Journal of Cellular Biochemistry 96:1003–1020 (2005)

Regulation of GTP Cyclohydrolase I Gene Transcription by Basic Region Leucine Zipper Transcription Factors

Jude Al Sarraj,[1] Charles Vinson,[2] Jiahuai Han,[3] and Gerald Thiel[1]*

[1]Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center, Homburg, Germany

[2]Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

[3]Department of Immunology, The Scripps Research Institute, La Jolla, California

Abstract Tetrahydrobiopterin is an essential cofactor for the phenylalanine, tyrosine and tryptophan hydroxylases, and the family of nitric oxide synthases. The initial and rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin is GTP cyclohydrolase I. The proximal promoter of the human GTP cyclohydrolase I gene contains the sequence motif 5′-TGACGCGA-3′, resembling a cAMP response element (CRE). The objective of this study was to analyze the regulation of GTP cyclohydrolase I gene transcription by basic region leucine zipper (bZIP) transcription factors. A constitutively active mutant of the cAMP response element binding (CREB) protein strongly stimulated GTP cyclohydrolase I promoter activity, indicating that the CRE in the context of the GTP cyclohydrolase I gene is functional. Likewise, GTP cyclohydrolase I promoter/luciferase gene transcription was stimulated following nuclear expression of the catalytic subunit of cAMP-dependent protein kinase. Constitutively active mutants of activating transcription factor 2 (ATF2) and c-Jun additionally stimulated GTP cyclohydrolase I promoter activity, but to a lesser extent than the constitutively active CREB mutant. The fact that stress-activated protein kinases target the GTP cyclohydrolase I gene was corroborated by expression experiments involving p38 and MEKK1 protein kinases. We conclude that signaling pathways involving either the cAMP-dependent protein kinase or stress-activated protein kinases converge to the GTP cyclohydrolase I gene. Hence, enzymatic reactions that require tetrahydrobiopterin as cofactor are therefore indirectly controlled by signaling cascades involving the signal-responsive transcription factors CREB, c-Jun, and ATF2. J. Cell. Biochem. 96: 1003–1020, 2005. © 2005 Wiley-Liss, Inc.

Key words: ATF2; CREB; c-Jun; MEKK1; p38 protein kinase; Egr-1 promoter; TNFα promoter

6(R)-5,6,7,8-tetrahydrobiopterin (BH_4) is synthesized from GTP by three enzymatic reactions, involving GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase. The rate-limiting enzyme for the synthesis of tetrahydrobiopterin (BH_4) is GTP cyclohydrolase I (EC 3.5.4.16), which catalyzes the formation of dihydroneopterin triphosphate from GTP. The GTP cyclohydrolase I holoenzyme is a homodecameric protein composed of two pentamers facing one another.

Tetrahydrobiopterin is as an essential cofactor for the monooxygenases phenylalanine, tyrosine, and tryptophan hydroxylases [Hufton et al., 1995]. A deficiency in tetrahydrobiopterin impairs therefore the synthesis of dopamine, noradrenaline, adrenaline, serotonin, and melatonin. In addition, for maximal activity, nitric oxide synthase, which synthesizes nitric oxide from L-arginine, requires tetrahydrobiopterin as cofactor [Gross et al., 2000]. Nitric oxide in turn regulates a vast number of biological processes, including immune modulation, neurotransmission, vasodilation, and programmed cell death. Mutations in the GTP cyclohydrolase I gene causing tetrahydrobiopterin deficiency are responsible for severe diseases including atypical phenylketonuria and the autosomal dominant DOPA-responsive dystonia [Ichinose

Grant sponsor: HOMFOR, Universität des Saarlandes; Grant sponsor: “Deutscher Akademischer Austauschdienst” (DAAD).

*Correspondence to: Gerald Thiel, Department of Medical Biochemistry and Molecular Biology, Building 44, University of Saarland Medical Center, D-66421 Homburg, Germany. E-mail: gerald.thiel@uniklinik-saarland.de

Received 24 March 2005; Accepted 2 June 2005

DOI 10.1002/jcb.20580

© 2005 Wiley-Liss, Inc.

et al., 1994], a hereditary progressive dystonia with marked diurnal fluctuation.

The expression of GTP cyclohydrolase I is regulated at the transcriptional level by extracellular signaling molecules, including the proinflammatory cytokines interferon-γ, interleukin 1β, and tumor necrosis factor (TNF) α [D'Sa et al., 1996; Plüss et al., 1996, 1997; Frank et al., 1998; Geller et al., 2000; Vann et al., 2000; Golderer et al., 2001]. As well, bacterial endotoxins such as lipopolysaccharide (LPS), phytohemagglutinin, the kit ligand, and epidermal growth factor have been shown to stimulate GTP cyclohydrolase I expression [Ziegler et al., 1993; D'Sa et al., 1996; Frank et al., 1998; Geller et al., 2000]. Signaling molecules that elevate the intracellular cAMP concentration may also be involved in the regulation of GTP cyclohydrolase I expression because cell-permeant analogs of cAMP and the adenylate cyclase activator forskolin activate GTP cyclohydrolase I gene transcription in PC12 and mesangial cells [Plüss et al., 1996, 1997; Hirayama et al., 2001]. A cyclic AMP response element (CRE)-like genetic element has been identified in the GTP cyclohydrolase I promoter and the basic region leucine zipper (bZIP) proteins CREB, ATF2, and ATF4 have been proposed to connect elevated intracellular cAMP concentrations with enhanced GTP cyclohydrolase I gene transcription [Kapatos et al., 2000; Hirayama et al., 2001]. Here, we have investigated the transcriptional regulation of a GTP cyclohydrolase I promoter/reporter gene by bZIP transcription factors, using constitutively active mutants. This experimental strategy enabled us to compare the transactivation potential of CREB, CREB2/ATF4, ATF2, c-Jun, and C/EBPα. Furthermore, the dimerization code of bZIP proteins was studied in living cells using a battery of dominant-negative mutants. Finally, expression experiments with constitutively active protein kinases supplemented the overall picture. The results show that CREB is of major importance in upregulation of GTP cyclohydrolase I gene transcription. In addition, we show that ATF2 and c-Jun transactivate the GTP cyclohydrolase I promoter containing reporter gene in a CREB-independent manner. Likewise, expression of the catalytic subunit of cAMP-dependent protein kinase or expression of MAP kinase kinase 6, together with p38 MAP kinase, upregulated reporter gene transcription. In contrast, the bZIP proteins CREB2/ATF4 and C/EBP played either no or only a marginal role in the regulation of GTP cyclohydrolase I promoter activity.

MATERIALS AND METHODS

Reporter Constructs

The reporter plasmid pGTPCHIluc that contains nucleotides from -60 to -97 of the human GTP cyclohydrolase I gene, was generated by subcloning of the synthetic annealed oligonucleotides 5′-TCG AGG GCC GTG ACG CGA GGC GGG GCC GGC CAA TGG GAG-3′ and 5′-TCG ACT CCC ATT GGC CGG CCC CGC CTC GCG TCA CGG CCC-3′ into the Xho I and Sal I-sites of plasmid pHIVTATA-CAT [Thiel et al., 1996]. This sequence was excised together with the minimal promoter and cloned into pGL3-Basic (Promega). Plasmid pGTPCHICREmut-luc was generated by the insertion of the annealed oligonucleotides 5′-CGA GGG CCG TAA TTC GAG GCG GGG CCG GCC AAT GGG AGC 3′ and 5′-TCG AGC TCC CAT TGG CCG GCC CCG CCT CGA ATT ACG GCC CTC GGT AC -3′ with KpnI/XhoI cohesive ends into the KpnI/XhoI sites of plasmid pHIVTATAluc. The transcription units present in the reporter plasmids contain a minimal promoter consisting of the human immunodeficiency virus TATA box and the adenovirus major late promoter initiator element. The TNFα promoter/luciferase construct pTNFα(-1311)luc [Rhoades et al., 1992] was a kind gift of James S. Economou, UCLA, Los Angeles. To construct the reporter plasmid pTNFα(-131)luc, we cloned a Bsp120I/HindIII fragment of plasmid pTNFα(-1311)luc into pGL3-Basic. The Egr-1 promoter/luciferase reporter plasmid pEgr-1.1luc has been described [Groot et al., 2000] as well as plasmid pUAS^{5}luc, containing five binding sites for GAL4 ('upstream activating sequence,' UAS) [Thiel et al., 2000]. Plasmid 2xC/EBPluc that has two C/EBP binding sites upstream of a minimal herpes simplex virus thymidine kinase promoter was a kind gift of Peter F. Johnson, NCI-Frederick Cancer Research and Development Center. The asparagine synthetase promoter nutrient-sensing response unit/luciferase reporter gene pASluc has been described [Thiel et al., 2005b].

Expression Vectors

The expression vector pCMV-FLAG-C2/c-Jun, encoding a constitutively active CREB2/c-Jun

chimera, was generated by cloning of an EheI/XbaI fragment of the c-Jun cDNA into plasmid pCMV-FLAG-C2N [Steinmüller and Thiel, 2003]. The encoded protein contains the transcriptional activation domain of CREB2/ATF4 (amino acids 1–187) linked to the C-terminal region of c-Jun (amino acids 188–331). The plasmid pCMVFLAG-C2/C/EBPα encodes the chimeric transcription factor C2/C/EBPα that consists of the N-terminal transcriptional activation domain of CREB2/ATF4 (amino acids 1–187) and the C-terminal bZIP domain of C/EBPα (amino acids 245–358). Expression vectors encoding FLAG-tagged C/EBPα, CREB2/ATF4, and constitutively active ATF2 and CREB mutants (C2/ATF2, C2/CREB) have been described [Steinmüller and Thiel, 2003; Averous et al., 2004; Thiel et al., 2005a,b].

The GAL4 expression plasmid pFA2ATF2 was purchased from Stratagene. The GAL4-c-Jun expression plasmid pGAL4-c-Jun was a kind gift of Michael Karin, University of California San Diego. Expression vectors encoding A-CREB, A-ATF2, A-ATF4, A-Fos, and A-C/EBP have been described [Olive et al., 1997; Ahn et al., 1998; Steinmüller et al., 2001; Vinson et al., 2002; Thiel et al., 2004]. The expression vectors pEGBN, pEBGN-CREB, and pEBGN-KCREB that encode GST or the GST fusion proteins GST-CREB and GST-KCREB have been described [Thiel et al., 2005b] as well as the expression vectors encoding truncated forms of ATF2 and c-Jun (ATF2ΔN and c-JunΔN) [Steinmüller and Thiel, 2003]. An expression vector encoding the catalytic subunit of PKA (pCMVCα) was a kind gift of Michael Uhler from the University of Michigan, Ann Arbor [Uhler and McKnight, 1987]. The expression vector encoding NLSCα has been described [Thiel et al., 2005b]. An expression plasmid encoding MEKK1Δ, a truncated form of MEK kinase 1 that lacks amino acids 1–351 [Minden et al., 1994] was a kind gift of Michael Karin, University of California, San Diego. Expression vectors encoding FLAG-tagged p38 MAP kinase and a constitutively active form of MAP kinase kinase termed MKK6b(E) have been described [Han et al., 1996; Jiang et al., 1996].

