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Inhibitory Effect of AP-1 Complex on 5-Aminolevulinate Synthase Gene Expression through Sequestration of cAMP-response Element Protein (CRE)-binding Protein (CBP) Coactivator*

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Activation protein-1 (AP-1) transcription factors are early response genes involved in a diverse set of transcriptional regulatory processes. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is often used to induce AP-1 activity. The purpose of this work was to explore the molecular mechanisms involved in the TPA regulation of ubiquitous 5-aminolevulinate synthase (ALAS) gene expression, the first and rate-controlling step of the heme biosynthesis. Previous analysis of the 5′-flanking sequence of ALAS revealed the existence of two cAMP-response elements (CRE) required for basal and cAMP-stimulated expression. The fragment −833 to +42 in the 5′-flanking region of rat ALAS gene was subcloned into a chloramphenicol acetyltransferase (CAT) reporter vector. The expression vector pALAS/CAT produced a significant CAT activity in transiently transfected HepG2 human hepatoma cells, which was repressed by TPA. Sequence and deletion analysis detected a TPA response element (TRE), located between −261 and −255 (TRE-ALAS), that was critical for TPA regulation. We demonstrated that c-Fos, c-Jun, and JunD are involved in TPA inhibitory effect due to their ability to bind TRE-ALAS, evidenced by supershift analysis and their capacity to repress promoter activity in transfection assays. Repression of ALAS promoter activity by TPA treatment or Fos/Jun overexpression was largely relieved when CRE protein-binding protein or p300 was ectopically expressed. When the TRE site was placed in a different context with respect to CRE sites, it appeared to act as a transcriptional enhancer. We propose that the decrease in ALAS basal activity observed in the presence of TPA may reflect a lower ability of this promoter to assemble the productive pre-initiation complex due to CRE protein-binding protein sequestration. We also suggest that the transcriptional properties of this AP-1 site would depend on a spatial-disposition-dependent manner with respect to the CRE sites and to the transcription initiation site.

Activation protein-1 (AP-1)[1] transcription factors are early response genes involved in a diverse set of transcriptional regulatory processes. AP-1 is a dimeric complex composed of members of the Fos and Jun family proteins (1). This complex binds the consensus DNA sequence TGA(G/C)TCA, termed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE) or AP-1, sites found in a variety of promoters of genes, such as growth factors, chemokines, and cytokines (2). The Fos family contains four proteins (c-Fos, Fos-B, Fra-1, and Fra-2), whereas the Jun family is composed of three (c-Jun, JunB, and JunD). Fos and Jun are members of the basic leucine zipper group of proteins, and this basic motif mediates the formation of homo- and heterodimers. c-Jun is the major component of the AP-1 complex, and c-Fos is its best known partner (3, 4). AP-1 is activated by mitogens, oncoproteins, cytokines, and stress agents such as ultraviolet light. The phorbol ester TPA is often used to induce AP-1 activity. The activation of this protein may be mediated both by transcriptionally independent and dependent mechanisms, which involve post-translational modifications of its components or increases in the expression of their corresponding genes, respectively (5, 6).

Transcription coactivators bridge transcription factors and the components of the basal transcriptional apparatus (7). One important class of coactivators includes the cAMP-response element protein (CREB)-binding protein (CBP) and the highly related p300 protein, which were originally identified for their ability to interact strongly with CREB (8). Subsequently, CBP and p300 were identified as essential cofactors for a number of nuclear transcription factors, including AP-1 complex (9), several components of the basal transcriptional machinery (TBP and TFIIB) (10), other histone acetyltransferases (SRC-1, ACTR and P/CAF) (11), developmental proteins (GATA-1, MEF-2, Pit-1) (12), viral oncoproteins (E1A, large T antigen, and Tax) (13), and nuclear receptors (14).

These coactivator proteins are important not only due to their role in positive transcriptional regulation from DNA binding sites but also due to their role as mediators of cross-talk between different signal transduction pathways (15). Although negative binding elements have been described, repression is mainly conducted by interference with other transcription factors, of which AP-1 is one of the most representative (16). CBP

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[1] The abbreviations used are: AP-1, activation protein-1; 4α PDA, 4α-phorbol 12,13-diacetate; ALAS, 5-aminolevulinate synthase; CAT, chloramphenicol acetyltransferase; CREB, cAMP-responsive element protein; CBP, CREB-binding protein; CRE, cyclic AMP-responsive element; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; HA, hemagglutinin; NHR, nuclear hormone receptors; PKA and PKC, protein kinase A and C, respectively; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element.

has been reported to play a significant role in the negative cross-talk between members of the nuclear receptor family, including glucocorticoid receptor, retinoic acid receptor, thyroid hormone receptor, and AP-1 activity, without inhibition of DNA binding (17–19). Several independent approaches revealed that CBP is necessary for the activation of both AP-1 and of nuclear hormone receptors (NHR) as well. As has been suggested, competition for limiting amounts of CBP may account for many of the inhibitory effects of NHR on AP-1 activation (17, 20). Thus, on genes containing NHR binding sites but lacking AP-1 binding sites, positive regulation by liganded NHR is inhibited by activation of AP-1. Conversely, liganded NHR can inhibit AP-1-mediated transcription.

In this paper, we present a distinct mechanism of negative cross-talk between CREB and AP-1 that involves competition by CBP on the transcriptional activity of 5-aminolevulinate synthase (ALAS) gene promoter. ALAS is a mitochondrial matrix enzyme that catalyzes the first and rate-limiting step of heme biosynthesis (21). There are two related ALAS isozymes that are encoded by two separate genes located on different chromosomes. The erythroid cell-specific enzyme or ALAS-2 is developmentally regulated, and it markedly increases during erythropoiesis to meet the demand for heme during hemoglobin production. The second enzyme, ubiquitous or liver type ALAS (ALAS-1), is probably expressed in all tissues to provide heme for cytochromes and other hemoproteins (21, 22).

Expression of ALAS in the liver was found to be subject to feedback regulation by heme (21). In addition to this major mechanism of regulation, we have demonstrated that cAMP induces and phorbol esters repress the expression of liver ALAS through protein kinase A (PKA) and protein kinase C (PKC) activation, respectively (23, 24). Studies carried out on the 5′-regulatory region of ALAS gene showed the presence of two functional CRE-like sites that are necessary not only for the cAMP-mediated induction but also for basal expression. These sites are bounded by CREB and recruit coactivator CBP (25).

The purpose of this study was to examine the molecular mechanism underlying TPA-inhibited expression of ALAS gene. Promoter deletion analysis were performed on the ALAS gene, which, as we have already demonstrated, is repressed by TPA (23). We found that an AP-1 binding site (TRE-ALAS) was crucial for the inhibition of the ALAS promoter despite its widely reported positive responsiveness to TPA in several promoters (26, 27). The nuclear heterodimeric complexes that bind to TRE-ALAS would be composed of c-Fos and c-Jun or c-Fos and JunD. In addition, our data indicate that overexpression of CBP relieved TPA repression, suggesting that sequestration of CBP prevents the downstream formation of the CREB·CBP complex necessary for basal transcription of ALAS gene. This competition for limiting the intracellular amount of a common coactivator could explain the bizarre inhibitory effect of TPA on ALAS gene expression. Finally, we observed different responses to TPA depending on the relative position of TRE and CRE sites on the ALAS promoter. Therefore, AP-1 complex on TRE-ALAS would interfere in a disposition-dependent manner with the transcription machinery through CBP sequestration during the inhibition of ALAS gene expression.

EXPERIMENTAL PROCEDURES

Reagents—Eagle's minimum essential medium, guanidine isothiocyanate, TPA, 4α-phorbol-12,13-diacetate (4αPDA), agarose, calphostin C, chloramphenicol, butyryl coenzyme A, 8-CPT-cAMP, and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma. [*ring*-3,5-^{3}H]Chloramphenicol (specific activity 1.1–2.2 GBq/mmol), [γ-^{32}P]ATP (specific activity 222 TBq/mmol), and [α-^{32}P]dCTP (specific activity 111 TBq/mmol) were purchased from PerkinElmer Life Sciences. Random primers kit, restriction endonucleases, and DNA modifying enzymes were from New England Biolabs, Inc. All other chemicals were of analytical grade. Oligodeoxynucleotides were chemically synthesized by Bio-Synthesis Inc. (Lewisville, TX).

