CREB (cAMP response element binding protein) and C/EBPα (CCAAT/enhancer binding protein) are required for the superstimulation of phosphoenolpyruvate carboxykinase gene transcription by adenoviral E1a and cAMP

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In the present study, we observed superstimulated levels of cAMP-stimulated transcription from the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter in cells infected with wild-type adenovirus expressing 12 S and 13 S E1a proteins, or in cells expressing 13 S E1a alone. cAMP-stimulated transcription was inhibited in cells expressing only 12 S E1a, but slightly elevated in cells expressing E1a proteins with mutations in conserved regions 1 or 2, leading us to conclude that the superstimulation was mediated by conserved region 3 of 13 S E1a. E1a failed to enhance cAMP-stimulated transcription from promoters containing mutations that abolish binding by cAMP response element binding protein (CREB) or CCAAT/enhancer binding proteins (C/EBPs). This result was supported by experiments in which expression of dominant-negative CREB and/or C/EBP proteins repressed E1a- and cAMP-stimulated transcription from the PEPCK gene promoter. In reconstitution experiments using a Gal4-responsive promoter, E1a enhanced cAMP-stimulated transcription when chimaeric Gal4–CREB and Gal4–C/EBPα were co-expressed. Phosphorylation of CREB on serine-133 was stimulated in cells treated with dibutyryl cAMP, whereas phosphorylation of C/EBPα was increased by E1a expression. Our data support a model in which cAMP agonists increase CREB activity and stimulate PEPCK gene transcription, a process that is enhanced by E1a through the phosphorylation of C/EBPα.

Key words: adenovirus, protein phosphorylation, transcriptional synergy, viral transforming protein.

INTRODUCTION

An intriguing conundrum in biochemistry is the ability of apparently similar intracellular signalling and gene regulatory systems to produce vastly different effects on various cell functions and activities. This is not only true for different cell types, but can readily be observed even within cells of the same lineage. Nowhere is this more apparent than in the regulation of gene transcription by the adenovirus E1a oncoproteins.

Two E1a mRNA transcripts, 243R and 289R, are derived from the E1a gene, from which proteins denoted 12 S and 13 S respectively are generated [1]. The two E1a proteins contain three regions which are highly conserved between different viral serotypes [2]. Conserved region 1 (CR1) and CR2 are common to both 12 S and 13 S E1a proteins, whereas CR3 is found only in the 13 S protein. In general, the 13 S E1a protein stimulates transcription of certain target genes, primarily through its ability to bind to various transcription factors, such as ATF2 (activating transcription factor 2), TFIID (transcription factor IID) and AP-1 (activator protein-1), through CR3 [3–7]. Alternatively, the 12 S protein has generally been regarded as a transcription repressor, a function that is mediated by interactions with cellular proteins such cAMP response element binding protein (CREB) binding protein (CBP) and/or p300 [8–10].

One perplexing aspect of E1a transcriptional activity is its differential ability to enhance cAMP-stimulated transcription in certain system while repressing it in others. In most systems, cAMP-stimulated gene transcription is mediated through the phosphorylation of CREB on a specific serine residue by cAMP-dependent protein kinase (PKA) [11]. Phosphorylation of CREB increases its ability to interact with the transcriptional co-activators CBP and p300 [12,13]. Arany et al. [9] and Lundblad et al. [10] have shown that 12 S E1a blocks cAMP-stimulated transcription by binding to CBP/p300 in U-2 OS osteosarcoma cells and F9 teratocarcinoma cells respectively. Likewise, 12 S E1a has been shown to block basal and cAMP-stimulated phosphoenolpyruvate carboxykinase (PEPCK) gene transcription in HepG2 cells [14]. Lee et al. [15] confirmed the ability of E1a to repress CREB/p300-regulated transcription in U-2 OS cells, but, surprisingly, found that E1a enhanced cAMP-stimulated gene transcription via CREB and p300 in HeLa cells. Likewise, we have reported that high-level E1a expression enhances cAMP-stimulated and basal PEPCK gene transcription in the HepG2 and H4IIE hepatoma cell lines [16]. Thus studies investigating the participation of E1a in cAMP-regulated gene expression have produced conflicting results.

We have continued to examine the role of E1a in cAMP-stimulated transcription using the PEPCK gene promoter as our model system. PEPCK expression, which is limited primarily to the liver, kidney cortex and adipose tissue, is regulated almost entirely at the level of gene transcription by a wide variety of hormones and extracellular signals [17–20]. Changes in PEPCK

Abbreviations used: Ad5, adenovirus type 5; AP-1, activator protein-1; ATF, activating transcription factor; Bt_2cAMP, dibutyryl cAMP; CREB, cAMP response element binding protein; CBP, CREB binding protein; C/EBP, CCAAT/enhancer binding protein; CR1 (etc.), conserved region 1 (etc.); GBF-F, dominant-negative C/EBP; KCREB, dominant-negative CREB; PEPCK, phosphoenolpyruvate carboxykinase; PKA, cAMP-dependent protein kinase; TFIID, transcription factor IID; TK, thymidine kinase.