Cell Culture, Transient Transfections and Reporter Gene Assays

Human HepG2 hepatoma cells were cultured and transfected as described [Steinmüller et al., 2001]. The amounts of expression vectors transfected are indicated in the figure legends. The luciferase reporter plasmids (1 μg/plate) and one of the internal reference plasmids pRSVβ or pSV40lacZ were transfected into cells grown on 60 mm plates. Cells were harvested 48 h post-transfection. Lysates were prepared using cell culture lysis buffer (Promega) and β-galactosidase and luciferase activities were measured [Thiel et al., 2000].

Normalizing Transfection Data and Statistics

Luciferase activity was normalized to β-galactosidase activity, which served as control for transfection efficiency. Data from a single experiment (transfection in quadruplicate) are reported as mean ± SD and are representative of at least three different experiments.

RESULTS

A Constitutively Active CREB2/CREB Mutant Transactivates a Reporter Gene Under Control of the Proximal Regulatory Region of the Human GTP Cyclohydrolase I Gene

GTP cyclohydrolase I gene expression has been shown to be regulated by the intracellular concentration of cAMP in renal mesanglial and neuroblastoma cells [Plüss et al., 1996, 1997; Hirayama et al., 2001], suggesting that the cAMP-activated transcription factor CREB may be responsible for the transactivation of the GTP cyclohydrolase I gene. In addition, the sequence motif 5′-TGACGCGA-3′, resembling a cAMP response element (CRE), has been identified in the core promoter of the human GTP cyclohydrolase I gene. To directly test whether CREB transactivates the GTP cyclohydrolase I gene via the CRE-like sequence, we used a constitutively active CREB mutant termed C2/CREB. Figure 1A shows the modular structure of the transcription factors CREB, CREB2/ATF4, and C2/CREB. All three proteins have a bZIP domain on their C-termini responsible for dimerization and DNA-binding. The N-termini of CREB and CREB2/ATF4 contain activation domains. While the activation domain of CREB2/ATF4 is constitutively active and transferable to heterologous DNA-binding domains [Schoch et al., 2001], the activation domain of CREB is controlled by phosphorylation. The C2/CREB fusion protein contains the bZIP domain of CREB, which is responsible for DNA binding and dimerization, fused to the

transcriptional activation domain of CREB2/ATF4. A reporter plasmid was used that contained the proximal region of the human GTP cyclohydrolase I gene, upstream of a minimal promoter (Fig. 1B). To study the regulation of this GTP cyclohydrolase I/luciferase reporter gene by C2/CREB, we transfected human HepG2 hepatoma cells with the pGTPCHIluc reporter plasmid together with either the "empty" expression vector pCMV5 (denoted "−") or an expression vector encoding C2/CREB (plasmid pCMVFLAG-C2/CREB) (denoted "+"). In addition, we transfected plasmid pRSVβ, encoding β-galactosidase under the control of the Rous sarcoma virus long terminal repeat, to correct for variations in transfection efficiencies. Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities. Expression of C2/CREB induced reporter gene transcription on the order of 77-fold (Fig. 1C), indicating that transcription of the GTP cyclohydrolase I/luciferase reporter gene was strongly transactivated by C2/CREB.

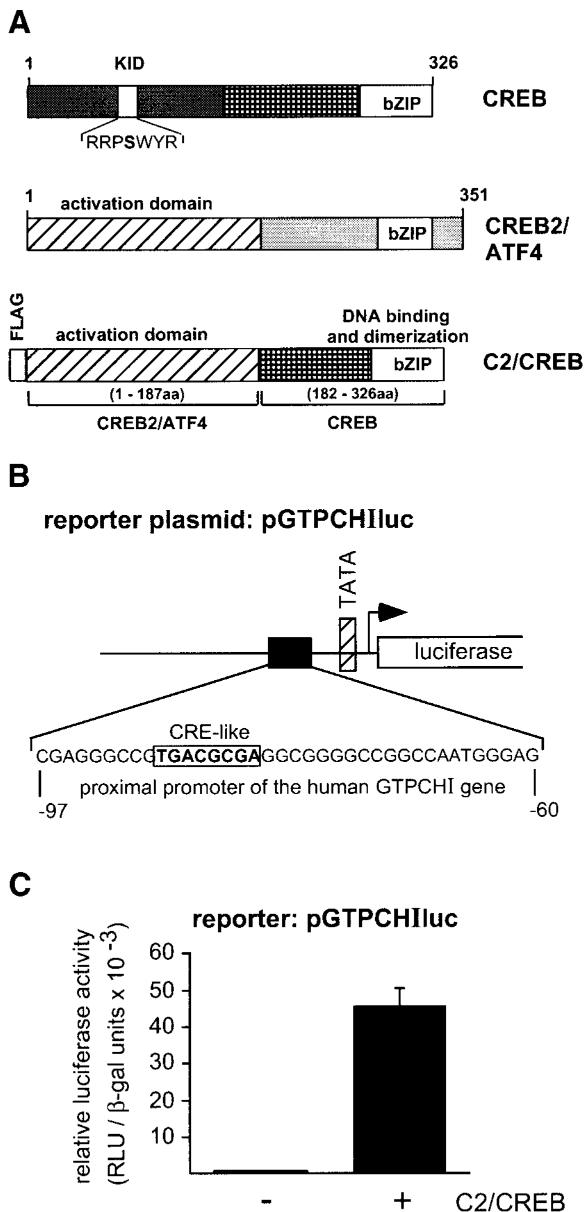

Fig. 1. The constitutively active CREB mutant C2/CREB stimulates transcription of a GTP cyclohydrolase I promoter/luciferase reporter gene. **A**: Schematic representation of the modular structure of CREB, CREB2/ATF4, and C2/CREB. The basic region leucine zipper (bZIP) domain is indicated. The chimeric bZIP protein C2/CREB consists of the constitutively active transcriptional activation domain of CREB2/ATF4 and the bZIP domain of CREB, responsible for dimerization and DNA-binding. **B**: The reporter plasmid pGTPCHIluc contains GTP cyclohydrolase I promoter sequences from −97 to −60 upstream of a minimal promoter consisting of the human immunodeficiency virus TATA box, the adenovirus major late promoter initiator element and the luciferase open reading frame. The position and sequence of the CRE-like motif is shown. **C**: The reporter plasmid pGTPCHIluc was transfected into HepG2 cells together with the pRSVβ internal standard plasmid, encoding β-galactosidase under the control of the Rous sarcoma virus long terminal repeat, and either the "empty" expression vector pCMV5 or an expression vector encoding C2/CREB (25 ng plasmid/plate). Lysates were prepared 48 h post-transfection using cell culture lysis buffer (Promega), and β-galactosidase and luciferase activities were measured. The data are presented as the ratio of luciferase activity (light units) to β-galactosidase units (OD units) measured in the cell extracts. The mean ± SD is depicted.

Activation of GTP Cyclohydrolase I Promoter/luciferase Reporter Gene Transcription Following Expression of a Nuclear Targeted Mutant of the Catalytic Subunit of cAMP-Dependent Protein Kinase

The modular structures of the catalytic subunit of cAMP-dependent protein kinase (Cα) and the modified kinase NLSCα are depicted in Figure 2A. The NLSCα mutant has a nuclear targeting signal derived from the SV40 large T antigen (NLS) and a FLAG-epitope. NLSCα has been shown to be highly active in enhancing CRE-mediated transcription in neuronal and hepatoma cells [Thiel et al., 2005a,b]. To supplement the data obtained with C2/CREB, we tested whether expression of NLSCα significantly augmented GTP cyclohydrolase I promoter/luciferase reporter gene transcription. Transfection of a nanomolar concentration of an expression vector encoding the wild-type form of CREB did not significantly change basal

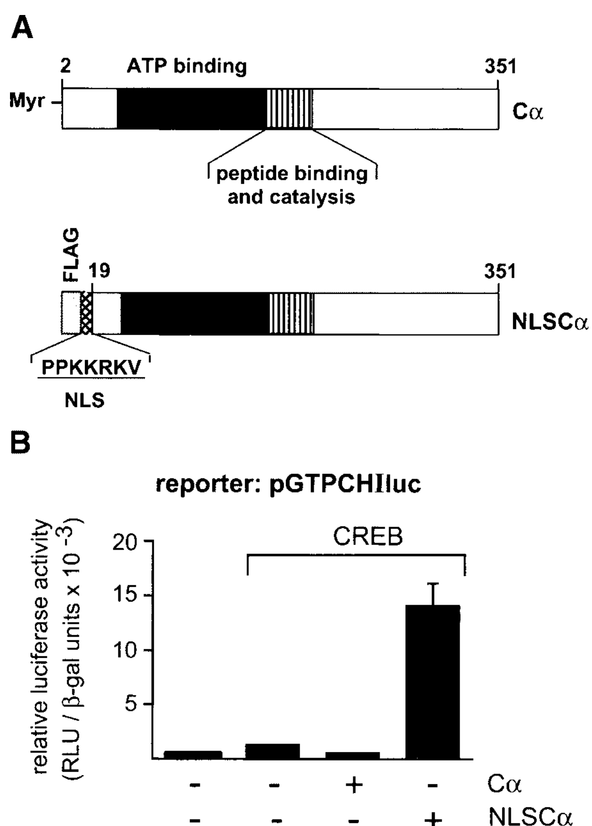

Fig. 2. Expression of the catalytic subunit of cAMP-dependent protein kinase in the nucleus stimulates GTP cyclohydrolase I promoter activity. **A**: Schematic representation of the modular structure of the catalytic subunit of cAMP-dependent protein kinase (Cα) and the mutant NLSCα. The wild-type enzyme is myristylated as indicated (Myr). The location of the nuclear localization signal derived from the SV40 large T antigen (sequence PPKKRKV) and the triple FLAG epitope in NLSCα are shown. **B**: The reporter plasmid pGTPCHIluc was transfected into HepG2 cells together with the pRSVβ internal standard plasmid and the "empty" expression vector pCMV5 or an expression vector encoding wild-type CREB (25 ng plasmid/plate). In addition, an expression vector encoding either Cα or NLSCα (100 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts were determined. The mean ± SD is depicted.

transcription of the reporter gene, as expected, because only the concentration of inactive unphosphorylated CREB was increased. However, additional transfection of an NLSCα expression vector stimulated reporter gene transcription on the order of ninefold (Fig. 2B). In contrast, no transcriptional activation was observed following overexpression of the wild-type form of the catalytic subunit. These results confirm the previous observations, obtained by overexpression experiments of C2/CREB, that the cAMP/PKA signaling pathway enhances GTP cyclohydrolase I gene expression via CREB.