Expression Vectors—The following expression vectors were used as indicated in each experiment. Plasmid pALAS/CAT contains the 5′-flanking region (−833 to +42 bp) of rat ubiquitous ALAS gene cloned upstream the CAT reporter gene in vector pBLCAT6. Deletion mutant plasmids p-459ALAS/CAT, p-354ALAS/CAT, p-156ALAS/CAT, and p-75ALAS/CAT were described previously (25). Vectors pSG5 encoding wild type forms of c-Fos, c-Jun, JunB or JunD, and a pRc/RSV vector encoding the cDNA for the wild type version of CBP were kindly provided by Dr. P. Sassone-Corsi (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Vector pCEFLp300 encoding cDNA for the wild type version of p300 was a gift from Dr. J. Silvio Gutkind (NIDCR, National Institutes of Health, Bethesda, MD). The pCMV/A-Fos (designated pA-Fos) is a cytomegalovirus-driven expression vector in which the normal basic region critical for DNA binding at the N terminus of the Fos leucine zipper was replaced for an acidic sequence (a generous gift from Dr. Charles Vinson, NCI, National Institutes of Health, Bethesda, MD) (28). The plasmid pTRE-tk-CAT contains two TRE consensus sequence upstream of the HSV thymidine kinase promoter (29). The heterologous pASCAT vector, in which a copy of the −354 to −156-bp sequence of ALAS gene was placed upstream of the thymidine kinase promoter, was generated by cloning the *Afl*II/*Stu*I fragment of pACAT into the *Sal*I site of pBLCAT2. Similarly, pAECATd or pAECATi vectors containing a copy of the −354 to −38-bp sequence of ALAS gene in the right or inverted position, respectively, upstream of the thymidine kinase promoter were generated by cloning the *Afl*II/*Bst*EII fragment of pACAT into the *Sal*I site of pBLCAT2. The mutations were generated by polymerase chain reaction-based site-directed mutagenesis (Stratagene, La Jolla, CA). In p-354ALAS/CATm, pAECATdm, and pAECATim vectors the TRE-ALAS site was mutated from wild-type TGACGCA (coding strand) to TGACGTG (coding strand). The fidelity of all mutated vectors was checked by DNA sequence. Plasmid pCEFL containing the β-galactosidase gene was also used.

To perform transfection assays, plasmids were purified using the Wizard *Plus* Maxipreps (Promega Co). DNA concentration was estimated spectrophotometrically.

Cell Culture and Treatments—The human hepatoma cell line HepG2 was grown as monolayer cultures in minimum essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 1% penicillin-streptomycin, 100 μM nonessential amino acids, and 2 mM glutamine. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. For RNA analysis, phenobarbital and TPA were added to HepG2 cells at 75% confluence in 100-mm tissue culture plates for the times and concentrations detailed in the figure legends. Phenobarbital was dissolved in 0.1 ml of the corresponding medium. Phorbol esters and calphostin C were dissolved in a small volume (less than 0.5% of the total volume of culture media) of Me_2SO.

RNA Extraction and Northern Blot Analysis—Total cellular RNA was isolated from cultured HepG2 cells according to Chomczinsky and Sacchi (30). 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 in 1% glyoxal-agarose gels, and transferred to nylon membranes (Hybond N, Amersham Biosciences). The membranes were sequentially hybridized with ^{32}P-labeled 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 ubiquitous ALAS mRNA (31). The oligodeoxynucleotide was purified and 5′-end-labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase. The resulting probe had a specific activity of about 5–6 × 10^3 cpm/fmol. Hybridization was carried out overnight at 70 °C in the same prehybridization solution by adding the ^{32}P-labeled oligodeoxynucleotide (3.0 × 10^5 cpm/cm^2) as previously described (24). To detect β-tubulin mRNA, chicken β-tubulin cDNA (a generous gift of Dr. J. Messina, Florida) was labeled by random priming using [α-^{32}P]dCTP and Klenow to a specific activity of about 6 × 10^8 cpm/μg. Membranes were stripped, prehybridized, hybridized, and washed in standard conditions described by Sambrook *et al.* (32). Autoradiographs were obtained by exposing these blots to Kodak XAR-5 film with an intensifying screen for 3–5 days at −70 °C.

Transient Transfection Experiments—HepG2 transient transfections were performed according to the standard calcium phosphate precipitation method as previously described (25). In brief, 4 μg of pALAS/CAT or its derivatives and 6 μg of pCEFLβgal were cotransfected into 5 × 10^5 cells plated on 35-mm Petri dishes. The β-galactosidase plasmid was used as the internal standard to normalize transfection efficacy. The use of other cotransfected plasmids is indicated in each experiment. The amount of Fos/Jun, CBP, and p300 expression vectors that have

been used in the different experiments was previously determined through dose-response curves. Different quantities of the plasmids mentioned were co-transfected into HepG2 cells together with p-354ALAS/CAT, and CAT expression was measured. The minimum amount of each plasmid that produced the maximum effect was used in later experiments. Final DNA concentration was adjusted to 30 μg/35-mm dish with nonspecific DNA carrier. Control transfections with carrier alone and carrier plus vector pBLCAT6 or pBLCAT2 were done in parallel. Sixteen hours later, the medium was replaced with 3 ml of serum-free medium containing the reagents indicated in each experiment and incubated for 24 h.

Then cells were collected, and CAT activity was measured in cell extracts as described previously (25) according to the Seed and Sheen phase-extraction method (33). The β-galactosidase activity was determined spectrophotometrically in the transfected cell extracts (32). CAT activity was expressed as the amount of radiolabeled chloramphenicol acetylated by 1 mg of protein in 1 min and normalized with β-galactosidase activity. β-Galactosidase activity was not modified by any of the treatments used. The protein concentration of the cell extracts was determined by the Bradford assay (34).

Electrophoretic Mobility Shift and Supershift Assays—Nuclear extracts were prepared from TPA-stimulated and unstimulated HepG2 cells as described by Andrews and Faller (35). Double-strand DNA probes and cold competitors used were TRE-ALAS (AP-1 site located at −261 bp), 5′-AGGAGTCTGACGCACAGGGCT-3′, and mutant, 5′-AGGAGTCTGACGTGCAGGGCT-3′, called TRE-ALASmut, in which the underlined bases have been mutated to disrupt the specific binding of AP-1-binding proteins. A positive control oligodeoxynucleotide containing a consensus AP-1 binding site, 5′-CGCTTGATGAGTCAGCCGGAA-3′, and an oligodeoxynucleotide containing a consensus CRE site corresponding to the −58 to −31 bp of rat somatostatin gene promoter 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′, were also used. Oligodeoxynucleotides were 5′-end-labeled with T_4 polynucleotide kinase and 222 TBq/mmol [γ-^{32}P]dATP at 37 °C for 60 min. Binding reactions were performed mixing 10 μg of the nuclear extract with 2 μg of poly(dI-dC) and 150,000 dpm of the labeled probe in TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol) to a final volume of 20 μl and incubated at room temperature for 30 min. When noted, the nuclear extract was incubated for 20 min at room temperature in a binding mixture with the indicated molar excess of unlabeled competitor DNA before the addition of labeled probe. After the binding reaction, electrophoresis was carried out through a 5% non-denaturating polyacrylamide gel containing 0.25× TBE (1× TBE: 50 mM Tris borate, pH 8.3, 1 mM EDTA). The gel was then dried, and autoradiography was performed. The supershift analysis was performed by incubating the nuclear extract with 3 μl of specific antibody at 4 °C for 4 h before the band shift assays already described. Antibodies against c-Fos, c-Jun, and JunD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation and Western Blot Analysis—HepG2 cells were plated on 100-mm dishes (2×10^6) and transfected with 2.5 μg of CBP expression vector, where CBP was tagged with HA, or the backbone vector using Escort transfection reagent according to the recommendations of the manufacturer (Sigma). Two days after transfection, total cell lysates were prepared in radioimmune precipitation assay buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride, 60 μg/ml aprotinin, and 1 mM sodium orthovanadate). The lysates were centrifuged at 10,000 × *g* for 10 min to remove cell debris. Cleared lysates (100 μg) were immunoprecipitated with monoclonal anti-HA antibody (Santa Cruz). The immune complexes were recovered on protein A/G-agarose beads (Santa Cruz) for 1 h at 4 °C and then washed 4 times with phosphate-buffered saline. The precipitated proteins were resuspended in sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 3 min, fractionated by SDS-PAGE on a 10% gel, and thereafter blotted to a nitrocellulose membrane. The membrane was then immunoblotted with polyclonal anti-rabbit anti-phospho-CREB (Cell Signaling Technology). The antibody was detected using horseradish peroxidase-linked goat anti-rabbit IgG (Sigma) and visualized by the Pierce Super Signal Ultra Chemiluminescence signaling systems and a Bio-Imaging Analyzer Fujifilm LAS-1000. For direct immunoblotting of cell proteins, total cell lysates of parallel samples were fractionated by electrophoresis in SDS-PAGE on a 10% gel, and the proteins were transferred to a nitrocellulose membrane. Protein CREB was detected using the rabbit polyclonal antibody anti-CREB (Cell Signaling). The immune complexes were visualized by chemiluminescence as describe above.

