-flanking region can regulate basal and cAMP-mediated induced expression, the proximal 833 bp was placed upstream of the CAT reporter gene, and the resulting plasmid (p-833ALAS/CAT) was transiently transfected into cultured HepG2 cells. As shown in Figure 1, the ALAS 5'-flanking region regulated high levels of CAT expression. We also carried out co-transfection experiments with pMtCα, a vector driving the expression of the PKA catalytic subunit. They resulted in a significant increase in ALAS-CAT expression (Figure 1), similar to that achieved by transfected cells incubated with 8-CPT-cAMP or adenylate cyclase activators like forskolin or norepinephrine (noradrenalin; results not shown). These results demonstrated that the 833 bp 5'-non-coding region of the ALAS gene is enough for constitutive and PKA-mediated induced expression. Then we performed 5' deletion analysis to identify specific sequences within the ALAS promoter that regulate constitutive and cAMP-induced promoter activity. Plasmids containing progressively decreasing lengths of ALAS 5'-flanking region upstream of the CAT reporter gene were transiently transfected into HepG2 cells. As shown in Figure 1, progressive deletion of sequences from –833 to –156 bp did not impair basal or PKA-induced promoter activity. A partial decrease of PKA stimulation was observed when the region between nucleotides –156 and –75 was deleted. Significant differences between levels of induction were obtained (*P* < 0.05). A complete loss of PKA stimulation was detected when nucleotides –75 to –38 were eliminated. The results suggest the presence of two PKA-responsive sites in the proximal 5'-non-coding region of the ALAS gene, located between –156 and –75 bp, and between –75 and –38 bp. On the other hand, there were slight differences in the basal levels of promoter activity among the deletion mutants tested, except for p-38ALAS/CAT, in which CAT activity fell to values 60% lower than those of p-833ALAS/CAT.

Characterization of CRE-like binding sites within the ALAS promoter

The search for consensus recognition patterns in the proximal 156 bp of the 5'-flanking region of the ALAS gene [26] revealed the presence of two CRE-like elements corresponding to nucleotides –142 (core similarity 1.000; matrix similarity 0.932) and –38 (core similarity 1.000; matrix similarity 0.925) of the lower strand that were named C2 and C1, respectively. Electrophoretic mobility-shift assays were undertaken using radiolabelled oligodeoxynucleotides containing C1- or C2-binding sites and incubated with nuclear extracts prepared from HepG2 cells. A

Figure 1 Deletion analysis reveals a region important for PKA-mediated induction of ALAS promoter activity

HepG2 cells were transiently transfected with 4 μ g/plate of p-833ALAS/CAT or equivalent amounts of deletion mutants containing the 5'-flanking region of the ALAS gene, illustrated on the left. Transfections included (grey bars) or did not include (black bars) 3 μ g/plate of expression vector for the PKA catalytic subunit. Results are expressed as relative CAT activity with respect to the basal value for p-833ALAS/CAT, which was set to 1. Fold induction represents the PKA-stimulated reporter activity divided by the unstimulated level. Bars represent means $\pm$ S.E.M. from five independent experiments performed in duplicate. Statistical analysis between p-833ALAS/CAT and mutant reporters applying Student's *t* test revealed some significant differences (**P* < 0.05 between unstimulated reporters; **P* < 0.05 and ***P* < 0.01 between PKA-stimulated reporters; **P* < 0.05 and ***P* < 0.01 between fold inductions).

Figure 2 Putative CRE-like sites form complexes with proteins expressed in HepG2 nuclear extracts

Protein (10 μ g) prepared from extracts of HepG2 cells was incubated with 32 P-labelled probes representing the putative CRE sites at -38 (C1) or -142 (C2) in the presence or absence of a 100-fold molar excess of unlabelled competitor oligonucleotides. The competitor oligonucleotides were the unlabelled CRE or Sp1 consensus sequences, CRE-like binding sites (C1 or C2) or mutant versions of these sites (C1m or C2m). The positions of DNA-protein complexes are indicated by arrows.

protein-DNA complex was visualized with both C1 and C2 oligodeoxynucleotides, as revealed by the presence of a retarded band (Figure 2). Competitive binding studies were carried out to determine whether these protein-DNA complexes represented specific interactions and whether the proteins binding to both sites were related. Addition of a 100-fold molar excess of unlabelled C1 or C2 oligodeoxynucleotides diminished the formation of the protein-DNA complex (Figure 2). However, addition of similar amounts of oligodeoxynucleotides C1m or C2m, in which two residues within the predicted CRE-recognition sequences had been mutated, failed to prevent complex formation, indicating that proteins were binding specifically to the potential CRE motif. Both bands also decreased when C1 or C2 oligodeoxynucleotides were incubated with an excess of a DNA fragment corresponding to a well-characterized CRE such as that of the rat somatostatin gene. Excess of an unlabelled oligodeoxynucleotide containing the Sp1 consensus sequence,

used as a negative control, had no effect whatsoever. As a whole, these results suggest that the C1 and C2 motifs are CRE-like binding sites.