C2/CREB-induced and NLSCα-Mediated Upregulation of GTP Cyclohydrolase I Promoter/Luciferase Reporter Gene Transcription is Selectively Impaired by a Dominant-Negative CREB Mutant

The DNA-binding and dimerization specificities of C2/CREB and CREB are identical because both proteins share an identical bZIP domain. To investigate whether other bZIP proteins are able to interfere with C2/CREB-induced transcriptional activation, we used dominant-negative mutants termed A-ZIPs. These reagents inhibit DNA-binding of the wild-type bZIP proteins in a leucine zipper-dependent fashion. A-ZIP proteins are amphipathic molecules that contain an acidic region instead of the natural basic domain N-terminal to the leucine zipper domain. This acidic extension of the leucine zipper forms a heterodimeric coiled coil structure with the basic region of its target that is more stable than the bZIP dimer bound to DNA. The heterodimer complexes formed between an A-ZIP mutant and a wild-type bZIP protein are defective for DNA-binding [Vinson et al., 2002; Thiel et al., 2004]. A-CREB was the only reagent effective in inhibiting C2/CREB-stimulated transcription of the reporter gene under these conditions (Fig. 3A). Expression of A-ATF2 did not change C2/CREB-induced reporter gene transcription, indicating that ATF2 does not heterodimerize with CREB. A-ATF4 (a dominant-negative of CREB2/ATF4), A-Fos (a dominant-negative of c-Jun), and A-C/EBP (a dominant-negative of C/EBP) even enhanced transcription of the reporter gene, presumably by sequestering inhibitory cofactors away from the transcription units. The competition experiments were repeated using the expression of NLSCα as an inducer of transcriptional upregulation. Expression of wild-type CREB did not stimulate reporter gene transcription, whereas expression of NLSCα strongly enhanced transcription of the reporter gene. The competition assay revealed that only A-CREB impaired CREB/NLSCα-mediated transcription (Fig. 3B). A-C/EBP again enhanced reporter gene transcription. Collectively, these data indicate that a homodimer of C2/CREB or phosphorylated CREB, respectively, mediated transcriptional activation of the GTP cyclohydrolase I promoter/luciferase reporter

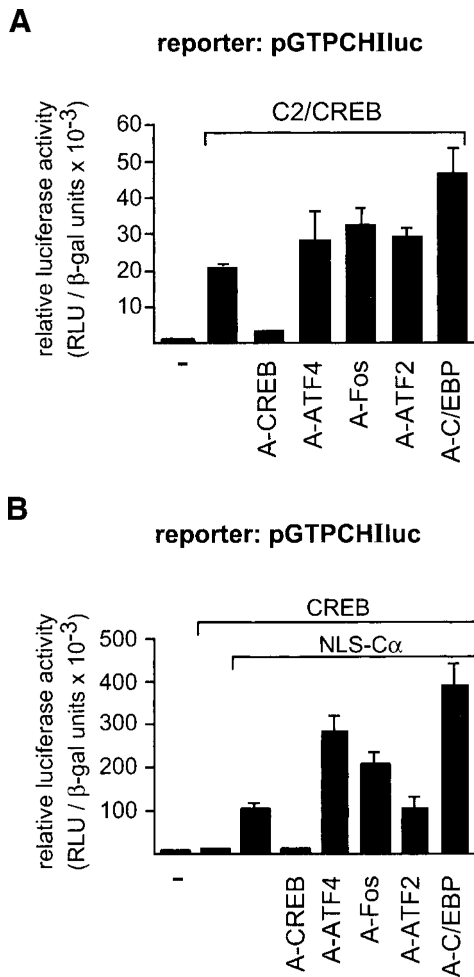

Fig. 3. A dominant-negative mutant of CREB impairs C2/CREB or NLSCα-induced transcriptional activation of the GTP cyclohydrolase I/luciferase reporter gene. **A**: HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, an expression vector encoding C2/CREB, the pRSVβ reference plasmid, and 100 ng/plate of the expression vectors encoding A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. **B**: HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, the pRSVβ reference plasmid, and expression vectors encoding wild-type CREB (25 ng/plate) and NLSCα (100 ng/plate). In addition, expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP (250 ng/plate) were transfected as indicated. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined.

gene, and that the bZIP proteins CREB2/ATF4, c-Jun, ATF2, and C/EBP were unable to heterodimerize with C2/CREB.

CREB2/ATF4 is Unable to Transactivate a GTP Cyclohydrolase I/Luciferase Reporter Gene

CREB2/ATF4 has been proposed to transactivate the GTP cyclohydrolase I promoter in PC12 cells, based on in vitro DNA/protein binding experiments [Kapatos et al., 2000]. We tested the biological effect of CREB2/ATF4 on GTP cyclohydrolase I promoter activity using an expression vector encoding CREB2/ATF4. As a positive control, we used a reporter gene containing the nutrient-sensing response unit of the asparagine synthetase (plasmid pASluc). While low concentrations of CREB2/ATF4 expression vector were sufficient to strongly activate transcription via the nutrient-sensing response unit, no enhancement of GTP cyclohydrolase I promoter activity was observed following overexpression of CREB2/ATF4 (Fig. 4A). Likewise, reporter genes under control of the TNFα (plasmid pTNFα(−131)luc) or the Egr-1 promoter (plasmid pEgr-1.1luc) depicted in Figure 4B, that both contained a CRE-like sequence, were transactivated by the chimeric C2/CREB transcription factor, but not by CREB2/ATF4 (Fig. 4C). We conclude that CREB2/ATF4 is unable to transactivate the genes encoding GTP cyclohydrolase I, TNFα, or Egr-1 via the CRE-like sequences in their proximal promoter regions.

Biological Activity of a Constitutively Active ATF2 Mutant on a GTP Cyclohydrolase I Promoter/Reporter Gene

Based on in vitro DNA/protein binding experiments it has been suggested that ATF2 binds to the GTP cyclohydrolase I core promoter [Hirayama et al., 2001]. To directly analyze whether ATF2 can transactivate a GTP cyclohydrolase I promoter/luciferase reporter gene, we performed expression experiments using the constitutively active ATF2 mutant C2/ATF2 (Fig. 5A). As a control, we tested the effect of ATF2 and C2/ATF2 on TNFα and Egr-1 promoter activity. Both the *TNFα* and the *Egr-1* gene have been described as targets for ATF2 [Tsai et al., 1996; Rolli et al., 1999]. The results show that C2/ATF2 transactivated all three-reporter genes (Fig. 5B). The transcriptional activation of the GTP cyclohydrolase I, TNFα, and Egr-1 promoter was ≈10-fold, 5.8-fold, and 2.8-fold, respectively. In contrast, the wild-type ATF2 was inactive. A competition assay using the expression of the A-ZIP mutants revealed that C2/ATF2-stimulated GTP cyclohydrolase I promoter/luciferase reporter was specifically impaired by A-ATF2 and A-Fos, a dominant-negative to c-Jun. Neither A-CREB nor A-ATF4 nor A-C/EBP were able to block the

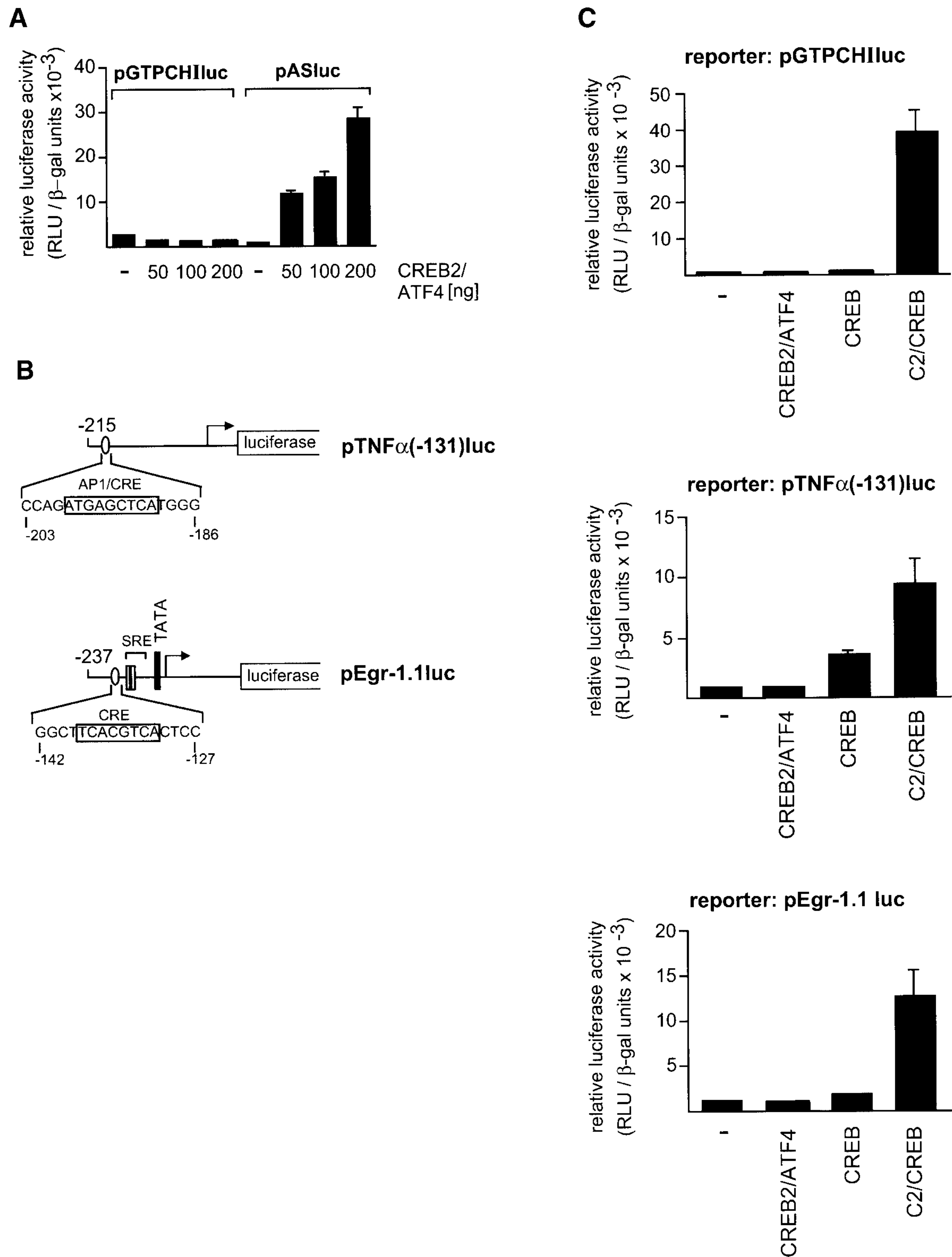

Fig. 4. CREB2/ATF4 does not upregulate GTP cyclohydrolase I, TNFα, and Egr-1 promoter activities. **A**: The reporter plasmids pGTPCHIluc or pASluc were transfected into HepG2 cells, together with plasmid pRSVβ, the expression vectors pCMV5 or an expression vector encoding CREB2/ATF4 (50, 100, 200 ng plasmid/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. **B**: Schematic representation of the TNFα promoter/luciferase and Egr-1 promoter/luciferase reporter plasmids pTNFα(−131)luc and pEgr-1.1luc. The sequence of the CRE-like motifs within these promoters is indicated. SRE, serum response element. **C**: One of the reporter plasmids pGTPCHIluc, pTNFα(−131)luc or pEgr-1.1luc was transfected into HepG2 cells, together with plasmid pRSVβ, the expression vector pCMV5 or expression vectors encoding either CREB or CREB2/ATF4 (25 ng plasmid/plate). For comparison, an expression vector encoding C2/CREB was transfected (25 ng plasmid/plate). Cell extracts were prepared forty-eight hours later and the relative luciferase activities of these extracts were determined. Data are mean ± SD.