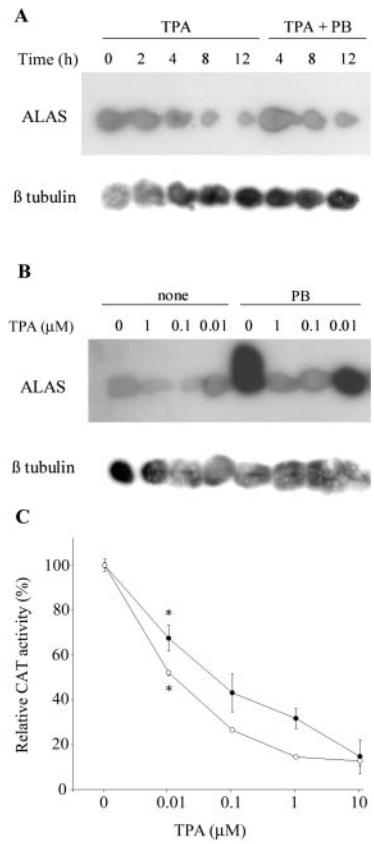

FIG. 1. **Effect of TPA on ALAS mRNA and promoter activity.** *A*, HepG2 cells were incubated in serum-free medium with 1 μM TPA in the presence or absence of 0.6 mM phenobarbital (*PB*) for the times indicated. *B*, HepG2 cells were incubated in serum-free medium with different concentrations of TPA in the presence or absence of 0.6 mM phenobarbital for 8 h. In *A* and *B* total RNA was extracted from the cells and subjected to Northern blot analysis using a ^{32}P-labeled probe specific for human ALAS mRNA and reprobed for β-tubulin mRNA as described under "Experimental Procedures." Each figure shows a representative autoradiograph of three independent experiments with similar results. *C*, HepG2 cells transiently transfected with 4 μg of p-ALAS/CAT plasmid were incubated in serum-free medium with different concentrations of TPA in the presence (●) or absence (○) of 0.6 mM phenobarbital. After 24 h, cells were harvested, and CAT activity was determined as described under "Experimental Procedures." Results are expressed as relative CAT activity with respect to samples non-treated with TPA, which were set to 100. Each point represents the mean ± S.E. of four different experiments performed in duplicate. Student's *t* test was used to compare samples treated with TPA with non-treated samples (*, $p < 0.05$, indicates the minimum TPA concentration that causes a significant reduction in CAT activity).

RESULTS

TPA Inhibits ALAS Gene Expression in HepG2 Cells—We first studied the effect of TPA on the expression of ALAS gene in HepG2 cells either in basal or induced conditions. These cells were incubated with 1 μM TPA for up to 24 h in the presence or the absence of 0.6 mM phenobarbital, a well known inducer of ALAS gene expression (36). Northern blot analysis showed that in both cases there was a time-dependent decrease in mRNA levels for ALAS, suggesting that TPA inhibited ALAS gene expression in HepG2 cells (Fig. 1*A*). The TPA concentration-response relationship for ALAS mRNA inhibition revealed a dose-dependent effect. TPA was effective over a concentration range of 10^{-8} to 10^{-5} M, and maximum inhibition was reached at 10^{-6} M and over (Fig. 1*B*). These results agree with previous observations made in primary cultures of rat hepatocytes (23). To determine whether sequences in the 5′-flanking region of the ALAS gene could confer TPA responsiveness, we fused about 870 bp of this region to the bacterial reporter gene for

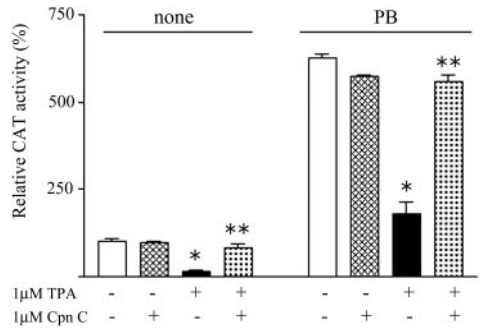

FIG. 2. **Calphostin C blocks the TPA inhibitory effect on ALAS promoter activity.** HepG2 cells transiently transfected with 4 μg of p-ALAS/CAT plasmid were incubated in serum-free medium with the indicated additions. After 24 h, cells were harvested, and CAT activity was determined as described under "Experimental Procedures." Results are expressed as relative CAT activity with respect to basal value without any addition, which was set to 100. *Bars* represent the mean ± S.E. of three independent experiments performed in duplicate. Student's *t* test was used to compare samples containing TPA with the respective basal value (*, $p < 0.05$) or samples containing TPA plus calphostin C with samples containing TPA alone (**, $p < 0.05$). *PB*, 0.6 mM phenobarbital; *Cpn*, calphostin C.

CAT. HepG2 cells were transiently transfected with this ALAS/CAT vector and then incubated with different amounts of TPA before harvesting and analysis of CAT activity (Fig. 1*C*). TPA caused a dose-dependent inhibition in ALAS/CAT expression with a maximum decrease of almost 5-fold in untreated cells and 8-fold in cells exposed to 0.6 mM phenobarbital. The action of 4αPDA, a TPA analog that is ineffective in tumor promotion and PKC activation, was tested to exclude the possibility of a general inhibitory effect due to incubation with phorbol esters. The addition of 1 μM 4αPDA failed to reduce ALAS/CAT expression (data not shown).

In many cell types, prolonged treatment with phorbol esters resulted in almost complete depletion of cellular PKC. Because PKC activation led to inhibition of ALAS gene expression, HepG2 cells transiently transfected with ALAS/CAT were pretreated with 1 μM TPA for 12 h to determine whether PKC diminution by translocation to the cell membrane prevented this inhibition. We observed that prolonged PKC stimulation resulted in the blockage of ALAS inhibition by TPA both in basal and phenobarbital-stimulated conditions (data not shown). Furthermore, incubation of ALAS/CAT-transfected HepG2 cells with 1 μM calphostin C, a PKC inhibitor, resulted in the blockage of the inhibitory effect of 1 μM TPA on ALAS promoter activity (Fig. 2). These results suggest that PKC is involved in the inhibitory effect of ALAS gene expression.

ALAS Gene Expression Is Inhibited by TPA due to a Major Responsive Site in the Proximal 5′-Noncoding Region—To identify *cis*-acting response elements in the 5′-flanking region of the rat ALAS gene that are TPA-responsive, a series of progressively longer deletion mutants of ALAS/CAT were constructed. As in the case of ALAS/CAT, these deletion promoter mutants were transiently transfected into HepG2 cells and assayed for CAT activity in the absence and presence of 1 μM TPA. As shown in Fig. 3, progressive deletion of sequences from −833 to −354 bp did not significantly impair TPA-mediated inhibition of promoter activity. Further deletion of the promoter from −354 to −156 bp almost completely blocked the TPA-inhibited ALAS/CAT expression, suggesting that an essential TPA-responsive element is contained within this region. There were only slight differences in the basal levels of promoter activity in the deletion mutants tested (Fig. 3, *inset*).