CRE-like elements are required for PKA-mediated activation of the ALAS promoter

To assess the role of the C1 and C2 sites in ALAS promoter function, we compared the activity in HepG2 cells of the wild-type ALAS promoter with mutants carrying changes in either or both of these sites. Mutations were tested in the context of p-156ALAS/CAT, which had been shown previously to support basal and PKA-inducible expression. The same mutations in sites C1 and C2 used in the gel-shift assays were introduced into the ALAS promoter either alone or in combination. Each construct was transfected into HepG2 cells, and CAT activity was measured in untreated cells and in cells transfected with an expression vector encoding the catalytic subunit of PKA. Wild-type -156 ALAS promoter (p-156ALAS/CAT) displayed low basal activity, which was stimulated 11-fold by PKA. Although mutations of either C1 or C2 site (-156 ALAS C1m and -156 ALAS C2m) resulted in unchanged basal activity; the C2 mutation caused a larger drop in PKA-induced activity than the C1 mutation (*P* < 0.05). On the other hand, mutation of both C1 and C2 (-156 ALAS C1m, C2m) resulted in a further decrease in PKA responsiveness to 20% of the wild-type activity (Figure 3). Basal activity in this dual mutant was nearly 50% lower than in the wild-type promoter. These results suggest that the C2-binding site is more effective for PKA responsiveness, although both C1 and C2 sites are necessary for complete PKA induction.

We determined basal and PKA-stimulated promoter activity in another construct, p-156ALAS110/38, in which the region spanning from -110 to -38 bp was deleted, including the C1 site. In this mutant, the C2 binding site was located in a relatively similar position to that occupied by the C1 site. When compared with p-156mC1ALAS/CAT, this deletion mutant showed a significant decrease in PKA-stimulated promoter activity. However, p-156ALAS110/38 activity was similar to p-75ALAS/CAT or p-156mC2ALAS/CAT (Figure 3). These results suggest that position rather than sequence would explain differences between C1 and C2 binding sites regarding their functional effectiveness.

To assess further the functional interaction between HepG2 nuclear proteins on putative CRE-sites C1 and C2 on the ALAS

Figure 3 Mutation of putative CRE-like sites impairs basal and PKA-stimulated ALAS promoter activity

HepG2 cells were transiently transfected with 4 μ g/plate of p-156ALAS/CAT or equivalent amounts of the mutants containing the 5'-flanking region of the ALAS gene, illustrated on the left. Transfections included (grey bars) or did not include (black bars) 3 μ g/plate of expression vector for PKA catalytic subunit. Results are expressed as relative CAT activity with respect to the basal value for p-156ALAS/CAT, which was set to 1. Fold induction represents the PKA-stimulated reporter activity divided by the unstimulated level. Bars represent means $\pm$ S.E.M. from four independent experiments performed in duplicate. Statistical analysis between p-156ALAS/CAT and mutant reporters applying Student's *t* test revealed some significant differences (**P* < 0.05 between unstimulated reporters; **P* < 0.05 and ***P* < 0.001 between PKA-stimulated reporters; **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 between fold inductions).

promoter, we performed competition experiments co-transfecting culture cells with p-156ALAS/CAT, the catalytic subunit of PKA, and increasing amounts of vector p4xCRELUC containing a tandem of four consensus CRE sites. When increasing amounts of the competitor plasmid were included in the transfection, the ability of PKA to induce ALAS promoter activity decreased in a dose-dependent manner (Table 1). This experiment strongly implies direct functional competition between CRE and C1 and C2 ALAS promoter sites.

CREB is involved in PKA-mediated induction of ALAS promoter activity

To examine the role of CREB in mediating PKA-stimulated ALAS gene expression, we co-transfected HepG2 cells with p-156ALAS/CAT or p-75ALAS/CAT constructs and with different amounts of an expression vector encoding CREB in the presence or absence of vector pMtC α expressing the PKA catalytic subunit. When CREB was co-expressed with PKA, both -156 and -75 ALAS promoter activities increased in a CREB-dose-dependent manner (Figure 4A). Surprisingly, when the CREB expression vector was co-transfected without PKA overexpression, CAT activity decreased consistently to 41 and 23% basal activity for -156 and -75 ALAS promoters, respectively (Figure 4A). A control p4xCRE/CAT construct containing four repetitive CRE sites located upstream of the CAT reporter gene was able to respond to PKA+CREB stimulation. However, unlike what happened with both ALAS/CAT promoters, CREB alone not only did not inhibit p4xCRE/CAT basal activity but also caused a slight but significant activation, probably due to CREB phosphorylation by endogenous PKA (Figure 4A). These results indicate that CREB has a functional effect on ALAS promoter transcriptional activity.