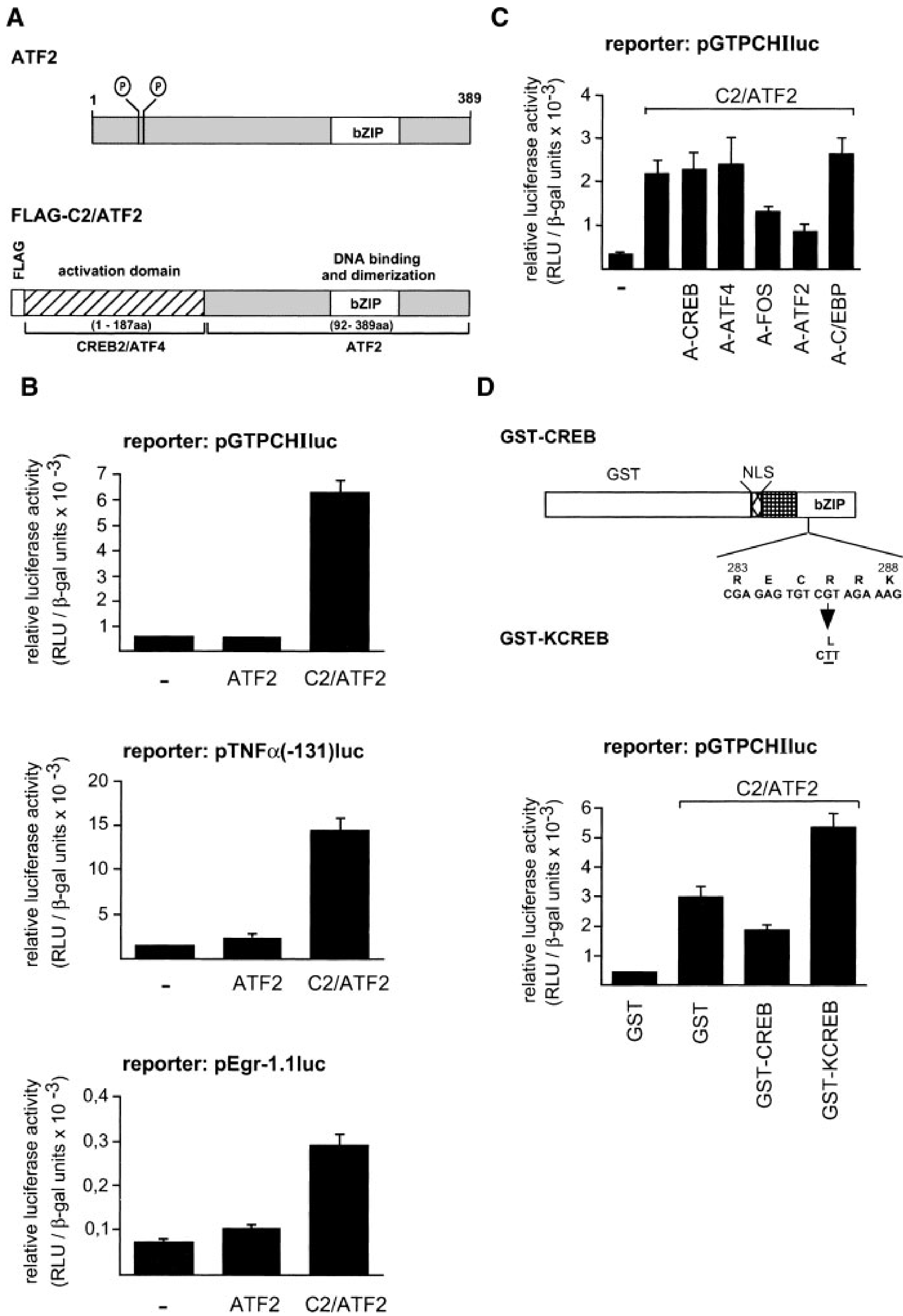

Fig. 5.

transcriptional activity of C2/ATF2 (Fig. 5C). These competition experiments, along with the A-ZIP-mediated competition experiments of C2/CREB-induced transcriptional activation (depicted in Fig. 3A), argue against a heterodimerization of CREB and ATF2. To confirm these results, we tested the effect of the dominant-negative CREB mutants GST-CREB and GST-KCREB upon C2/ATF2-mediated GTP cyclohydrolase I promoter/luciferase reporter gene transcription. GST-CREB has an intact bZIP domain derived from CREB and is therefore able to bind to the cognate sites of CREB, making these sites unavailable for wild-type bZIP proteins. In addition, GST-CREB may also form inactive heterodimers with wild-type CREB. In contrast, GST-KCREB is unable to bind to DNA due to a point mutation in the basic region. GST-CREB impaired C2/ATF2-stimulated transcription of the reporter gene (Fig. 5D). GST-KCREB did not show any negative effect on C2/CREB-mediated transactivation due to its inability to bind to the CRE. Thus, constitutively active mutants of CREB and ATF2 are independently able to transactivate the GTP cyclohydrolase I gene without forming heterodimers, suggesting that ATF2 and CREB compete for the same DNA-binding site.

Stimulation of GTP Cyclohydrolase I Promoter/Luciferase Gene Transcription by p38 MAP Kinase and a Constitutively Active Mutant of MAP Kinase Kinase 6

ATF2 is activated by phosphorylation of the threonine residues 69 and 71, catalyzed by the protein kinases p38 MAP kinase or c-Jun N-terminal protein kinase. To activate p38 protein kinase, we expressed a constitutively active mutant of p38 MAP kinase kinase, MAP kinase kinase 6 (MKK6). p38 MAP kinase is phosphorylated by MKK6 on residues Ser^{207} and Thr^{211}. These residues have been exchanged to glutamic acid residues, generating a constitutively active protein kinase MKK6(E) [Jiang et al., 1996]. To measure the transactivation potential of ATF2, we expressed an ATF2-GAL4 fusion protein, encompassing the phosphorylation-dependent activation domain of ATF2 (amino acids 1–96) and the DNA-binding domain of the yeast transcription factor GAL4 (Fig. 6A). Transcriptional activation was monitored by co-transfection of the reporter plasmid $pUAS^5luc$ that contains five copies of the GAL4 binding site (UAS) upstream of a luciferase reporter gene (Fig. 6B). As a control, plasmid pM1 encoding only the DNA binding domain of GAL4 ($GAL4_{DBD}$) was used. Figure 6C (upper panel) shows that the transcriptional activation potential of the GAL4-ATF2 fusion protein was 2.5-fold elevated following expression of MKK6(E). Transfection of a nanomolar concentration of an expression vector encoding wild-type p38 MAP kinase did not change reporter gene transcription, as expected, because only the concentration of the inactive, unphosphorylated p38 protein kinase was increased. However, a striking increase in the ATF2 transactivation potential was observed when we co-expressed the p38 protein kinase together with the constitutively active MKK6 kinase (Fig. 6C, lower panel). The protein consisting solely of the GAL4 DNA-binding domain ($GAL4_{DBD}$) did not stimulate transcription of

Fig. 5. A constitutively active ATF2 mutant transactivates the GTP cyclohydrolase I promoter/luciferase reporter gene. **A**: Schematic representation of the modular structure of C2/ATF2. This chimeric bZIP protein consists of the transcriptional activation domain of CREB2/ATF4 and the bZIP domain of ATF2, responsible for dimerization and DNA-binding. **B**: HepG2 cells were transfected with one of the reporter plasmids pGTPCHIluc, pTNFα(-131)luc, or pEgr-1.1luc, the pRSVβ internal reference plasmid and either the ''empty'' expression vector pCMV5 or an expression vector encoding wild-type ATF2 or the chimeric C2/ATF2 mutant (500 ng expression plasmid/plate). Lysates were prepared 48 h post-transfection and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted. **C**: HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, an expression vector encoding C2/ATF2, the pRSVβ reference plasmid and 50 ng/plate of the expression vectors encoding A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. Data are mean ± SD. **D**: Schematic representation of the modular structure of the CREB mutants GST-CREB and GST-KCREB. The GST mutants consist of amino-terminal glutathione-S-transferase, a nuclear localization signal derived from the SV40 large T antigen, and the bZIP domains of CREB or KCREB on the C-termini. The GST-KCREB protein contains a point mutation R286L in the basic domain as indicated, rendering it incapable of binding to DNA [Walton et al., 1992]. HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, an expression vector encoding C2/ATF2 (500 ng/plate) to upregulate reporter gene transcription and the pRSVβ reference plasmid. In addition, expression vectors encoding GST (control), GST-CREB, or GST-KCREB (250 ng/plate) were transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. Data are mean ± SD.

the luciferase reporter gene under any condition (Fig. 6C and data not shown).

Next, we tested whether an activation of the p38 MAP kinase signaling pathway influenced GTP cyclohydrolase I promoter/luciferase reporter gene transcription. Expression of MKK6(E) was not sufficient to increase reporter gene transcription (Fig. 6D, upper panel). Expression of MKK6(E) in the presence of p38 MAP kinase elevated GTP cyclohydrolase I promoter/luciferase gene transcription on the order of 9.6-fold (Fig. 6D, lower panel). We