Characterization of AP-1 Binding Site within the ALAS Promoter—In silico analysis of the 200-bp region (−354 to −156 bp) included in our 5′-deletion analysis in the TPA-mediated inhibition of ALAS promoter activity revealed a potential TRE/AP-1 regulatory element (TRE-ALAS) corresponding to nucleotides −261 to −255 bp (core similarity 1.000; matrix similarity 0.925) (37). Consistent with 5′ deletion analysis, mutation of the TRE-ALAS site impaired the ability of TPA to decrease promoter activity, revealing a role for this element in the regulation of ALAS gene expression (Fig. 3). However, basal promoter activity was unaffected by the TRE-ALAS mutation (Fig. 3, *inset*). To determine whether AP-1 proteins can bind the TRE-ALAS motif, we performed electrophoretic mobility shift assays with oligonucleotides containing this sequence and nuclear extracts from HepG2 cells treated or not treated with 1 μM TPA. As shown in Fig. 4, a probe containing the TRE-ALAS motif (TGACGCA) formed a shifted complex that increased several times with the addition of TPA-treated extracts. This complex was competed in a dose-dependent manner by the same unlabeled oligonucleotide but not by unlabeled oligonucleotide containing a mutated TRE-ALAS binding site (TGACGTG). Moreover, the DNA-protein complex was competed by unlabeled oligonucleotides containing the consensus AP-1 sequence but not by the consensus CRE sequence. Likewise, a shifted complex formed by a probe containing the AP-1 consensus motif was competed in a dose-dependent manner with unlabeled oligonucleotides containing the TRE-ALAS sequence (data not shown). Taken together, these data clearly show that the TRE-ALAS element is an AP-1 binding site.

Heterodimers c-Fos/c-Jun and c-Fos/JunD Inhibit ALAS Promoter Activity—The AP-1 protein, which is a complex consisting of proteins from Jun and Fos families, binds to TREs and modifies TPA-regulatable genes. To determine the effects of these AP-1-related proteins on ALAS transcription, several expression vectors of the Jun and Fos protein families were transfected into HepG2 cells. Co-transfection of p-354ALAS/CAT with c-Fos and c-Jun or c-Fos and JunD expression vectors resulted in a reduced expression of the fusion gene similar to the level achieved when p-354ALAS/CAT-transfected cells were incubated with TPA (Fig. 5). None of the other Fos/Jun combinations or Jun dimers modified CAT activity. No changes in promoter activity were observed in similar co-transfection experiments performed with p-156ALAS/CAT, a reporter vector that does not contain the TRE-ALAS site (data not shown), thus discarding unspecific effects of Fos/Jun-overexpressed proteins. We next performed supershift analysis to determine the binding of these proteins to the TRE-ALAS site. As shown in Fig. 4*C*, incubating HepG2 nuclear extracts with either anti-c-Fos, anti-c-Jun, or anti-JunD antibodies caused a supershifted band.

Because TRE, present in the promoter regions of several eukaryotic genes, is known to increase the transcription of these genes in response to TPA, in contrast to what happens with ALAS promoter, we challenged the ability of TPA and Fos/Jun expression vectors under our experimental conditions to induce CAT activity of a reporter vector containing two TRE sites with a minimum thymidine kinase promoter (pTRE-tk-CAT). Results in Table I clearly show that any Fos/Jun or Jun/Jun heterodimer was able to induce CAT expression as TPA did.

To confirm the functional contribution of AP-1 factors to the regulation of ALAS transcription by TPA, a dominant negative variant of c-Fos (A-Fos) was used in co-transfection experiments. This factor contains, instead of the DNA binding domain, an acidic domain complementary in charge distribution to the basic region of the targeted factor. As a result, when A-Fos dimerizes with a wild type factor to form a coiled coil through the leucine zipper region, the respective acidic and basic regions continue the formation of a very stable factor and

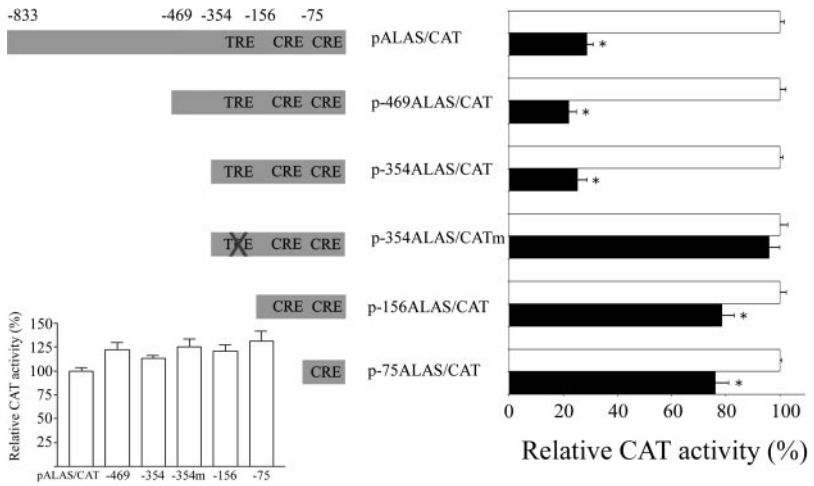

FIG. 3. **Deletion analysis reveals a region important for TPA-mediated inhibition of ALAS promoter activity.** HepG2 cells transiently transfected with 4 μg/plate of p-ALAS/CAT or equivalent amounts of deletion mutants containing the 5′-flanking region of ALAS gene illustrated on the *left* were incubated in serum-free medium alone (*white bars*) or with the addition of 1 μM TPA (*black bars*). After 24 h, cells were harvested, and CAT activity was determined as described. Results are expressed as relative CAT activity with respect to basal value for each construction, which was set to 100. *Bars* represent mean ± S.E. of four independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated and non-treated samples (*, $p < 0.05$). *Inset*, relative basal CAT activity of each promoter mutant with respect to the basal activity of p-ALAS/CAT, which was set to 100. *Bars* represent the mean ± S.E. of four independent experiments performed in duplicate.

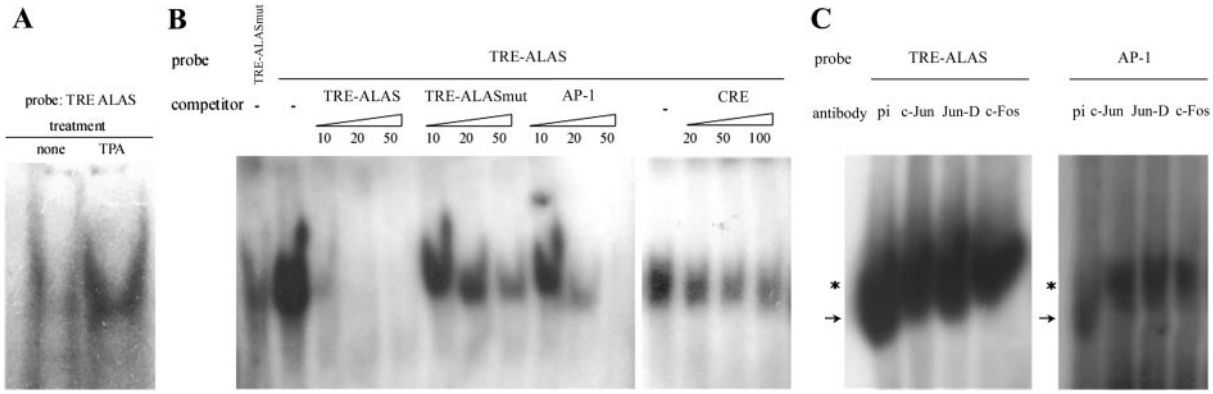

FIG. 4. **TRE-ALAS site forms a complex with c-Fos/c-Jun or c-Fos/JunD.** *A*, 10 μg of protein prepared from extracts of HepG2 cells previously treated or not treated with 1 μM TPA for 6 h were incubated with a ^{32}P-labeled probe representing the TRE-ALAS site. *B*, 10 μg of protein prepared from extracts of HepG2 cells previously treated with 1 μM TPA for 6 h were incubated with ^{32}P-labeled probes representing the TRE-ALAS or the mutated TRE-ALAS (TRE-ALASmut) in the presence or absence of increased quantities of unlabeled competitor oligonucleotides as indicated. The competitor oligonucleotides were either the unlabeled TRE-ALAS, mutated TRE-ALAS, AP-1, or CRE consensus sequences. *C*, 10 μg of nuclear extract prepared from the HepG2 cells treated with 1 μM TPA for 6 h were subjected to supershift assays. Nuclear proteins were preincubated with 3 μl of each antibody against c-Fos, c-Jun, or JunD proteins, and then ^{32}P-labeled TRE-ALAS or AP-1 probe was added. Controls were incubated with preimmune immunoglobulins (*pi*). In *A*, *B*, and *C*, protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.25× TBE buffer with 120 V at room temperature.

prevent it from binding to DNA (28). As seen in Fig. 6, the overexpression of A-Fos significantly diminished the inhibitory effect caused by TPA or by the overexpression of c-Fos/c-Jun or c-Fos/JunD on ALAS promoter activity. Similarly, overexpressed A-Fos blocked the induction effect obtained on pTRE-tk-CAT expression by TPA or by the mentioned heterodimers Fos/Jun (Table II).