To determine whether CREB needs to bind to DNA to modify ALAS/CAT promoter activity, we conducted transient-transfection experiments with a CREB mutant (CREB K) containing a lysine-to-glutamic acid substitution at position 304 in the DNA-binding domain, which abolishes its ability to bind DNA. As shown in Figure 4(B), mutant CREB K expression inhibited PKA stimulation of p-156ALAS/CAT and p4xCRE/CAT promoter activities. In fact, CAT activities fell to almost basal levels when CREB K was overexpressed. In the absence of PKA

overexpression, co-transfected CREB K was unable to diminish basal p-156ALAS/CAT activity. Similar results were obtained with p-75ALAS/CAT construct (results not shown). Therefore, DNA-binding ability is necessary for CREB to modify ALAS promoter activity.

To establish a causal link between PKA catalytic subunit and CREB *trans*-activation on the ALAS promoter, we used a CREB mutant (CREB M1) containing a conservative serine-to-alanine substitution at position 133 that destroys the PKA phosphorylation site. Results in Figure 4(B) show that CREB M1 was completely unable to activate transcription of p-156ALAS/CAT and p4xCRE/CAT. In the absence of PKA catalytic subunit, and unlike DNA-binding mutant CREB K, expression of CREB M1 repressed basal p-156ALAS/CAT promoter activity, although p4xCRE/CAT expression remained invariable. Similar results were observed with p-75ALAS/CAT (results not shown). Analysed together, these results suggest strongly that CREB binds to the ALAS promoter and that its phosphorylation activates ALAS expression. On the other hand, PKA non-activated CREB could specifically repress ALAS promoter activity.

p-156ALAS/CAT expression in PKA- and CREB-deficient F9 teratocarcinoma cells

We then performed a series of transient expression experiments in F9 teratocarcinoma cells. These cells are unresponsive to cAMP, becoming inducible only after differentiation with retinoic acid. The PKA and CREB levels are reduced 4–5-fold in undifferentiated versus differentiated F9 cells, thus suggesting that a deficiency in both components may account for the lack of cAMP responsiveness [18]. When co-transfected with a plasmid expressing CREB or a vector expressing the PKA catalytic subunit, p-156ALAS/CAT activity was not induced. Co-transfection of both PKA and CREB genes, however, caused a dramatic 20-fold induction in p-156ALAS/CAT activity, suggesting that both PKA and CREB are critical for the induction of ALAS transcription (Table 2). A control p4xCRE/CAT reporter gene was able to respond in a similar way. Furthermore, PKA catalytic subunit and CREB activities appear to be specific for CRE sequence, since neither CREB nor the catalytic subunit, alone or in combination, could stimulate CAT expression when co-expressed with pRSV/CAT reporter gene (Table 2).

Table 1 CRE-containing plasmid competes with ALAS promoter activity

HepG2 cells were transiently transfected with either 4 μ g/plate of p-156ALAS/CAT or 4 μ g/plate of p4xCRE/CAT and co-transfected with 8 or 16 μ g/plate of p4xCRELUC. Transfections included (PKA) or did not include (Basal) 3 μ g/plate of expression vector for the PKA catalytic subunit. Results are expressed as relative CAT activity with respect to basal values for p-156ALAS/CAT or p4xCRE/CAT, which were set to 1. Values represent means $\pm$ S.E.M. of three separate experiments performed in duplicate. Student's *t* test was applied to compare samples co-transfected with p4xCRELUC and non-cotransfected samples (**P* < 0.01).

CAT construct	Competitor p4xCRELUC (μ g)	Relative CAT activity	
		Basal	PKA
p-156ALAS/CAT	0	1.0 $\pm$ 0.023	11.162 $\pm$ 0.474
	8	0.613 $\pm$ 0.067*	5.589 $\pm$ 0.198*
	16	0.489 $\pm$ 0.119*	3.766 $\pm$ 0.392*
p4xCRE/CAT	0	1.0 $\pm$ 0.029	10.536 $\pm$ 0.183
	8	0.908 $\pm$ 0.044	8.034 $\pm$ 0.12*
	16	0.833 $\pm$ 0.072	5.459 $\pm$ 0.072*

Phosphorylation-site mutant CREB M1 or DNA-binding mutant CREB K were completely unable to activate transcription of p-156ALAS/CAT or p4xCRE/CAT when co-transfected with catalytic subunit of PKA. However, in the absence of co-transfected PKA, overexpression of CREB M1, like wild-type CREB, diminished p-156ALAS/CAT basal activity (Table 2). The co-transfection experiments described above were also performed with p-75ALAS/CAT and similar results were obtained (results not shown). These data indicate that CREB is both necessary and sufficient to stimulate transcription of the ALAS gene in response to PKA, and confirm that Ser 133-unphosphorylated CREB inhibits its expression.