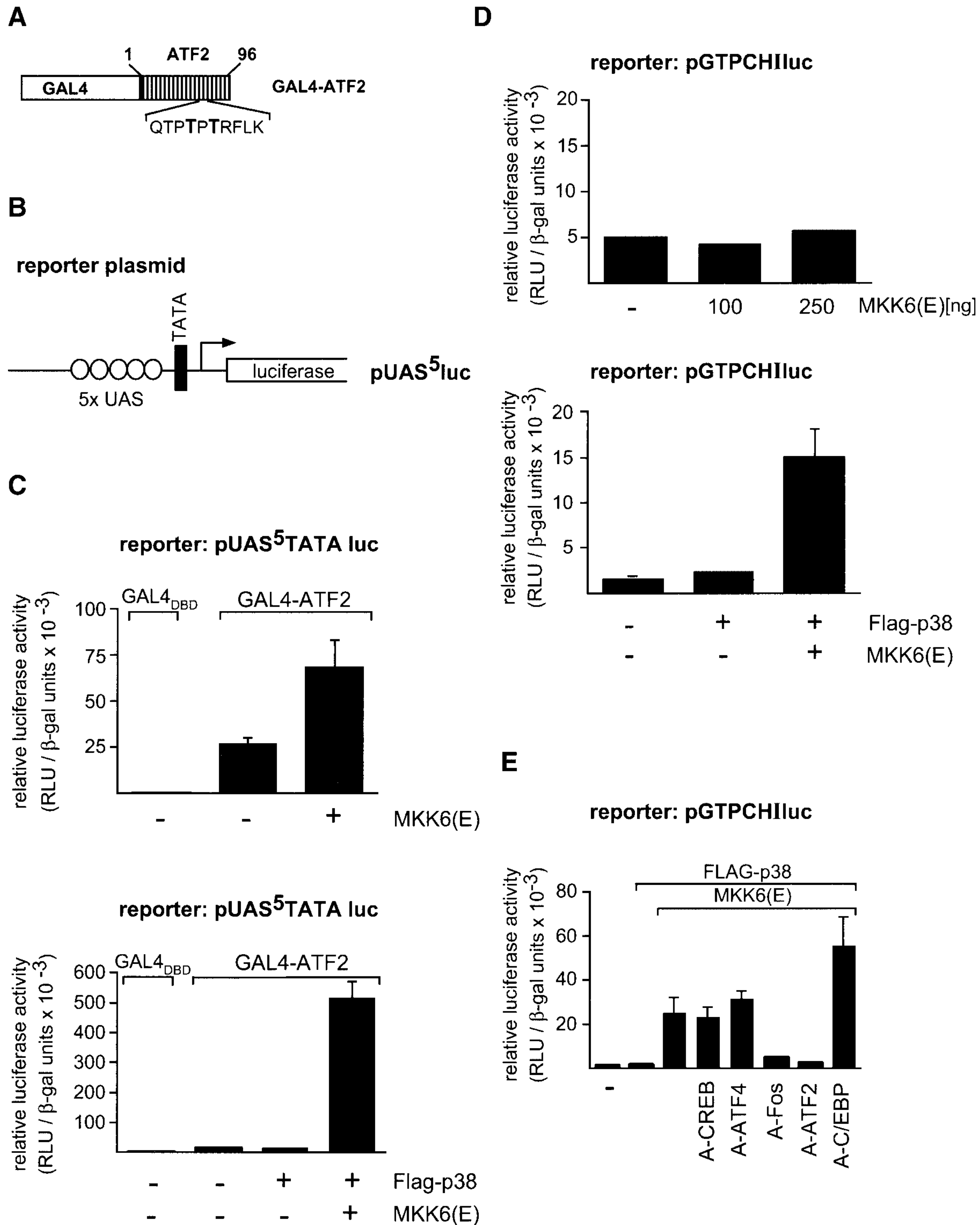

Fig. 6.

conclude that the activation of p38 protein kinase stimulated GTP cyclohydrolase I gene transcription. We would like to emphasize that the reporter plasmid pUAS^{5}luc, used to monitor GAL4-ATF2 transcriptional activity, contained five binding sites for the GAL4 fusion protein, giving raise to a synergistic effect on gene transcription, while the proximal GTP cyclohydrolase I promoter contains, in contrast, only a single CRE-like element. Thus, on quantitative terms the two experiments depicted in Figure 6C,D are not directly comparable. Competition experiments using the A-ZIP mutants confirmed the involvement of ATF2 in p38/MKK6(E)-stimulated GTP cyclohydrolase I/luciferase reporter activity. Figure 6E shows that A-ATF2 and A-Fos inhibited p38/MKK6(E)-stimulated transcription, whereas A-CREB, A-ATF4, and A-C/EBP had no effect.

Biological Activity of a Constitutively Active c-Jun Mutant on a GTP Cyclohydrolase I Promoter/Reporter Gene

ATF2 preferentially forms heterodimers through the leucine zipper with c-Jun, and ATF2/c-Jun heterodimers have been described to regulate c-Jun and TNFα gene transcription [van Dam et al., 1993; Tsai et al., 1996]. The transcription factor c-Jun contains a phosphorylation-regulated activation domain. Phosphorylation of the serine residues 63 and 73 of c-Jun by c-Jun N-terminal kinase (JNK) is necessary to turn c-Jun into a biologically active transcriptional activator. To analyze the impact of c-Jun on the GTP cyclohydrolase I promoter/luciferase reporter gene, we performed expression experiments with the constitutively active mutant C2/c-Jun (Fig. 7A). As a control, we tested the effect of c-Jun and C2/c-Jun on TNFα and Egr-1 promoter activity. The results show that C2/c-Jun transactivated all three-reporter genes (Fig. 7B). The transcriptional activation of the GTP cyclohydrolase I, TNFα, and Egr-1 promoters was 2.4-fold, 20-fold, and 2.3-fold, respectively. Thus, the TNFα promoter/luciferase reporter gene was by far the best c-Jun target, while GTP cyclohydrolase I and Egr-1 promoter activities were only weakly elevated. The unphosphorylated wild-type c-Jun protein was inactive. A competition assay employing the CREB mutants GST-CREB and GST-KCREB revealed that C2/c-Jun competes with GST-CREB for binding (Fig. 7C).

The Constitutively Active MAP3 Kinase Mutant MEKK1Δ Stimulates GTP Cyclohydrolase I Promoter/Luciferase Gene Transcription Involving ATF2 and c-Jun

The activity of the c-Jun phosphorylating kinase, c-Jun N-terminal kinase (JNK), is controlled by the MAP3 kinase mitogen- activated/extracellular signal responsive kinase kinase (MEK) kinase-1 (MEKK1). To stimulate JNK activity, we transfected an expression vector encoding a truncated form of MEK kinase 1 (MEKK1Δ). Experimentally, MEKK1Δ has been shown to strongly enhance the transcriptional activation potential of fusion protein consisting of the GAL4 DNA-binding domain and the c-Jun transactivation domain (data not shown). In addition, MEKK1Δ was able to elevate the transcriptional activation potential of ATF2 (data not shown). Expression of MEKK1Δ stimulated transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene approximately fourfold (Fig. 8A). This activation was blocked by cotransfection of A-Fos or A-ATF2 encoding expression vectors

Fig. 6. Activation of GTP cyclohydrolase I promoter activity by co-expression of p38 MAP kinase together with a constitutively active MAP kinase kinase 6. **A**: Modular structure of GAL4 fusion protein GAL4-ATF2. The protein contains the NH_2-terminal 147 amino acids of the yeast transcription factor GAL4 including the DNA-binding domain and the indicated amino acids of the activation domain of ATF2. The sequence encompassing the phosphorylation sites is depicted. **B**: Reporter plasmid pUAS^{5}luc containing the luciferase reporter gene, a TATA box and five binding sites for GAL4 upstream of the TATA box. **C**: The reporter plasmid pUAS^{5}luc, the GAL4 expression vector pM1, encoding amino acids 1–147 of GAL4 ($GAL4_{DBD}$) or an expression vector encoding GAL4-ATF2 were transfected into HepG2 cells. In addition, expression vectors encoding the constitutively active MKK6(E) mutant (25 ng plasmid/plate) and p38 MAP kinase (50 ng plasmid/plate) were transfected as indicated. Transfection efficiency was monitored by co-transfecting pSV40lacZ. We noticed an effect of p38 and MKK6(E) upon Rous sarcoma virus long terminal repeat controlled β-galactosidase expression. We therefore employed pSV40lacZ as the reference plasmid together with these expression vectors. **D**: The GTP cyclohydrolase I promoter/luciferase reporter plasmid pGTPCHIluc was transfected into HepG2 cells together with MKK6(E) or p38 MAP kinase expression vector. **E**: HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, the pSV40lacZ reference plasmid, and expression vectors encoding MKK6(E) and p38 MAP kinase as indicated. In addition one of the expression vectors encoding A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP (50 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase, and luciferase activities of these extracts determined. Data are mean ± SD.

(Fig. 8B). In addition, we performed experiments involving dominant-negative ATF2 and c-Jun mutants ATF2ΔN and c-JunΔN, depicted in Figure 8C. These mutants contained the intact bZIP domains of ATF2 or c-Jun, respectively. They were therefore able to bind to the cognate sites of ATF2 and c-Jun, making these sites unavailable for wild-type bZIP proteins.

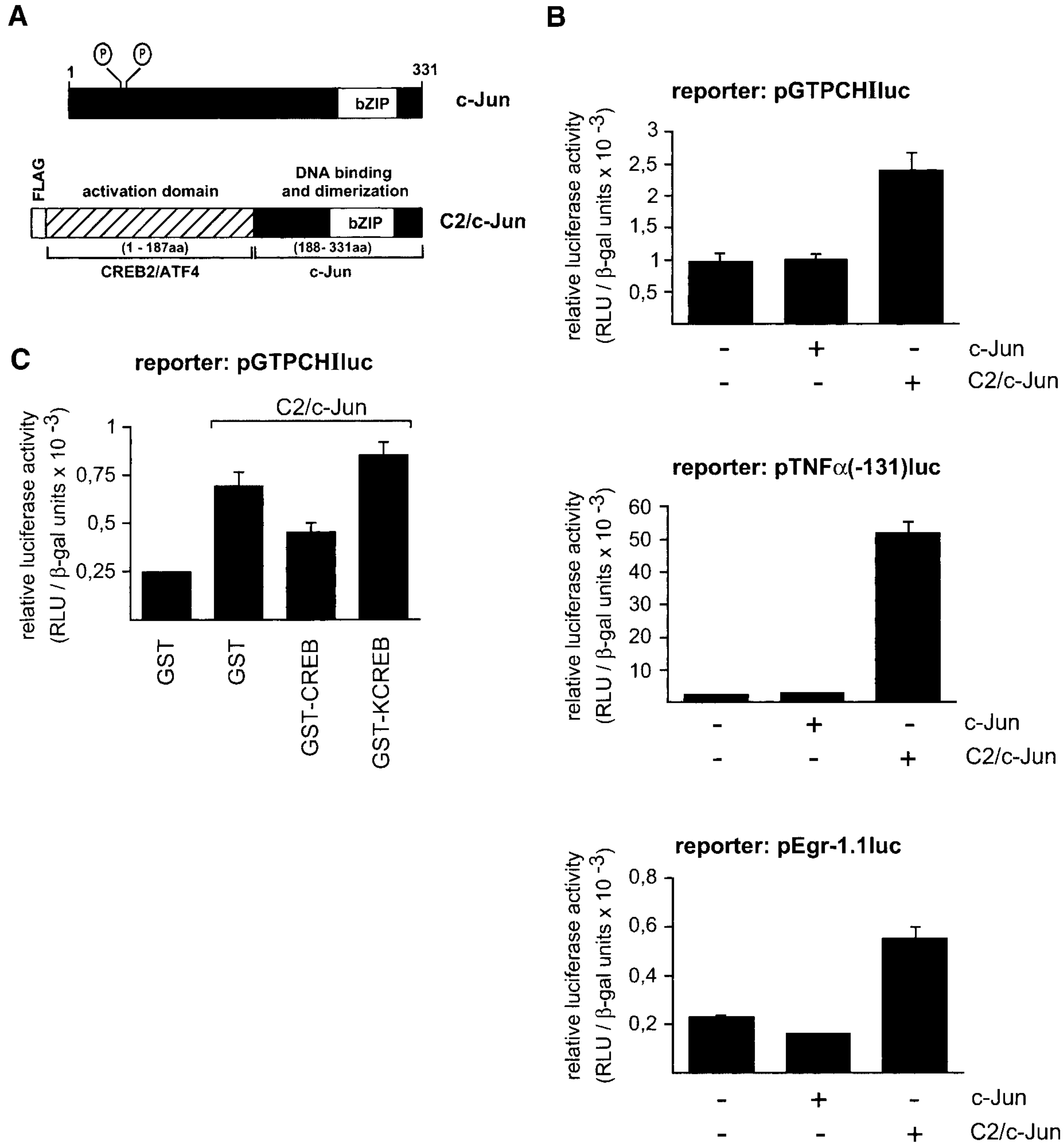

Fig. 7. Biological activity of a constitutively active c-Jun mutant towards the GTP cyclohydrolase I promoter/luciferase reporter gene. **A**: Schematic representation of the modular structure of c-Jun and C2/c-Jun. The chimeric C2/c-Jun bZIP protein consists of the transcriptional activation domain of CREB2/ATF4 and the bZIP domain of c-Jun, responsible for dimerization and DNA-binding. **B**: HepG2 cells were transfected with one of the reporter plasmids pGTPCHIluc, pTNFα(−131)luc, or pEgr-1.1luc, the pRSVβ internal reference plasmid, and either the "empty" expression vector pCMV5, or an expression vector encoding wild-type c-Jun or the chimeric C2/c-Jun mutant (500 ng expression plasmid/plate). Lysates were prepared 48 h post-transfection and β-galactosidase and luciferase activities were measured. **C**: HepG2 cells were transfected with the pGTPCHI-luc reporter plasmid, an expression vector encoding C2/c-Jun, the pRSVβ reference plasmid and expression vectors encoding GST (control), GST-CREB, or GST-KCREB (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities of these extracts determined. Data are mean ± SD.