All these results strongly indicate that the nuclear proteins interacting with the TRE-ALAS region are most likely composed of c-Fos and c-Jun or c-Fos and JunD and that these AP-1 complexes are responsible for the TPA-mediated inhibition of ALAS transcriptional activity.

Overexpression of CBP Counteracts the TPA Inhibitory Effect on ALAS Promoter Activity—In previous work we demonstrated the presence of two CRE-like sites on the regulatory region of ALAS gene, located downstream TRE-ALAS site, necessary not only to confer cAMP/PKA responsiveness but for also ALAS basal expression (25). These findings allow us to hypothesize that the competition for a common co-activator like CBP that prevents the downstream formation of the CREB·CBP complex necessary for basal transcription of ALAS gene could explain the bizarre inhibitory effect of TPA. To evaluate this hypothesis, HepG2 cells were co-transfected with p-354ALAS/CAT and CBP expression vector and treated or not with TPA. Overexpression of CBP severely curtailed the TPA-mediated inhibition of ALAS promoter activity (Fig. 7). Consistent with this result, when the inhibition of ALAS transcription was achieved by co-transfection of c-Fos and c-Jun or c-Fos and JunD expression vectors, CBP overexpression partially reversed this effect. Furthermore, the addition of TPA blocked the CBP-mediated reversion (Fig. 7). Similar experiments were performed with an expression vector encoding co-activator p300. Again, TPA-mediated inhibition of ALAS promoter activity was avoided by the overexpression of the transcriptional integrator (data not shown).

CBP Interacts with Endogenous CREB in Vivo—To challenge our hypothesis, we next asked whether CBP associates with CREB in HepG2 cells in basal conditions. To address this, an expression vector for CBP, harboring a HA tag, was transfected into the HepG2 cells. Immunoprecipitation of tagged CBP fol-

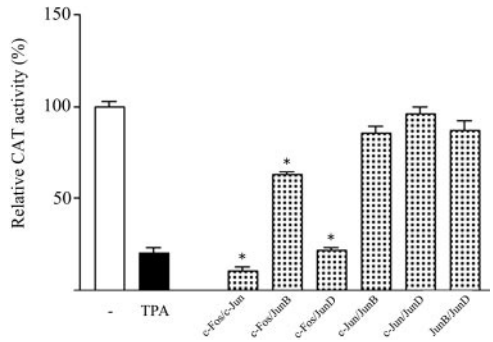

FIG. 5. **Heterodimers of Fos and Jun proteins inhibit ALAS promoter activity.** HepG2 cells were transiently transfected with 4 μg/plate of p-354ALAS/CAT and cotransfected or not with 3 μg/plate of each of the indicated expression vectors for Fos and Jun proteins. Non-cotransfected sample was incubated in serum-free medium containing 1 μM TPA during 24 h. Results are expressed as relative CAT activity with respect to p-354ALAS/CAT without any treatment, which was set to 100. *Bars* represent the mean ± S.E. of four independent experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected with some Fos/Jun expression vector and non-cotransfected and non-treated sample. *, $p < 0.05$.

TABLE I
TPA or Fos and Jun heterodimers stimulate the expression of a reporter vector containing TRE

HepG2 cells were transiently transfected with 4 μg/plate of TRE-tk-CAT or tk-CAT and cotransfected or not with 3 μg/plate of each of the indicated expression vectors for Fos and Jun proteins. Non-cotransfected sample was incubated in serum-free medium containing 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to the basal value of tk-CAT, which was set to 1. Values are the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was applied to compare samples cotransfected with a Fos and/or Jun expression vector or a TPA-treated sample with basal value of each reporter vector. ND, not determined.

Treatment	TRE-tk-CAT	Tk-CAT
	% Relative CAT activity	
None	4.66 ± 0.33	1.00 ± 0.14
TPA	20.12 ± 0.43[a]	1.46 ± 0.23
c-Fos/pSG5	5.64 ± 0.27	ND
c-Fos/c-Jun	20.34 ± 2.98[a]	1.34 ± 0.13
c-Fos/Jun-B	22.37 ± 1.95[a]	1.35 ± 0.23
c-Fos/Jun-D	19.30 ± 0.18[a]	1.34 ± 0.40
c-Jun/Jun-B	22.86 ± 0.10[a]	2.12 ± 0.07[a]
c-Jun/Jun-D	18.30 ± 1.44[a]	1.77 ± 0.50
Jun-B/Jun-D	12.57 ± 0.70[a]	1.13 ± 0.22

[a] $p < 0.05$.

lowed by Western blot analysis of the precipitants for the presence of phospho-CREB indicated an interaction between the proteins (Fig. 8*A*, *upper panel*). As expected the CREB-CBP interaction was increased in HepG2 cells previously treated with 100 μM 8-CPT-cAMP. Likewise, incubating cells with 100 μM H-7, a protein kinase inhibitor, partially blocked the protein association. No binding to CREB was observed when HA-tagged empty vector was used as the control. None of the treatments had any effect on endogenous levels of CREB in HepG2 cells (Fig. 8*A*, *lower panel*). These results strongly suggest that the presence of a basal level of phosphorylated CREB is able to recruit CBP in HepG2 cells despite the absence of exogenous cAMP stimulus.

It was of interest to determine whether ALAS promoter activity in HepG2 cells transfected with p-354ALAS/CAT correlates with CREB-CBP interaction in the conditions tested in the immunoprecipitation assay. As shown in Fig. 8*B*, cAMP-treated cells displayed the highest CAT activity, whereas the presence of the inhibitor H-7 diminished it below the basal levels, suggesting that the extent of CREB and CBP association is critical for ALAS promoter activity.

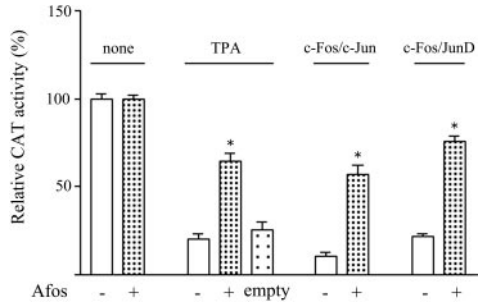

FIG. 6. **Effect of a dominant negative variant of c-Fos on the TPA or Fos/Jun-mediated inhibition of ALAS promoter activity.** HepG2 cells were transiently transfected with 4 μg/plate of p-354ALAS/CAT and cotransfected or not with 3 μg/plate of each of the indicated expression vectors for Fos and Jun proteins. Non-cotransfected samples were incubated in serum-free medium containing 1 μM TPA during 24 h. Transfections may include 6 μg/plate of expression vector for A-Fos or the empty vector as indicated. Results are expressed as relative CAT activity with respect to basal value of p-ALAS/CAT, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to compare in each group samples cotransfected and non-cotransfected with A-Fos. *, $p < 0.05$.

TABLE II
Effect of a dominant negative variant of c-Fos on the expression of a reporter vector containing TRE

HepG2 cells were transiently transfected with 4 μg/plate of TRE-tk-CAT and cotransfected or not with 3 μg/plate of each of the indicated expression vectors for Fos and Jun proteins. A non-cotransfected sample was incubated in serum-free medium containing 1 μM TPA for 24 h. Transfections may include 6 μg/plate of expression vector for A-Fos as indicated. Results are expressed as relative CAT activity with respect to basal value of TRE-tk-CAT, which was set to 100. Values are the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected or not with A-Fos.