The -142 and -38 CRE-like elements in the ALAS gene bind CREB

To assess further that CREB binds specifically to -38 (C1 site) and -142 (C2 site) elements in the 5'-flanking region of the

Figure 4 Effects of CREB and CREB mutations on basal and PKA-induced ALAS promoter activities

(A) HepG2 cells were transfected with 4 μ g/plate of p-156ALAS/CAT (○), p-75ALAS/CAT (●) or p4xCRE/CAT (■), and co-transfected with various amounts of pCREB3. Transfections included or did not include 3 μ g/plate of expression vector for the PKA catalytic subunit as indicated. Each point represents the mean $\pm$ S.E.M. from three separate experiments performed in duplicate. Student's *t* test was applied to compare samples co-transfected with pCREB3 and non-cotransfected samples (**P* < 0.05 and ***P* < 0.01). (B) HepG2 cells were transfected with 4 μ g/plate of p-156ALAS/CAT or p4xCRE/CAT, and co-transfected with 6 μ g/plate of pCREB3, pCREB M1 or pCREB K. Non-cotransfected cells were used as a control. Transfections included (grey bars) or did not include (black bars) 3 μ g/plate of expression vector for the PKA catalytic subunit. Bars represent means $\pm$ S.E.M. from three different experiments performed in duplicate. Student's *t* test was used to compare samples co-transfected with some CREB expression vector and non-cotransfected samples (**P* < 0.01 between unstimulated reporters; **P* < 0.01 between PKA-stimulated reporters). In A and B, results are expressed as relative CAT activity with respect to the basal values of each CAT reporter, which were set to 1.

Table 2 Effect of PKA and CREB on ALAS promoter activity in undifferentiated F9 teratocarcinoma cells

F9 cells were transfected with 4 μ g/plate of p-156ALAS/CAT, p4xCRE/CAT or pRSV/CAT and co-transfected with 6 μ g/plate of pCREB3, pCREB M1 or pCREB K. Transfections included (PKA) or did not include (Basal) 3 μ g/plate of expression vector for the PKA catalytic subunit. Results are expressed as relative CAT activity with respect to basal values for p-156ALAS/CAT, p4xCRE/CAT or pRSV/CAT, which were set to 1. Values are means $\pm$ S.E.M. from three different experiments performed in duplicate. Student's *t* test was applied to compare samples co-transfected with some CREB expression vector and non-cotransfected samples (**P* < 0.05 between unstimulated reporters, and †*P* < 0.01 between PKA-stimulated reporters).

CAT construct	CREB vector	Relative CAT activity	
		Basal	PKA
p-156ALAS/CAT	None	1.0 $\pm$ 0.036	2.005 $\pm$ 0.213
	CREB	0.756 $\pm$ 0.063*	21.486 $\pm$ 0.593†
	CREB M1	0.52 $\pm$ 0.058*	0.989 $\pm$ 0.201
	CREB K	0.989 $\pm$ 0.201	1.389 $\pm$ 0.018
p4xCRE/CAT	None	1.0 $\pm$ 0.026	1.848 $\pm$ 0.091
	CREB	1.927 $\pm$ 0.142*	23.037 $\pm$ 1.779†
	CREB M1	1.255 $\pm$ 0.104	1.33 $\pm$ 0.044
	CREB K	0.927 $\pm$ 0.034	1.251 $\pm$ 0.059
pRSVCAT	None	1.0 $\pm$ 0.129	1.135 $\pm$ 0.025
	CREB	0.899 $\pm$ 0.034	1.153 $\pm$ 0.052

Table 3 Dual effect of CREB overexpression on ALAS promoter activity

HepG2 cells were transiently transfected with 4 μ g/plate of p-156ALAS/CAT or equivalent amounts of the mutants indicated and co-transfected with 6 μ g/plate of pCREB3. Transfections included or did not include 3 μ g/plate of expression vector for the PKA catalytic subunit. Results are expressed as relative CAT activity with respect to the basal values for p-156ALAS/CAT, which were set to 1. Values represent means $\pm$ S.E.M. from four different experiments performed in duplicate. Statistical analysis between p-156ALAS/CAT and mutant reporters using the Student's *t* test revealed some significant differences (**P* < 0.01 between untreated samples; †*P* < 0.01 between CREB-treated samples; ‡*P* < 0.05, §*P* < 0.01 and ¶*P* < 0.001 between CREB + PKA-stimulated reporters).

ALAS/CAT construct	Relative CAT activity		
	None	CREB	PKA + CREB
p-156ALAS/CAT	1.0 $\pm$ 0.006	0.615 $\pm$ 0.012	16.862 $\pm$ 0.292
p-75ALAS/CAT	1.027 $\pm$ 0.065	0.503 $\pm$ 0.032	12.041 $\pm$ 0.578§
p-110/38ALAS/CAT	0.708 $\pm$ 0.038*	0.361 $\pm$ 0.002	8.422 $\pm$ 0.171¶
p-156mC2ALAS/CAT	1.222 $\pm$ 0.192	0.469 $\pm$ 0.091	12.970 $\pm$ 1.355‡
p-156mC1ALAS/CAT	1.071 $\pm$ 0.068	1.085 $\pm$ 0.082†	15.618 $\pm$ 1.385
p-156mC1C2ALAS/CAT	0.566 $\pm$ 0.033*	1.014 $\pm$ 0.332†	1.622 $\pm$ 0.08¶