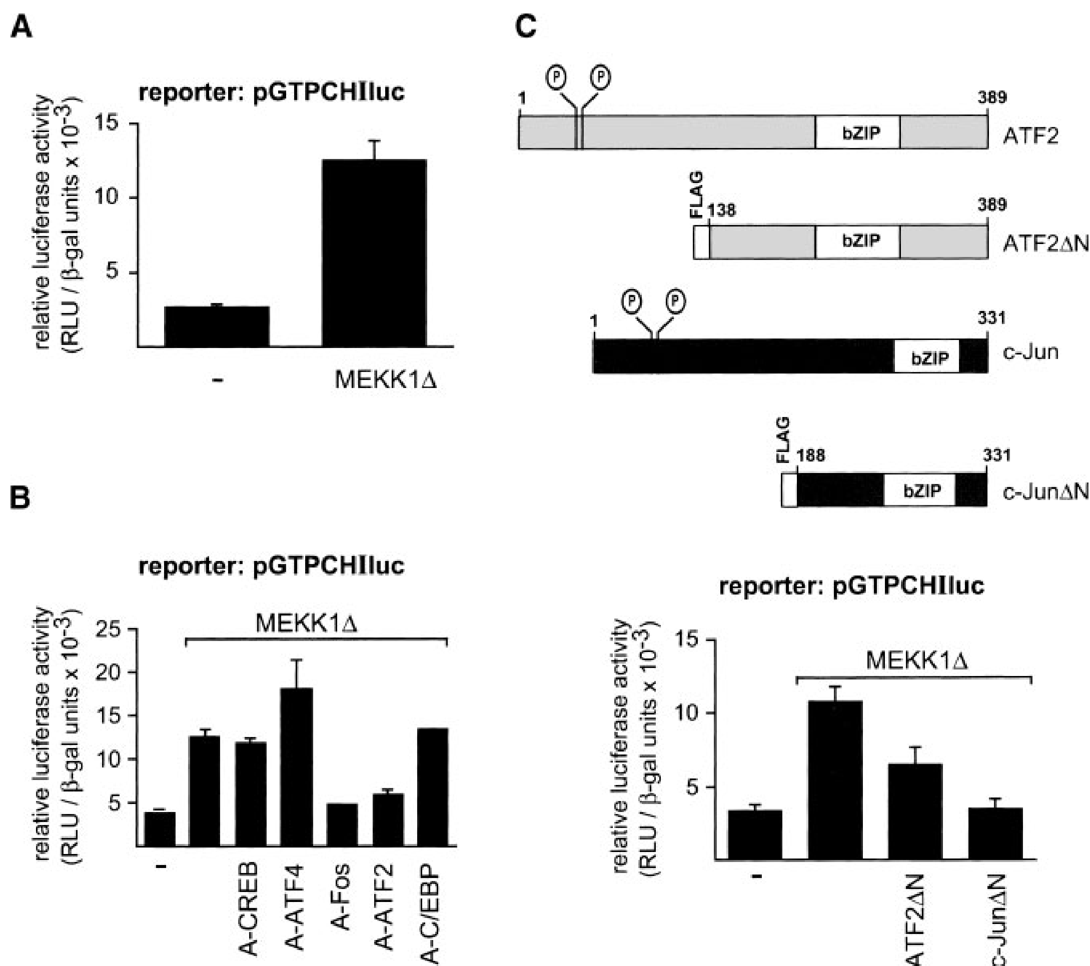

Fig. 8. Expression of a constitutively active mutant of MEKK1 increased GTP cyclohydrolase I promoter activity. **A**: The reporter plasmid pGTPCHIluc was transfected into HepG2 cells together with the pSV40lacZ internal standard plasmid and an expression vector encoding MEKK1Δ (2 ng plasmid/plate). **B**: HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, an expression vector encoding MEKK1Δ, the pSV40lacZ reference plasmid and 50 ng/plate of the expression vectors encoding A-CREB, A-ATF4, A-Fos, A-ATF2, or A-C/EBP. Forty-eight hours post-transfection cell extracts were prepared, and the β-galactosidase and luciferase activities of these extracts determined. Data are mean ± SD. **C**: Schematic representation of the modular structure of ATF2 and c-Jun and the dominant-negative mutants ATF2ΔN and c-JunΔN. HepG2 cells were transfected with the pGTPCHIluc reporter plasmid, an expression vector encoding MEKK1Δ, the pSV40lacZ reference plasmid and expression vectors encoding ATF2ΔN or c-JunΔN (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared, and the β-galactosidase and luciferase activities of these extracts determined. Data are mean ± SD.

The results confirmed the previous observation that either c-Jun or ATF2 or a heterodimer of ATF2 and c-Jun mediated the MEKK1Δ-induced upregulation of GTP cyclohydrolase I promoter/luciferase reporter gene transcription (Fig. 8C).

Mutation of the CRE-Like Sequence of the GTP Cyclohydrolase I Promoter Impairs Transactivation by CREB, ATF2, and c-Jun

To analyze the importance of the CRE-like sequence for CREB, ATF2, and c-Jun mediated transactivation, we generated a GTP cyclohydrolase I promoter/luciferase reporter gene containing three point mutations within the CRE-like sequence (Fig. 9A). In vitro DNA/protein binding experiments with annealed oligonucleotides carrying these point mutations showed a decreased binding of nuclear proteins, although these oligonucleotides retained the ability to compete with a probe having the intact CRE-like sequence [Kapatos et al., 2000]. Transfection experiments of HepG2 cells employing reporter genes carrying the intact or the mutated CRE-like site, together with expression vectors encoding C2/CREB, C2/ATF2, or C2/c-Jun revealed that the CRE-like sequence is of major importance for the

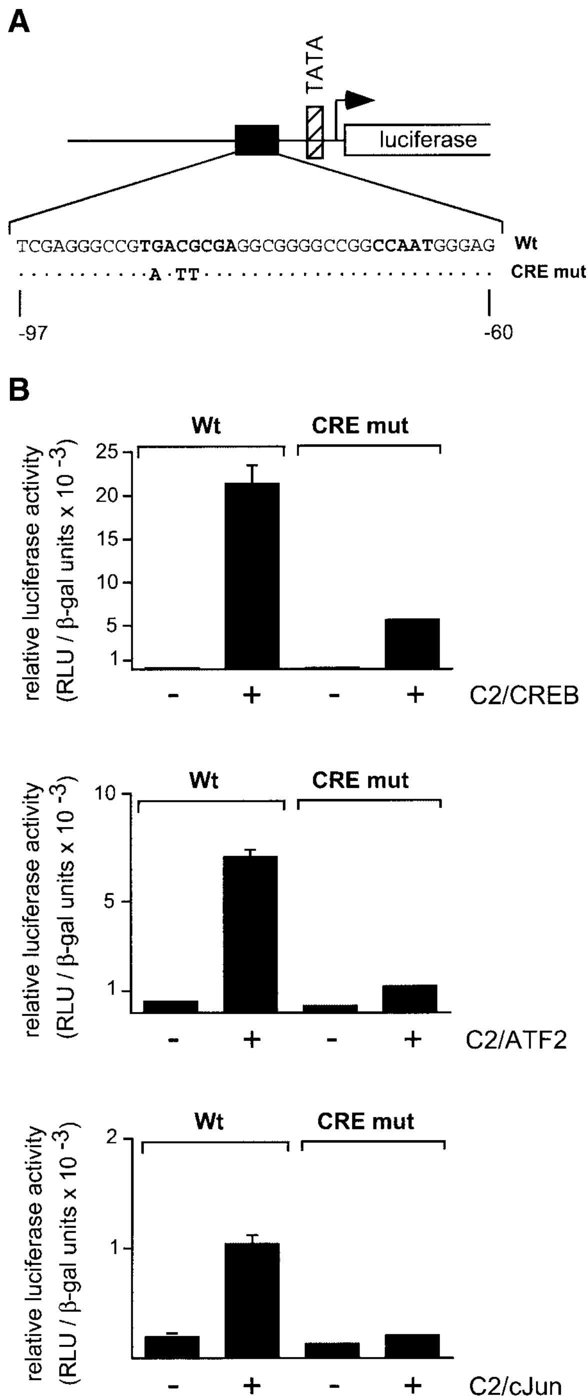

Fig. 9. Point mutations within the CRE-like motif of the GTP cyclohydrolase I promoter impair the transactivation mediated by constitutively active mutants of CREB, ATF2, and c-Jun. **A**: Schematic representation of the GTP cyclohydrolase I reporter plasmids containing an intact or a mutated CRE-like sequence. **B**: HepG2 cells were transfected with one of the reporter plasmids, containing an intact or mutated CRE-like motif, the pRSVβ internal reference plasmid, and either the "empty" expression vector pCMV5 or an expression vector encoding C2/CREB, C2/ATF2, or C2/c-Jun. Lysates were prepared 48 h post-transfection, and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

constitutively active CREB, ATF2, and c-Jun mutants to transactivate the GTP cyclohydrolase I/luciferase reporter gene (Fig. 9B).

CAAT/Enhancer Binding Protein (C/EBP) Transcription Factors Only Marginally Influence GTP Cyclohydrolase I Promoter Activity

The proteins of the C/EBP family of bZIP proteins bind specifically to the dyad symmetrical sequence 5′-ATTGCGCAAT-3′ [Agre et al., 1989], a motif quite distinct from the canonical CRE sequence 5′-TGACGTCA-3′. However, C/EBP proteins have also been reported to bind to ATF/CRE motifs [Bakker and Parker, 1991], and C/EBPα has been proposed to functionally substitute for CREB in the cAMP response unit of the phosphoenolpyruvate carboxykinase gene [Roesler et al., 1998]. We compared the transcriptional activity of C/EBPα and C2/C/EBPα, a chimeric transcription factor containing the CREB2/ATF4 activation domain and the C/EBPα bZIP domain, using the C/EBP-specific reporter gene 2xC/EBPluc (Fig. 10A,B). Both C/EBPα and C2/C/EBPα proteins strongly transactivated the reporter gene on the order of 800- and 1200-fold, respectively. In contrast, the GTP cyclohydrolase I promoter/luciferase reporter gene was only marginally transactivated by C/EBPα (1.8-fold). The chimeric C2/C/EBPα transcription factor increased reporter gene transcription on the order of 3.6-fold (Fig. 10C). We conclude that the C/EBP proteins play, if anything, only a marginal role in the regulation of GTP cyclohydrolase I gene transcription.