Treatment	None	A-Fos
	% Relative CAT activity	
None	100 ± 2	95 ± 7
TPA	365 ± 43	124 ± 13[a]
c-Fos/c-Jun	338 ± 13	135 ± 5[a]
c-Fos/Jun-D	313 ± 43	131 ± 1[a]

[a] $p < 0.05$.

Relative Position of CRE-ALAS and TRE-ALAS in a Heterologous Promoter Drives TPA Responsiveness—To strengthen the previous analysis we constructed three expression vectors cloning two different regions of ALAS promoter in the heterologous reporter vector pBLCAT2. As shown in Fig. 9*A*, vector pASCAT comprises the region between −354 and −156 bp containing the critical TRE-ALAS for TPA-mediated inhibition, vector pAECATd comprises the −354 to −38-bp region including TRE-ALAS and the two CRE-ALAS sites, and vector pAECATi contains the same sequence but in the inverse direction. In agreement with our hypothesis, when the TRE-ALAS region was attached to the thymidine kinase promoter bound to the CAT gene (pASCAT), it expressed high levels of CAT activity upon transfection in HepG2 cells treated with 1 μM TPA. This induction was slightly increased by CBP co-transfection (Fig. 9*B*). On the contrary, transfection of the fusion gene containing TRE and CRE sites (pAECATd) in TPA-treated cells resulted in a decreased CAT expression that was blocked by CBP overexpression, in agreement with the results obtained with the ALAS promoter (Fig. 9*B*). Interestingly, TPA induced CAT activity when vector pAECATi, with TRE and CRE sites in inverted position, was transfected.

In a previous work we demonstrate that the effect of TPA in reducing ALAS expression was dominant over the stimulatory action of cAMP (23). To assess the possibility that the stimu-

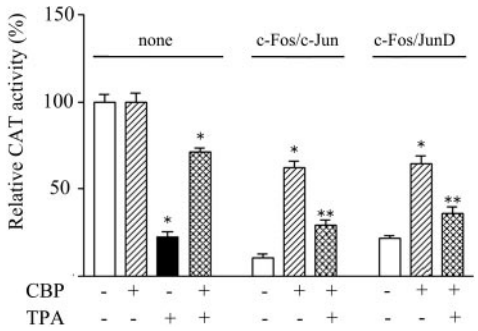

FIG. 7. **CBP overexpression blocks the inhibitory effect of TPA or Fos/Jun-mediated inhibition of ALAS transcriptional activity.** HepG2 cells were transiently transfected with 4 μg/plate of p-354ALAS/CAT and cotransfected or not cotransfected with 3 μg/plate of each of the indicated expression vectors for Fos and Jun proteins. Transfections may include 4 μg/plate of expression vector for CBP coactivator as indicated. Some cell cultures were treated with 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to non-cotransfected sample, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to reveal significant differences in each group. *, $p < 0.05$, between samples cotransfected and non-cotransfected with CBP; **, $p < 0.05$, between samples cotransfected with CBP plus TPA-treated and samples cotransfected with CBP only.

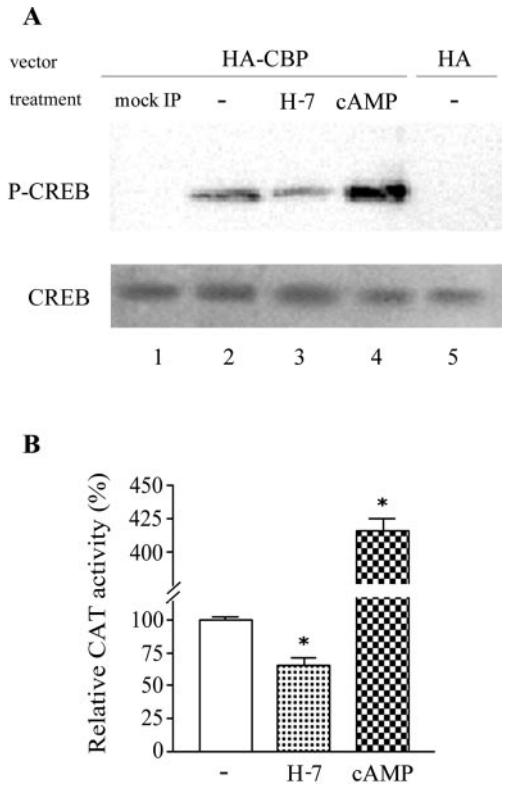

FIG. 8. **CBP interacts with CREB under basal conditions in HepG2 cells.** *A*, HA-tagged CBP protein was expressed in HepG2 cells before treatment with 100 μM H-7 or 100 μM 8-CPT-cAMP (*cAMP*). Equal amounts of proteins from cell lysates were immunoprecipitated (*IP*) using a monoclonal anti-HA antibody. The immune complexes were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by Western blot with an anti-phospho (Ser-133)-CREB antibody (*upper panel*). Parallel samples of HepG2 cells used in immunoprecipitation assay were electrophoresed, transferred to membranes, and probed with an anti-CREB antibody that recognizes total CREB (*lower panel*). Immunoprecipitation and direct immunoblotting experiments were performed twice and showed similar results. *B*, HepG2 cells were transiently transfected with 4 μg/plate of p-354ALAS/CAT and treated with 100 μM H-7 or 100 μM 8-CPT-cAMP (*cAMP*). After 24 h, cells were harvested, and CAT activity was determined as described. Results are expressed as relative CAT activity with respect to non treated sample, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to compare treated with non treated samples. *, $p < 0.05$.

latory and inhibitory effects of cAMP and TPA, respectively, would be a consequence of the relative positions of the sites for its specific binding factors, we tested the action of 100 μM 8-CPT-cAMP and 1 μM TPA in cotransfection experiments with pAECATd or pAECATi. As shown in Fig. 10, TPA inhibited the transcription activity of the ALAS promoter in the right orientation, whereas the phorbol ester induced ALAS/CAT expression when the promoter was located in the inverted position. Conversely, cAMP induced the expression of the fusion gene when the promoter was in the right orientation but did not display any significant stimulation when the regulatory region was located in the inverted position. We then tested the effect of mutation of the TRE-ALAS in these constructions. TPA inhibitory or stimulatory effects were impaired when TRE-ALAS was mutated in pAECATdm or pAECATim, respectively, whereas the effect of cAMP was not modified. Coincident with the above hypothesis, when cAMP and TPA were jointly added to HepG2 cells, the dominant inhibitory effect of TPA was observed when the promoter was located in the right position. In this case, mutation of TRE-ALAS in the direct orientation promoter alleviated the TPA dominant inhibition and allowed cAMP to induce CAT activity, whereas mutation in the inverted orientation promoter did not modify reporter activity, suggesting that increasing the distance between the CRE and the transcription initiation sites impairs the ability of cAMP to induce ALAS promoter activity. To support these findings, a similar experiment was performed in HepG2 cells cotransfected with the expression vector for CBP co-activator. As shown in the same figure, overexpression of CBP either blocked the inhibitory effects or enhanced the stimulatory action observed in the absence of the coactivator.

These results strongly suggest that the relative position of TRE with respect to CREs could drive the response to TPA and also reinforce the idea that competition by co-activator CBP could be involved in TPA-mediated inhibition of ALAS transcriptional activity.

DISCUSSION

The aim of this work was to determine the molecular mechanism involved in the TPA-mediated inhibition of the rat ubiquitous ALAS gene in human hepatoma HepG2 cells. Previous analysis of the 5′-flanking sequence of ALAS gene revealed the existence of binding sites for nuclear respiratory factor I (39) and for transcription factor CREB (25). We describe for the first time a DNA sequence (TRE-ALAS) that binds AP-1 proteins and is necessary for the inhibitory effect of phorbol esters on ALAS gene expression. Based on Northern blot analysis and transient transfection experiments with a promoter-reporter fusion gene containing a 870-bp fragment of the 5′ region of the ALAS gene, we conclude that the effect of TPA occurs at the transcriptional level. We have previously demonstrated that phorbol esters do not modify the ALAS mRNA turnover (23).

The well documented interaction of phorbol esters with PKC (40) suggests that protein phosphorylation is involved in the mechanism by which TPA suppresses ALAS mRNA transcription. The blockage of TPA inhibitory effect caused by a specific inhibitor of PKC such as calphostin C (41), the desensitization developed after pretreatment of HepG2 cells with TPA, and the fact that 4αPDA was ineffective in reducing ALAS promoter activity support the hypothesis that PKC activation mediates TPA inhibitory effect on ALAS gene expression.