Opposite effects of CREB on ALAS gene expression

In view of the preceding results and in an effort to better understand the functional interaction between CREB and ALAS promoter, the expression vector CREB was co-transfected with different p-156ALAS/CAT fusion genes, either in the presence or absence of PKA catalytic subunit expression vector. Table 3 shows changes in CAT expression caused by overexpression of CREB or CREB + PKA when sites C1 or C2 were mutated or deleted. Whereas C1 mutation (p-156mC1ALAS/CAT) did not modify the induction of ALAS promoter activity in HepG2 cells co-transfected with expression vectors CREB and PKA, C2 mutation (p-156mC2ALAS/CAT) or deletion (p-75ALAS/CAT) significantly decreased this induction. When site C2 was placed close to site C1 and the latter was eliminated (p-110/38ALAS/CAT), ALAS promoter activity fell to 50%. On the other hand, mutation of both sites (p-156mC1C2ALAS/CAT) blocked the induction of activity almost completely. These results confirm that CREB mediates the PKA inductive effect on the activity of the ALAS gene promoter through sites C1 and C2.

On the other hand, when HepG2 cells transfected with plasmids derived from p-156ALAS/CAT were co-transfected with the CREB expression vector alone, transcriptional activity of the ALAS promoter was inhibited below basal activity (Table 3). Although this inhibitory effect seemed to depend only on the presence of site C1, when site C2 was placed near C1 and the latter was eliminated, ALAS promoter activity suffered strong inhibition. These data confirm that CREB exerts an inhibitory effect in the absence of overexpressed PKA phosphorylating activity. However, this inhibitory action would be effective only when CREB binds to a site near the transcription-initiation site, no further than 70 or 80 bp.

To confirm that endogenous CREB plays a similar role in ALAS promoter activity, we examined the effect of oligodeoxynucleotide antisense CREB on p-156ALAS/CAT expression. The presence of antisense CREB impaired PKA-mediated induction of CAT activity when ALAS/CAT or p4xCRE/CAT fusion genes were transfected in HepG2 cells (Table 4 and results not shown). On the other hand, antisense CREB significantly increased the basal level of ALAS/CAT expression but it did not modify p4xCRE/CAT activity.

Figure 5 Putative CRE-like sites form complexes with purified recombinant CREB

CREB protein (1 μ g) was incubated with 32 P-labelled probes representing the putative CRE sites at -38 (C1) or -142 (C2) in the presence or absence of a 100-fold molar excess of unlabelled competitor oligonucleotide. The competitor oligonucleotides were the unlabelled CRE or Sp1 consensus sequences, CRE-like binding sites (C1 or C2) or mutant versions of these sites (C1m or C2m). The positions of DNA-protein complexes are indicated by arrows.

ALAS gene, radiolabelled C1 and C2 oligodeoxynucleotides were incubated with purified recombinant CREB. This resulted in the appearance of a single retarded band in the gel-shift assays for both C1 and C2 probes (Figure 5). Binding specificity was shown by the efficient competition between excess of either rat somatostatin CRE or unlabelled C1 or C2 oligodeoxynucleotides. However, addition of an excess of oligodeoxynucleotides C1m or C2m or an oligodeoxynucleotide containing the Sp1 consensus sequence showed no effect.

Table 4 CREB antisense oligodeoxynucleotide impairs PKA stimulation of ALAS promoter activity

HepG2 cells were co-transfected with 4 μ g/plate of p-833ALAS/CAT or p4xCRE/CAT, and co-transfected (PKA) or not (Basal) with 3 μ g/plate of expression vector for the PKA catalytic subunit. After 16 h, the medium was replaced, 10 μ g of CREB antisense oligodeoxynucleotide were added, and incubation continued for another 24 h. Results are expressed as relative CAT activity with respect to the basal values for p-833ALAS/CAT or p4xCRE/CAT, which were set to 1, except for the samples transfected with PKA and treated with CREB antisense, which are expressed in relation to their respective non-treated samples. Values represent means $\pm$ S.E.M. from four different experiments performed in duplicate. Student's *t* test was used to compare samples treated with CREB antisense oligodeoxynucleotide and untreated samples (**P* < 0.05).

CAT construct	CREB antisense (μ g)	Relative CAT activity	
		Basal	PKA
p-833ALAS/CAT	0	1.0 $\pm$ 0.11	4.661 $\pm$ 0.47
	10	1.679 $\pm$ 0.221*	2.536 $\pm$ 0.325*
p4xCRE/CAT	0	1.0 $\pm$ 0.034	8.574 $\pm$ 1.823
	10	0.898 $\pm$ 0.159	8.956 $\pm$ 2.318

DISCUSSION

The purpose of this study was to explore the molecular mechanisms involved in the cAMP regulation of the rat housekeeping gene ALAS. Promoter regions of eukaryotic genes are generally composed of multiple binding sites for transcriptional activators and repressors that act in combination to regulate the expression of a linked gene [35,36]. Previous analyses of the 5'-flanking sequence of ALAS had revealed the existence of binding sites for nuclear respiratory factor 1 [24]. We report here the identification of two novel elements required for basal and cAMP-stimulated expression from the ALAS promoter in hepatoma human cells.