DISCUSSION

Many extracellular signaling molecules including interferon-γ, interleukin 1β, TNFα, LPS, epidermal growth factor, and agents that elevate the intracellular cAMP concentration have been described to regulate GTP cyclohydrolase I expression on the transcriptional level. The fact that elevated cAMP concentration stimulates GTP cyclohydrolase I transcription, together with the identification of a cyclic AMP response element (CRE)-like motif in the proximal GTP cyclohydrolase I promoter, indicates that CREB or related bZIP proteins are involved in the stimulus-transcription coupling. However, it has been stated that "although a putative CREB consensus element is identified within the rat GTPCHI promoter, the function

has not been verified" [Gross et al., 2000]. The objective of this study was to analyze the regulation of GTP cyclohydrolase I gene transcription by bZIP transcription factors using constitutively active and dominant-negative mutants. Strategically, we decided to measure transcriptional activation of a GTP cyclohydrolase I promoter/luciferase reporter gene instead of in vitro transcription factor/DNA binding. Although DNA-binding is required for a subsequent transcriptional activation, enhanced binding activity of a transcription factor to DNA, monitored by an in vitro binding assay, does not necessarily prove enhanced transcriptional activation potential of this protein.

The use of a constitutively active mutant of CREB allowed us to uncouple the investigation of transcriptional targets of CREB from the signaling pathways in the cell that lead to activation of CREB. Using this strategy, we avoided the use of dibutyryl cAMP or forskolin that may trigger other biological responses. Here, we show that only nanomolar concentrations of the expression vector encoding C2/CREB were required to strongly activate transcription of a GTP cyclohydrolase I promoter/reporter gene, containing an intact CRE-like motif. Mutations within this sequence significantly impaired transcriptional upregulation by C2/CREB. Moreover, the experiments involving overexpression of NLSCα, a mutant of the catalytic subunit of cAMP-dependent protein kinase, showed an enhanced transcription of the GTP cyclohydrolase I promoter/reporter gene. Taken together, this study clearly shows that the CRE-like motif within the regulatory region of the human GTP cyclohydrolase I gene functions as a *bona fide* CRE.

Overexpression experiments of CREB2/ATF4 in PC12 cells showed a moderately increase in transcription of a GTP cyclohydrolase I

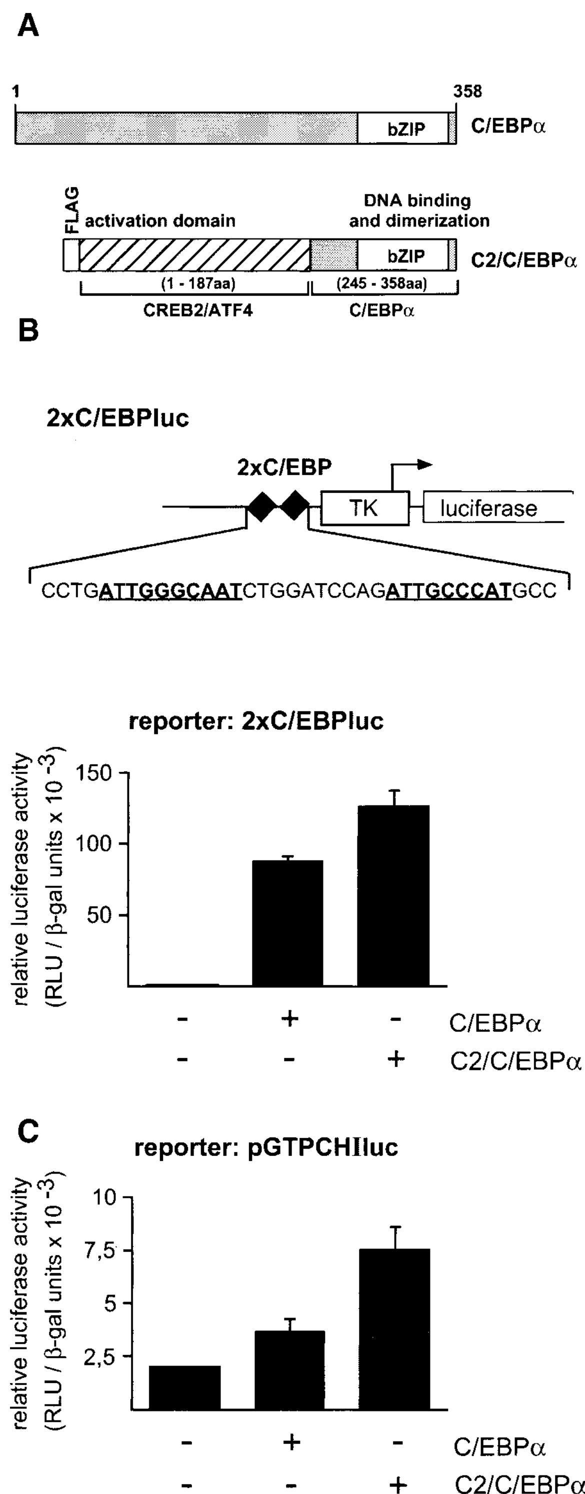

Fig. 10. C/EBPα and the C2/C/EBPα mutant marginally transactivated the GTP cyclohydrolase I promoter/reporter gene. **A**: Schematic representation of the modular structure of C/EBPα and the C2/C/EBPα. The chimeric C2/C/EBPαb protein consists of the transcriptional activation domain of CREB2/ATF4 and the bZIP domain of C/EBPα, responsible for dimerization and DNA-binding. **B**: Schematic representation of the reporter plasmid 2xC/EBPluc. The transcription unit includes two C/EBP binding sites as depicted upstream of a minimal thymidine kinase promoter. HepG2 cells were transfected with the reporter plasmid 2xC/EBPluc, the pSV40lacZ internal reference plasmid, and either the "empty" expression vector pCMV5 or an expression vector encoding either C/EBPα or C2/C/EBPα (100 ng expression plasmid/plate). **C**: HepG2 cells were transfected with the reporter plasmid pGTPCHIluc, the pSV40lacZ internal reference plasmid, and either the "empty" expression vector pCMV5 or an expression vector encoding either C/EBPα or C2/C/EBPα (100 ng expression plasmid/plate). Lysates were prepared 48 h post-transfection, and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

promoter/luciferase reporter gene in the presence of 8-Br-cAMP [Kapatos et al., 2000]. In addition, CREB2/ATF4 was identified in DNA/protein binding experiments as a binding partner to the CRE-like sequence of the GTP cyclohydrolase I gene [Kapatos et al., 2000]. Based on these observation, it has been concluded that CREB2/ATF4 belongs to the bZIP transcription factors that are recruited by the CRE-like motif of the GTP cyclohydrolase I gene. We have analyzed the impact of CREB2/ATF4 on the activity of the proximal GTP cyclohydrolase I promoter. The results clearly show that CREB2/ATF4 is not able to transactivate a reporter gene containing the CRE-like motif derived from the GTP cyclohydrolase I gene. Likewise, CREB2/ATF4 did not show any effect on the regulation of the TNFα or Egr-1 promoter that both are targets of the C2/CREB transcription factor. We would like to emphasize that CREB2/ATF4 and C2/CREB contain an identical transcriptional activation domain and differ only in their bZIP DNA-binding and dimerization domains. Thus, the differences of CREB2/ATF4 and C2/CREB in transactivating reporter genes containing CRE-like motifs can be attributed to their different DNA-binding abilities. Taken together, these data show that CREB2/ATF4 has no transactivation potential towards the GTP cyclohydrolase I gene via the CRE-like motif.

The expression experiments involving a constitutively active mutant of ATF2 (C2/ATF2) showed a 10-fold stimulation of GTP cyclohydrolase I promoter/luciferase reporter gene transcription. Point mutations within the CRE-like sequence of the GTP cyclohydrolase I gene almost abolished this effect, indicating that the transactivation of C2/ATF2 requires binding to this genetic element. Competition experiments between C2/CREB and dominant-negative bZIP mutants or C2/ATF2 and dominant-negative bZIP mutants revealed that both proteins do not heterodimerize. Instead, they compete for binding to the CRE-like motif of the GTP cyclohydrolase I promoter. We further compared the transactivation ability of C2/ATF2 upon the GTP cyclohydrolase I, TNFα, and Egr-1 promoters. The TNFα and the Egr-1 gene have been described to be transactivated by ATF2 [Tsai et al., 1996; Rolli et al., 1999]. The results showed that expression of C2/ATF2 upregulated TNFα promoter/luciferase reporter gene transcription, but to a lesser extent than transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene. These data support the view that the GTP cyclohydrolase I gene is a *bona fide* target of ATF2. In contrast, the transcription of the Egr-1 promoter/luciferase reporter gene was only weakly stimulated by C2/ATF2. In contrast, expression of a constitutively active c-Jun mutant (C2/c-Jun) preferentially stimulated TNFα promoter/luciferase gene transcription. In comparison, transcriptional upregulation of the GTP cyclohydrolase I promoter/reporter gene by C2/c-Jun was approximately 10%–20% of that measured for the TNFα promoter/reporter gene.

The biological activity of ATF2 is regulated by stress-activated protein kinases, and ATF2 plays an important role in the cellular stress response. In fact, overexpression of a constitutively active mutant of the MAP2 kinase MKK6, together with the expression of the p38 MAP kinase, upregulated transcription of a GTP cyclohydrolase I promoter/reporter gene. Reporter gene transcription was impaired by dominant-negative ATF2 and c-Jun mutants, confirming the previous results that ATF2 regulates GTP cyclohydrolase I gene transcription. In liver, it has been reported that p38 MAP kinase is constitutively active [Mendelson et al., 1996]. However, our observation that the constitutively active protein kinase MKK6 was inactive towards the GTP cyclohydrolase I promoter/reporter gene, unless the concentration of the p38 protein kinase was experimentally elevated, suggests that HepG2 hepatoma cells do not contain a constitutive p38 MAP kinase activity. Expression of a truncated form of MEKK1 (MEKK1Δ) induced an upregulation of GTP cyclohydrolase I promoter/reporter gene, and experiments involving dominant-negative mutants revealed that either c-Jun or ATF2 or heterodimers of these bZIP proteins are responsible for the transcriptional effect of MEKK1Δ expression. We conclude that the activation of stress-activated protein kinases, together with the activation of cAMP-dependent protein kinase, converge to the GTP cyclohydrolase I gene, involving the transcription factors CREB, ATF2, and c-Jun.