Deletion analysis of the 5′-regulatory region of the ALAS gene allowed us to identify a major TPA-responsive region located at −354 to −156 bp. Transfection assays with plasmids containing sequences shorter than −156 bp of the ALAS pro-

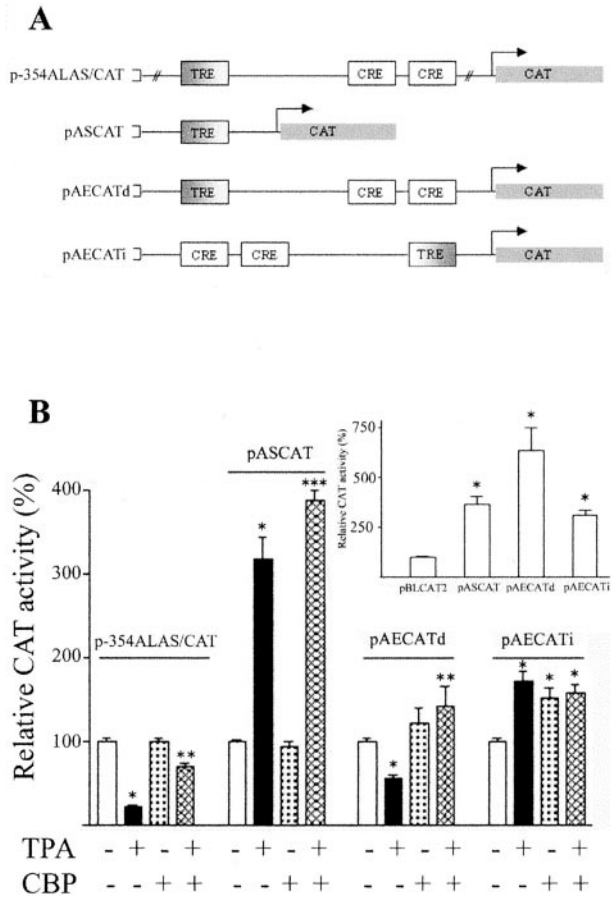

FIG. 9. **TRE-ALAS site linked to heterologous promoter confers TPA responsiveness.** *A*, maps of the p-354ALAS/CAT and chimerical ALAS promoters indicating the elements that are present. *B*, HepG2 cells were transiently transfected with 6 μg/plate of p-354ALAS/CAT or the depicted chimerical expression vectors and cotransfected or not with 4 μg/plate of expression vector for CBP coactivator as indicated. Some cell cultures were treated with 1 μM TPA for 24 h. For each ALAS/CAT expression vector, results are expressed as relative CAT activity with respect to non-cotransfected and non-TPA-treated sample, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to reveal significant differences. *, $p < 0.05$, between samples treated with TPA, samples cotransfected with CBP, or samples cotransfected with CBP plus TPA-treated, non-treated, and non-cotransfected samples; **, $p < 0.05$, between samples cotransfected with CBP plus TPA-treated and samples treated with TPA; ***, $p < 0.05$, between samples cotransfected with CBP plus TPA-treated and samples cotransfected with CBP). *Inset*, relative basal CAT activity of each chimerical ALAS/CAT expression vector respect to empty vector (pBLCAT2), which was set to 100. *, $p < 0.05$.

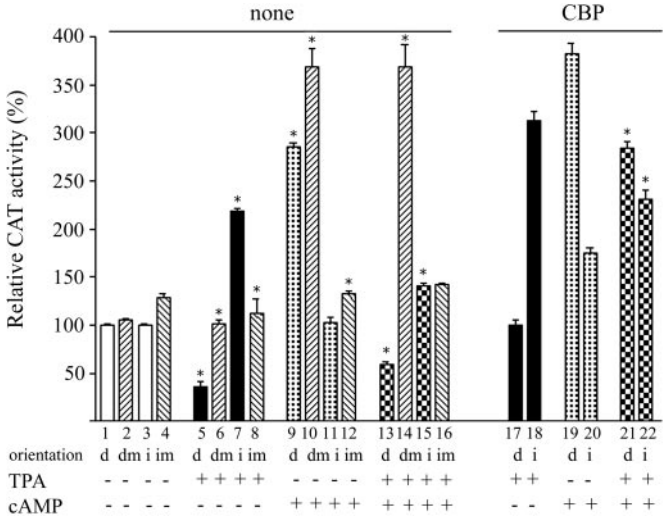

FIG. 10. **Relative position of CRE and TRE sites in a heterologous promoter drives TPA and cAMP responsiveness.** HepG2 cells were transiently transfected with 6 μg/plate of pAECATd (*d*) or pAECATi (*i*) or their mutated version (pAECATdm (*dm*) or pAECATim (*im*)) and cotransfected or not with 4 μg/plate of CBP expression vector as indicated. Some cell cultures were treated with 1 μM TPA and/or 100 μM 8-CPT-cAMP (*cAMP*) for 24 h. For each ALAS/CAT expression vector, results are expressed as relative CAT activity with respect to the wild type version of non-cotransfected and non-treated sample, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to reveal significant differences. *, $p < 0.05$, between samples 5, 9, and 13 and sample 1; *, $p < 0.05$, between sample 7 and sample 3; *, $p < 0.05$, between sample 13 and sample 9; *, $p < 0.05$, between sample 15 and sample 7; *, $p < 0.05$, between sample 21 and sample 13; *, $p < 0.05$, between sample 22 and sample 15. In all cases, relative CAT activity of mutants was compared with their respective wild type version.

moter in HepG2 cells abrogate the response to the influence of TPA. This sequence contains a TRE motif at −261 bp that differs from the consensus sequence in only 1 nucleotide (42). The mutation of this TRE-ALAS site abolished the response to TPA in the p-354ALAS/CAT vector.

Additional evidence for functional TRE in the ALAS gene is as follows. First, CAT activity was reduced when a reporter gene containing the TRE region was cotransfected with a combination of vectors that expressed c-Fos and c-Jun or c-Fos and JunD, proteins that belong to the AP-1 family. Second, cotransfection with the dominant negative A-Fos abolished the response of the ALAS promoter to the inhibitory effect of either TPA or c-Fos/c-Jun or c-Fos/JunD. The suggested effect is the titration of c-Jun and JunD factors (28). Finally, TRE-ALAS conferred sensitivity to the thymidine kinase promoter on the action of TPA when this heterologous plasmid was transfected to HepG2 cells.

The results of gel-shift experiments merit some comments. Incubation of HepG2 nuclear extracts with an oligonucleotide containing the TRE-ALAS sequence of ALAS determined a complex that was highly increased with extracts from TPA-treated HepG2 cells. This complex was competed with the unlabeled probe in a simultaneous incubation but not with a mutated version. Likewise, competition with an unlabeled consensus AP-1 probe indirectly confirmed the TRE-ALAS identity. It has been reported that all Jun·Jun and Jun·Fos complexes have the same binding specificity but exhibit different binding affinities (4). Thus, Jun and Fos proteins have the ability to interact among themselves and activate different targets that contain AP-1-response elements in their promoters. In addition to transfection experiments, supershift assays confirm that nuclear proteins that bind to the TRE-ALAS are most likely composed of c-Fos and c-Jun or c-Fos and JunD. It has been reported that c-Fos, c-Jun, and JunD are expressed in HepG2 cells (43). Therefore, our finding is compatible with the expression pattern of the AP-1 protein family in this cell line. The binding of c-Jun and c-Fos to the TRE-ALAS site after treatment of HepG2 cells with TPA is not surprising given that these nuclear proteins can be induced rapidly and transiently and display enhanced AP-1 binding activity upon treatment of cultured cells with an array of compounds, including TPA (44). Conversely, JunD appears to be constitutively expressed as relatively high levels in cultured cells, including hepatomas, with only modest increases in mRNA levels after treatment with TPA or growth factors (45). Formation of heterodimeric and homodimeric complexes between Fos and Jun family members and their association with DNA depends on their zipper domains. Thus, the Fos zipper avidly binds to the Jun zipper but does not bind to itself. Members of the Jun family can weakly homodimerize or form stronger heterodimeric com-

plexes with Fos family members (46). This could be the reason for the presence of Fos in the two forms of AP-1 that bind TRE-ALAS site. Because TRE-ALAS does not exactly match with the consensus TRE site, the stronger AP-1 factors could establish the most stable complexes on the DNA and would be the most effective inhibitors.