Deletion analysis of the 5'-flanking region from the housekeeping ALAS gene allowed us to identify a major PKA-responsive region located at -156/-38. This region contains two CRE-like motifs at -38 and -142, able to bind CREB, named C1 and C2 respectively. We also found that the -75 to -38 proximal region of the ALAS gene is crucial for the basal transcriptional activity of the promoter (Figure 1). Although the two putative CRE sites in the ALAS promoter differed from each other and from the proposed consensus sequence, gel-shift assays showed that each of these elements and the CRE-binding sites from the somatostatin promoter bound a similar protein. Moreover, authentic CRE specifically competed to bind to these ALAS elements, and vice versa, in competition assays. Additionally, both C1 and C2 sites are bound strongly by recombinant CREB (Figures 2 and 5). On the basis of these results the two ALAS promoter elements were considered to be CREB-binding sites.

Transient-transfection experiments in HepG2 cells established that the two CRE-like binding sites are important for PKA-stimulated transcriptional activity of the ALAS promoter. Following mutation of both CRE-like sites in the promoter, PKA-mediated induction of CAT activity was virtually abolished in HepG2 cells, and basal expression was dramatically impaired, confirming the critical roles played by the two sites in directing gene expression (Figure 3). According to these results, the C1 site appears to promote to a lesser extent the response to PKA. However, when the C2 element was placed downstream, replacing C1, CAT expression was concomitantly diminished. This observation indicates that contextual sequences or spatial disposition exert profound influences on the transcriptional activities mediated by these elements [37].

The well-documented interaction of CREB/ATF proteins with

CRE suggest that one member of this family mediates the PKA stimulation of ALAS promoter activity [38,39]. In addition to the results of the gel-shift assays, several other pieces of evidence support the idea that CREB is the protein involved in this effect. The co-transfection experiments with wild-type and negative mutants CREB expression vectors (Figure 4), the results obtained with F9 cells (Table 2) and the use of CREB antisense oligodeoxynucleotide (Table 4) strongly suggest that CREB is the transcription factor involved in PKA-mediated stimulation of ALAS promoter activity.

We also studied the interaction and subsequent effects of the transcription factor CREB on ALAS promoter basal activity. Using transient-transfection assays in HepG2 cells, we demonstrated that CREB specifically inhibits ALAS promoter basal activity and that this repression appears to be relieved by co-transfection with the catalytic subunit of PKA. Our results indicate that this inhibition of ALAS promoter activity requires a CREB that can bind to a CRE-like site (Figure 4B). The data presented here suggest that CREB has a dual function. On the one hand, it can function as a repressor of a promoter containing a CRE-like site, whereas on the other it works as a transcriptional activator of the same promoter. Negative and positive CREB regulation appears to be modulated by its phosphorylation state. Transcriptional transactivation by CREB is controlled by PKA-induced phosphorylation of a specific serine residue located in a consensus PKA phosphorylation site of CREB [40]. Results obtained with wild-type CREB and CREB M1 mutant, which is unable to be phosphorylated by PKA, support the idea that the dual effect of CREB is controlled by PKA-induced phosphorylation (Figure 4B). Similar conclusions might be inferred from data of transient-transfection experiments in undifferentiated F9 cells (Table 2).

On the basis of the results obtained in co-transfection experiments with p-156ALAS/CAT mutants and CREB expression vector it is clear that the inhibitory effect depends on the presence of the CRE-like site C1 (Table 3). However, as it happened with the stimulatory effect, when the C2 site was situated downstream, replacing the C1 site, a similar inhibitory effect was observed. These data support the idea that intrinsic differences between C1 and C2 sequences are not determinant on ALAS promoter activity. Clearly, the relative strength of CREB as a basal repressor and PKA-activable transcription factor depends not only on PKA-mediated phosphorylation but also on the particular surrounding sequences of the binding site or the spatial promoter context. Using CREB antisense oligodeoxynucleotide, we observed that CREB constitutively inhibits ALAS promoter activity, since disruption of its endogenous level increases gene expression (Table 4).

Our observations reinforce the notion suggested in earlier studies [21-23,41] that dephospho-CREB could act as a repressor of gene transcription. Thus, relative ratios of nuclear dephospho- and phospho-CREB would be likely to determine the relative transcriptional transactivation potency of CREB. This hypothesis is consistent with the reporter findings, in which CREB dimers consisting of one phosphorylated monomer and one mutated unphosphorylated monomer give 50 % of the transactivational activity of the wild-type phosphorylated dimer [42]. Lack of CREB phosphorylation, however, does not fully explain the lack of basal activity of CREB, because CREB has a transcriptional transactivation domain that works in the absence of PKA-mediated phosphorylation [20,43]. This domain, the constitutive activation domain (CAD), interacts with the general transcription factor TFIID through one or more TATA-binding protein-associated factors (TAFs). Recently, Felinski and Quinn [44] have presented evidence supporting a mechanism for CREB

activation of basal transcription that uses TAF 110 as a co-activator to recruit TFIID to the promoter and facilitate the pre-initiation complex assembly. On this basis, we propose that the decrease in basal activity observed in the presence of CREB may reflect a diminished ability of this CREB-bound promoter to assemble the pre-initiation complex. Dephosphorylated CREB would interfere in a spatial-disposition-dependent manner with the transcriptional machinery driving inhibition of gene expression.