In vitro DNA/protein binding experiments suggested that the C/EBPβ transcription factor interacts with the CRE-like motif of the GTP cyclohydrolase I core promoter [Kapatos et al., 2000]. However, no functional data concerning the biological activity of C/EBP proteins on GTP

cyclohydrolase I promoter activity had been presented in this study. Recently, we showed that CREB and C/EBPα recognize different genetic elements [Thiel et al., 2005b]. Moreover, the biochemical basis for the binding of C/EBPα to its cognate site has been elucidated. The Val^{296} residue within the C/EBPα molecule is of primary importance in restricting the interactions with C/EBP sites. C/EBPα strongly binds to CRE sequences when Val^{296} is mutated to alanine [Miller et al., 2003]. The data presented here confirm the previous observations that a dominant-negative C/EBP mutant (A-C/EBP) is unable to block C2/CREB-induced transcription. Rather, we observed an activation of reporter gene transcription, suggesting that the C/EBP mutant removes inhibitory constraints on CRE-regulated transcription units. Our data are complemented by in vitro DNA/protein binding experiments showing that A-C/EBP did not compete with CREB bound to its cognate site [Greenwel et al., 2000; Ménard et al., 2002]. In addition, expression experiments involving C/EBPα or a CREB2/C/EBPα mutant revealed that both proteins contain a powerful transactivation activity towards a C/EBP-sensitive reporter gene, but showed only a marginal activity on the GTP cyclohydrolase I core promoter.

In summary, we have shown that CREB, ATF2, and c-Jun are independently able to transactivate a reporter gene containing the GTP cyclohydrolase I core promoter. CREB was by far the most potent transactivator. In addition, a constitutively active ATF2 mutant upregulated transcription of a reporter gene under control of the GTP cyclohydrolase I promoter to a larger extent than a reporter gene controlled by the TNFα gene promoter, a well-known target of ATF2. In contrast, CREB2/ATF4 and C/EBPα showed either no or only a marginal transactivation activity towards the GTP cyclohydrolase I promoter controlled reporter gene, indicating that these proteins are not belonging to the key transcriptional regulators of the GTP cyclohydrolase I gene.

ACKNOWLEDGMENTS

We thank Karl Bach for excellent technical help, James S. Economou, Peter F. Johnson, Michael Karin, and Michael Uhler for their generous gifts of plasmids, and Libby Guethlein and Oliver Rössler for critical reading of the manuscript. This work was supported by the Medical Faculty of the University of Saarland via HOMFOR and by a fellowship from the "Deutscher Akademischer Austauschdienst" (DAAD) to Jude Al Sarraj.

REFERENCES

Agre P, Johnson PF, McKnight SL. 1989. Cognate DNA binding specificity retained after leucine zipper exchange between HCN4 and C/EBP. Science 246:922–926.

Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. 1998. A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol 18:967–977.

Averous J, Bruhat A, Jousse C, Carraro V, Thiel G, Fafournoux P. 2004. Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. J Biol Chem 279:5288–5297.

Bakker O, Parker MG. 1991. CAAT/enhancer binding protein is able to bind to ATF/CRE elements. Nucl Acids Res 19:1213–1217.

D'Sa C, Hirayama K, West A, Hahn M, Zhu M, Kapatos G. 1996. Tetrahydrobiopterin biosynthesis in C6 glioma cells: Induction of GTP cyclohydrolase I gene expression by lipopolysaccharide and cytokine treatment. Mol Brain Res 41:105–110.

Frank S, Kolb N, Werner ER, Pfeilschifter J. 1998. Coordinated induction of inducible nitric oxide synthase and GTP-cyclohydrolase I is dependent on inflammatory cytokines and interferon-γ in HaCaT keratinocytes: Implications for the model of cutaneous wound repair. J Invest Dermatol 111:1065–1071.

Geller DA, Di Silvio M, Billiar TR, Hatakeyama K. 2000. GTP cyclohydrolase I is coinduced in hepatocytes stimulated to produce nitric oxide. Biochem Biophys Res Comm 276:633–641.

Golderer G, Werner ER, Heufler C, Strohmaier W, Gröbner P, Werner-Felmayer G. 2001. GTP cyclohydrolase I mRNA: Novel splice variants in the slime mould *Physarum polycephalum* and in human monocytes (THP-1) indicate conservation of mRNA processing. Biochem J 355:499–507.

Greenwel P, Tanaka S, Penkov D, Zhang W, Olive M, Moll J, Vinson C, Di Liberto M, Ramirez F. 2000. Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. Mol Cell Biol 20:912–918.

Groot M, Boxer LM, Thiel G. 2000. Nerve growth factor and epidermal growth factor regulated gene transcription in PC12 pheochromocytoma and INS-1 insulinoma cells. Eur J Cell Biol 79:924–935.

Gross SS, Jones CL, Hattori Y, Raman CS. 2000. Tetrahydrobiopterin: An essential cofactor of nitric oxide synthase with an elusive role. In: Lancaster JR, editor. Nitric oxide. Orlando: Academic Press. pp 167–185.

Han J, Lee J-D, Jiang Y, Li Z, Feng L, Ulevitch RJ. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). J Biol Chem 271:2886–2891.

Hirayama K, Shimooji M, Swick L, Meyer A, Kapatos G. 2001. Characterization of GTP cyclohydrolase I gene expression in the human neuroblastoma SKN-BE(2)M17:

Enhanced transcription in response to cAMP is conferred by the proximal promoter. J Neurochem 79:576–587.
Hufton SE, Jennings IG, Cotton RGH. 1995. Structure and function of the aromatic amino acid hydrolases. J Biochem 311:353–366.
Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, Nomura Y, Endo K, Tanaka H, Tsuji S, Fujita K, Nagatsu T. 1994. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat Genet 8:236–242.
Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J. 1996. Characterization of the structure and function of a new mitogen-activated protein kinase (p38B). J Biol Chem 271:17920–17926.
Kapatos G, Stegenga SL, Hirayama K. 2000. Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat GTP cyclohydrolase I gene. J Biol Chem 275:5947–5957.
Mendelson KG, Contois L-R, Tevosian SG, Davis RJ, Paulson KE. 1996. Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. Proc Natl Acad Sci USA 93:12908–12913.
Miller M, Shuman JD, Sebastian T, Dauter Z, Johnson PF. 2003. Structural basis for DNA recognition by the basic region leucine zipper transcription factor CCAAT/enhancer binding protein α. J Biol Chem 278:15178–15184.
Minden A, Lin A, McMahon M, Lange-Carter C, Dérijard B, Davis RJ, Johnson GL, Karin M. 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 266:1719–1794.
Ménard C, Hein P, Paquin A, Savelson A, Yang XM, Lederfein D, Barnabé-Heider F, Mir AA, Sterneck E, Peterson AC, Johnson PF, Vinson C, Miller FD. 2002. An essential role for a MEK-C/EBP pathway during growth factor-regulated cortical neurogenesis. Neuron 36:597–610.
Olive M, Krylov D, Echlin DR, Gardner K, Taparowsky E, Vinson C. 1997. A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. J Biol Chem 272:18586–18594.
Plüss C, Werner ER, Blau N, Wachter H, Pfeilschifter J. 1996. Interleukin 1β and cAMP trigger the expression of GTP cyclohydrolase I in rat renal mesangial cells. Biochem J 318:665–671.
Plüss C, Werner ER, Wachter H, Pfeilschifter J. 1997. Differential effect of dexamethasone on interleukin 1β- and cyclic AMP-triggered expression of GTP cyclohydrolase I in rat renal mesanglial cells. Brit J Pharmacol 122:534–538.
Rhoades KL, Golub SH, Economou JS. 1992. The regulation of the human tumor necrosis factor α promoter region in macrophages, T cell, and B cell lines. J Biol Chem 267:22102–22107.
Roesler WJ, Park EA, McFie PJ. 1998. Characterization of CCAAT/Enhancer-binding protein α as a cyclic AMP-responsive nuclear regulator. J Biol Chem 273:14950–14957.
Rolli M, Kotlyarov A, Sakamoto KM, Gaestel M, Neininger A. 1999. Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase-2 independent manner. J Biol Chem 274:19559–19564.
Schoch S, Cibelli G, Magin A, Steinmüller L, Thiel G. 2001. Modular structure of cAMP response element binding protein 2 (CREB2). Neurochem Int 38:601–608.
Steinmüller L, Thiel G. 2003. Regulation of gene transcription by a constitutively active mutant of activating transcription factor 2 (ATF2). Biol Chem 384:667–672.
Steinmüller L, Cibelli G, Moll JR, Vinson C, Thiel G. 2001. Regulation and composition of activator protein 1 (AP-1) transcription factors controlling collagenase and c-Jun promoter activities. Biochem J 360:599–607.
Thiel G, Petersohn D, Schoch S. 1996. pHIVTATA-CAT, a versatile vector to study transcriptional regulatory elements in mammalian cells. Gene 168:173–176.
Thiel G, Kaufmann K, Magin A, Lietz M, Bach K, Cramer M. 2000. The human transcriptional repressor protein NAB1: Expression and biological activity. Biochim Biophys Acta 1493:289–301.
Thiel G, Lietz M, Hohl M. 2004. How mammalian transcriptional repressors work. Eur J Biochem 271: 2855–2862.
Thiel G, Al Sarraj J, Stefano L. 2005a. cAMP response element binding protein (CREB) activates transcription via two distinct genetic elements of the human glucose-6-phosphatase gene. BMC Mol Biol 6:2.
Thiel G, Al Sarraj J, Vinson C, Stefano L, Bach K. 2005b. Role of basic region leucine zipper transcription factors CREB, CREB2, activating transcription factor 2 and CAAT/enhancer binding protein α in cAMP response element-mediated transcription. J Neurochem 92:321–336.
Tsai EY, Jain J, Pesavento PA, Rao A, Goldfeld AE. 1996. Tumor necrosis factor alpha gene regulation in activated T cells involves ATF2/Jun and NFATp. Mol Cell Biol 16:459–467.
Uhler MD, McKnight GS. 1987. Expression of cDNAs for two isoforms of the catalytic subunit of camp-dependent protein kinase. J Biol Chem 262:15202–15207.
van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P, Zantema A, Angel P, van der Eb AJ. 1993. Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus protein. EMBO J 12:479–487.
Vann LR, Twitty S, Spiegel S, Milstien S. 2000. Divergence in regulation of nitric-oxide synthase and its cofactor tetrahydrobiopterin by tumor necrosis factor-α. J Biol Chem 275:13275–13281.
Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. 2002. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 22:6321–6335.
Walton KM, Rehfuss RP, Chrivia JC, Lochner JE, Goodman RH. 1992. A dominant repressor of cyclic adenosine 3′, 5′-Monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter in vivo. Mol Endocrinol 6:647–655.
Ziegler I, Hültner L, Egger D, Kempkes B, Mailhammer R, Gillis S, Rödl W. 1993. In a concert action kit ligand and interleukin 3 control the synthesis of serotonin in murine bone marrow-derived mast cells. Up-regulation of GTP cyclohydrolase I and tryptophan 5-monooxygenase activity by the kit ligand. J Biol Chem 268:12544–12551.