The inhibitory effect of TRE-ALAS on the transcriptional activity of the ALAS promoter is rather interesting since the presence of the same sequence in a heterologous promoter enhanced TPA transcription. 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 (47). Previous analysis of the 5′-flanking sequence of ALAS revealed the existence of two CRE sites. This elements are required for basal and cAMP-stimulated expression of the ALAS gene in hepatoma human cells (25). Our results show that TPA inhibitory effect can be blocked by CBP overexpression. Thus, our hypothesis to explain TPA inhibitory effect is based on the competition between AP-1 and CREB factors for limiting amounts of CBP. There are several evidences on the interaction of CBP with CREB and Fos/Jun proteins (8, 48) as well as on the competition mechanisms that regulate the action of different transcription factors, which involve CBP and other coactivators. Fronsdal *et al.* (49) provide evidence that the transcriptional interference between androgen receptors and AP-1 may be mediated through competition for limiting amounts of CBP. Similarly, DiSepio *et al.* (19) suggest that the mutual transrepression of the retinoic acid-receptor and AP-1 might be due to the competition for limiting coactivators, including CBP. In this regard, our demonstration that CBP interacts with CREB under basal conditions in HepG2 cells and that the extent of this association is correlated with the ALAS promoter activity provides a strong support for our hypothesis.

Even though the competition between different transcription factors for CBP is well known, there is no conclusive evidence that coactivator levels are limiting, as has been proposed (17, 50). Karin and Chang (51) suggest that, because the amounts of nuclear CBP/p300 seem to exceed those of AP-1 or glucocorticoid receptor and CBP/p300 is also a common target for many other sequence-specific transactivators, which do not transrepress AP-1 activity, it is unlikely that simple competition for a limiting amount of CBP/p300 can explain the transrepression of AP-1 activity by glucocorticoid receptor. Despite this, our results present a cross-talk between different signal pathways that regulate gene expression, like cAMP and TPA, and that occur at the level of a coactivator.

Finally, our work highlights the importance of the natural promoter context when studying AP-1-mediated gene expression. It seems likely that a completely orientation-dependent silencer or enhancer acts by presenting its specific binding factor in a particular position or direction relative to other regulatory sequences or factors (52). We suggest that the contribution of the AP-1 binding factors to the promoter activity might be determined mainly by the promoter context (*e.g.* surrounding binding sites for other transcription factors) rather than by the element itself. Results depicted in Figs. 9 and 10, in which the TRE-ALAS site was placed in different contexts with respect to CRE sites, thus acting as a transcriptional enhancer, support this view.

In addition to the feedback regulation by heme (21) and in addition to the transcription inhibitory effect described in this paper, ALAS is also repressed by insulin (53). Recently, the postreceptor-signaling mechanisms for insulin regulation of ALAS gene expression has begun to be uncovered. We suggest that both pathways, phosphatidylinositol 3-kinase/PKB and

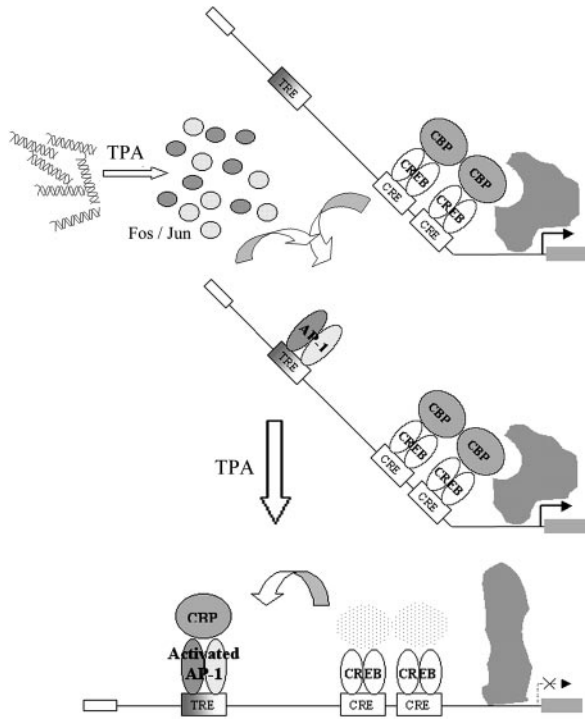

FIG. 11. **Model of positive and negative cross talk modulated by the transcriptional coactivator CBP in ALAS promoter.** ALAS basal expression requires the interaction between CREB and the promoter CRE sites, thus facilitating the incorporation of coactivator CBP. The formation of this complex would have a stabilizing effect on the transcription mechanism in basal conditions (25). TPA stimulates the synthesis of proteins from the AP-1 family, which would occupy TRE sites located upstream CRE sites. The subsequent activation of Fos/Jun factors due to post-transductional changes would lead to CBP shift from the CRE·CREB complex to further TRE·AP-1 complex, thus destabilizing the transcription mechanism and causing the inhibition of ALAS gene expression.

Ras/mitogen-activated protein kinase/p90^{RSK} are jointly required for insulin-mediated inhibition of ALAS gene expression in rat hepatocytes and human hepatoma cells (54). Also, the activation of PKC is indispensable for the transduction of the insulin effect to the ALAS promoter, although the connection with the mentioned pathways remains unclear (53). In a number of instances investigators have described that phorbol esters mimic the action of insulin (55). On the other hand, there is evidence that insulin increases diacylglycerol concentrations and PKC activity (56). Because PKC is a primary target of phorbol esters, one could speculate that this protein kinase acts as a common factor linking insulin and TPA signaling. However, there are several reports demonstrating that genes that are acutely inhibited by insulin present different responses to this hormone after inhibition or down-regulation of PKC (57–59). In the particular case of ALAS, beyond the involvement of PKC in both insulin and TPA signaling, the phorbol ester inhibits transcription through *cis*-acting elements, the TRE site, and *trans*-acting factors, AP-1 proteins, that differ from those involved in insulin regulation of ALAS promoter activity, which would include hepatocyte nuclear factor-3 and NF-1 sites (this paper and Ref. 54).

There are many signals that activate PKC in hepatocytes. Various prostaglandins, metabolites of arachidonic acid, and other lipid mediators produced by phospholipases C and D are thought to play important roles in hepatocyte proliferation through PKC activation (60). The proliferative action of transforming growth factor-α is mediated by phospholipase C and PKC, among other effectors (61). Moreover, mechanisms involving diacylglycerol and PKC seem to play a role in the

mitogenic effects of various agents that bind to G protein-coupled receptors and activate cells in early G_1, such as norepinephrine, angiotensin II, and vasopressin (62).

The inhibition of ALAS expression can have important physiologic outcomes. Its imbalanced repression results in a deficit of vital hemeproteins, such as hemoglobin, enzymes taking part in cellular respiration, reduction of sulfite and nitrite, neutralization of reactive oxygen species, and the oxidative metabolism of lipophilic xenobiotics, as a consequence of an impaired synthesis of heme (21, 63). The inhibitory effect of TPA-mediated PKC activation on the ALAS transcription could explain the apoptotic cellular response driven by phorbol ester treatment (64, 65). One of the reasons could be the increase of free radicals produced by TPA and the decrease in the amount of peroxisomal hemeproteins involved in neutralizing reactive oxygen species. A similar mechanism could be utilized by prostaglandin A_2 to induce apoptosis in human hepatocarcinoma Hep3B and HepG2 cells (66).

In summary, our results demonstrate the presence of a functional TRE site on the ALAS promoter that, upon TPA stimulation, interacts with c-Fos/c-Jun or c-Fos/JunD heterodimers. We propose that the decrease in ALAS basal activity observed in the presence of TPA may reflect a reduction in the capability of this promoter to assemble the productive pre-initiation complex involving the interaction of CREB-CBP with the CRE sites through sequestration of the coactivator (Fig. 11). We also demonstrate that the transcriptional properties of this AP-1 site would depend on a spatial-disposition-dependent manner with respect to CRE sites and/or the transcription initiation site.

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