In summary, we propose that transcription from ALAS gene CRE-like sites is the result of a complex interaction between positive- and negative-acting CREB, although the participation of other transcription factors cannot be discarded. Furthermore, these observations emphasize the critical importance of the availability of nuclear PKA-catalytic-subunit activity in determining the balance between activation and repression of CRE-mediated transcription by phospho- and dephospho-CREB, respectively. Alterations in the cAMP second-messenger signalling pathway and the existence of cross-talk between different families of CRE-binding transcription factors may have important functional consequences for gene expression, switching from a regulated to a constitutive pattern. A crucial role would be played by the three-dimensional structure of the promoter with respect to CRE-like sites and the transcription-initiation site.

We thank Dr Paolo Sassone-Corsi and Dr Michael Greenberg for their generous gift of plasmids and Dr Moisés Grinstein for critical comments on the work. We also thank Laura Gutiérrez for help with manuscript preparation and linguistic advice. This work was supported by research grants from the Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Técnica (ANPCYT), Argentina. L.E.G. is a Research Fellow of the ANPCYT.

REFERENCES

- May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C. and Bottomley, S. S. (1995) Molecular regulation of heme biosynthesis in higher vertebrates. *Progr. Nucleic Acids Res. Mol. Biol.* **51**, 1–51
- Ades, Y. Z. (1990) Heme production in animal tissues: the regulation of biogenesis of δ -aminolevulinic synthase. *Int. J. Biochem.* **22**, 565–578
- Bottomley, S. S., May, B. K., Cox, T. C., Cotter, P. D. and Bishop, D. F. (1995) Molecular defects of erythroid 5-aminolevulinic synthase in X-linked sideroblastic anemia. *J. Bioenerg. Biomembr.* **27**, 161–168
- Bishop, D. F., Henderson, A. S. and Astrin, K. H. (1990) Human delta-aminolevulinic synthase: assignment of the house keeping gene to 3p21 and erythroid specific gene to the X chromosome. *Genomics* **7**, 207–214
- Kappas, A., Sassa, S., Galbraith, R. A. and Nordmann, Y. (1995) The porphyrias, in *The Metabolic and Molecular Basis of Inherited Diseases*, 7th edn (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 2103–2159, McGraw-Hill, New York
- Surinya, K. H., Cox, T. C. and May, B. K. (1997) Transcriptional regulation of the human erythroid 5-aminolevulinic synthase gene. *J. Biol. Chem.* **272**, 26585–26594
- Sadlon, T. J., Dell'Oso, T., Surinya, K. H. and May, B. K. (1999) Regulation of erythroid 5-aminolevulinic synthase expression during erythropoiesis. *Int. J. Biochem. Cell Biol.* **31**, 1153–1167
- Ponka, P. (1999) Cell biology of heme. *Am. J. Med. Sci.* **318**, 241–256
- Moore, M. R. (1993) Biochemistry of porphyria. *Int. J. Biochem.* **25**, 1353–1368
- Srivastava, G., Hansen, A. J., Bawden, M. J. and May, B. K. (1990) Hemin administration to rats reduces levels of hepatic mRNAs for phenobarbital-inducible enzymes. *Mol. Pharmacol.* **38**, 486–493
- DeMatteis, F. and Marks, G. S. (1996) Cytochrome P450 and its interactions with the heme biosynthetic pathway. *Can. J. Physiol. Pharmacol.* **74**, 1–8
- Jover, R., Hoffman, K. and Meyer, U. A. (1996) Induction of 5-aminolevulinic synthase by drugs is independent of increased apocytochrome P450 synthesis. *Biochem. Biophys. Res. Commun.* **226**, 152–157
- Varone, C. L., Cánepa, E. T., Llambías, E. B. C. and Grinstein, M. (1994) Cyclic AMP regulation of phenobarbital mediated induction of δ -aminolevulinic synthase mRNA in hepatocytes from normal and experimental-diabetic rats. *Biochem. Cell Biol.* **72**, 381–390
- Varone, C. L. and Cánepa, E. T. (1997) Evidence that protein kinase C is involved in δ -aminolevulinic synthase expression in rat hepatocytes. *Arch. Biochem. Biophys.* **341**, 259–266
- Montminy, M. R. (1997) Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem.* **66**, 807–822
- Brindle, P. K. and Montminy, M. R. (1992) The CREB family of transcription activators. *Curr. Opin. Genet. Dev.* **2**, 199–204
- de Groot, R. P. and Sassone-Corsi, P. (1993) Hormonal control of gene expression: multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators. *Mol. Endocrinol.* **7**, 145–153
- Gonzalez, G. A. and Montminy, M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675–680
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414
- Brindle, P., Linke, S. and Montminy, M. R. (1993) Protein-kinase-A-dependent activator in transcription factor CREB reveals a new role for CREM repressors. *Nature (London)* **364**, 821–824
- Ofir, R., Dwarki, V. J., Rashid, D. and Verma, I. M. (1991) CREB represses transcription of fos promoter: role of phosphorylation. *Gene Expr.* **1**, 55–60
- Vallejo, M., Gosse, M. E., Beckman, W. and Habener, J. F. (1995) Impaired cyclic AMP-dependent phosphorylation renders CREB a repressor of C/EBP-induced transcription of the somatostatin gene in an insulinoma cell line. *Mol. Cell. Biol.* **15**, 415–424
- Lemaigre, F. P., Ace, C. I. and Green, M. R. (1993) The cAMP response element binding protein, CREB, is a potent inhibitor of diverse transcriptional activators. *Nucleic Acids Res.* **21**, 2907–2911
- Braidotti, G., Borthwick, I. A. and May, B. K. (1993) Identification of regulatory sequences in the gene for 5-aminolevulinic synthase from rat. *J. Biol. Chem.* **268**, 1109–1117
- Yomogida, K., Yamamoto, M., Yamagami, T., Fujita, H. and Hayashi, N. (1993) Structure and expression of the gene encoding rat nonspecific form of δ -aminolevulinic synthase. *J. Biochem.* **113**, 364–371
- Quandt, K., French, K., Karas, H., Wingender, H. and Werner, T. (1995) MatInd and MatInspector-New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878–4884
- Varone, C. L., Giono, L. E., Ochoa, A., Zakín, M. M. and Cánepa, E. T. (1999) Transcriptional regulation of 5-aminolevulinic synthase by phenobarbital and cAMP-dependent protein kinase. *Arch. Biochem. Biophys.* **372**, 261–270
- Mellon, P. L., Clegg, C. H., Correll, L. A. and McKnight, G. S. (1989) Regulation of transcription by cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4887–4891
- Dwarki, V. J., Montminy, M. R. and Verma, I. M. (1990) Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. *EMBO J.* **9**, 225–232
- Seed, B. and Sheen, J. Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67**, 271–277
- Chomczynsky, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **71**, 341–350
- Bishop, D. F. (1990) Two different genes encode delta-aminolevulinic synthase in humans: nucleotide sequences of cDNAs for the housekeeping and erythroid genes. *Nucleic Acids Res.* **18**, 7187–7188
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn, Cold Spring Harbor Press, Cold Spring Harbor
- Andrews, N. C. and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499–2503
- Roesler, W. J. and Park, E. A. (1998) Hormone response units: one plus one equals more than two. *Mol. Cell. Biochem.* **178**, 1–8
- Ogbourne, S. and Antalis, T. M. (1998) Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem. J.* **331**, 1–14
- Habener, J. F., Meyer, T. E., Yun, Y., Waeberg, G. and Hoeffler, J. P. (1990) Characterization of a cAMP-regulated enhancer-binding protein. *Metabolism* **39**, 13–16
- Kingsley-Kallesse, M. L., Kelly, D. and Rizzino, A. (1999) Transcriptional regulation of the transforming growth factor-beta2 promoter by cAMP-responsive element-binding protein (CREB) and activating transcription factor-1 (ATF-1) is modulated by protein kinases and the coactivators p300 and CREB-binding protein. *J. Biol. Chem.* **274**, 34020–34028
- Wolff, S., Martínez, C. and Majzoub, J. A. (1999) Inducible binding of cyclic adenosine 3',5'-monophosphate (cAMP)-responsive element binding protein (CREB) to a cAMP-responsive promoter *in vivo*. *Mol. Endocrinol.* **13**, 659–669
- De Cesare, D., Fimia, G. M. and Sassone-Corsi, P. (1999) Signaling routes to CREM and CREB: plasticity in transcriptional activation. *Trends Biochem. Sci.* **24**, 281–285

- 41 Lamph, W. H., Dwarki, V. J., Ofir, R., Montminy, M. R. and Verma, I. M. (1990) Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4320–4324
- 42 Loriaux, M. M., Rehfuess, R. P., Brennan, R. G. and Goodman, R. H. (1993) Engineered leucine zippers show that hemiphosphorylated CREB complexes are transcriptionally active. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9046–9050
- 43 Quinn, P. G. (1993) Distinct activation domains within cAMP response element-binding protein (CREB) mediate basal and cAMP-stimulated transcription. *J. Biol. Chem.* **268**, 16999–17009
- 44 Felinski, E. A. and Quinn, P. G. (1999) The CREB constitutive activation domain interacts with TATA-binding protein-associated factor 110 (TAF110) through specific hydrophobic residues in one of the three subdomains required for both activation and TAF110 binding. *J. Biol. Chem.* **274**, 11672–11678

Received 16 June 2000/29 September 2000; accepted 2 November 2000