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gene transcription are controlled by several tissue-specific factors, including CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ [21–27] and hepatic nuclear factors 1, 3 and 4 [28,29], as well as multiple hormone-responsive factors such as CREB [22,24,26,30,31], the glucocorticoid retinoic acid, retinoid X and thyroid hormone receptors [32–37], and a poorly defined insulin-responsive system [25,38,39]. These factors bind to at least 12 well-defined *cis*-acting elements within 600 bp of the transcription start site [17].

cAMP stimulation of PEPCK gene transcription was originally thought to be mediated primarily through CREB, based on its constitutive presence in relevant tissues, its ability to bind to the CRE1 site in the PEPCK gene promoter and its phosphorylation by PKA in response to cAMP agonists [22,24,26,30]. However, several reports have indicated that multiple regions in the PEPCK gene promoter are required for a robust response to cAMP, especially those bound by C/EBP family members. Park et al. [22] suggested that C/EBPβ is involved in the cAMP responsiveness of the PEPCK gene promoter, based on its increased expression and nuclear translocation in response to cAMP agonists, its pericentral expression in the liver (the same as PEPCK) and its ability to bind to the CRE1 site of the promoter with higher apparent affinity than either CREB or C/EBPα. More recently, Roesler et al. [23] have presented evidence that C/EBPα, but not C/EBPβ, supports a considerable cAMP response with the PEPCK gene promoter, and with a Gal4-responsive promoter in the presence of a chimaeric Gal4–C/EBPα protein.

In the present paper we show that wild-type adenovirus type 5 (Ad5) expressing both 12 S and 13 S E1a proteins, or a mutant virus expressing the 13 S protein alone, enhances cAMP-stimulated gene transcription, implicating CR3 in this process. This concept was confirmed in experiments in which deletion of CR3, but not deletion of CR1 or CR2, from the 13 S E1a protein completely abrogated the enhancement of cAMP-stimulated gene transcription. Further, we found that mutations in CREB and C/EBP binding sites in the PEPCK gene promoter designated CRE1, P3-I and P4 block the ability of cAMP agonists and E1a to modulate transcription. Introduction of dominant-negative CREB (KCREB) or C/EBP (GBF-F) proteins also inhibited the responsiveness of the PEPCK gene promoter to cAMP and E1a. We were able to recapitulate the superstimulation of cAMP-stimulated transcription by E1a with an enhancerless promoter linked to four Gal4 response elements in cells expressing both chimaeric Gal4–CREB and Gal4–C/EBPα proteins. No superstimulation was observed in cells expressing C/EBPβ alone, or in conjunction with Gal4–CREB or Gal4–C/EBPα. No interactions between CREB, C/EBPα or E1a were noted in co-immunoprecipitation assays, but C/EBPα recovered from E1a-expressing cells was highly phosphorylated compared with C/EBPα from control cells. Our data indicate that the response of PEPCK gene transcription to cAMP and E1a is mediated in large part through interactions between CREB and C/EBPα, but not C/EBPβ. The ability of E1a to potentiate cAMP-stimulated transcription from the PEPCK gene promoter may be due in large part to its role in augmenting the phosphorylation of C/EBPα.

EXPERIMENTAL

Materials

The Ad5 E1a mutant DL520, which expresses only the 12 S form of E1a, was provided by Arnold Berk (University of California, Los Angeles, CA, U.S.A.). E1a-1104 and E1a-1108, which are Ad5 E1a CR1 and CR2 deletion mutants respectively, were provided by Stanley Bayley (McMaster University, Hamilton, Ontario, Canada). Wild-type Ad5 was grown and titred in A549 cells, and the mutant adenoviruses were grown and titred in 293 cells.

Plasmids containing the full-length, wild-type PEPCK gene promoter, or versions of the promoter in which the CRE1, P3-I, P3-II or P4 protein-binding sites were disrupted via site-directed mutagenesis, were provided by Richard Hanson (Case Western Reserve University, Cleveland, OH, U.S.A.). The expression vector pRSV-KCREB, from which the dominant-negative CREB inhibitor protein is expressed, was a gift from Richard Goodman (Vollum Institute, Portland, OR, U.S.A.), and the plasmid pRGX-GBF-F, from which the dominant-negative C/EBP protein was expressed, was obtained from Charles Vinson (National Institutes of Health, Bethesda, MD, U.S.A.). A luciferase reporter plasmid containing an enhancerless thymidine kinase (TK) promoter linked to four copies of the Gal4 DNA-binding sequence, pGal4TKLuc, was provided by James Hoeffler (Invitrogen, Carlsbad, CA, U.S.A.). A chimaeric protein consisting of the Gal4 DNA-binding domain linked to the CREB transactivation region was expressed from the plasmid pRSV-Gal4-CREB 327, a gift from Joel Habener (Massachusetts General Hospital, Boston, MA, U.S.A.). William Roesler (University of Saskatchewan, Saskatoon, Saskatchewan, Canada) kindly provided the expression vectors pSV-Gal4-C/EBPα and pSV-Gal4-C/EBPβ, from which chimaeric proteins containing the Gal4 DNA-binding domain linked to the C/EBPα or C/EBPβ transactivation domains respectively were expressed.

Polyclonal antibodies to CREB, and to CREB phosphorylated on serine-133, were purchased from New England Biolabs (Beverly, MA, U.S.A.), and polyclonal antibodies to C/EBPα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell lines, and transfection and infection procedures

HepG2 human hepatoma cells were passaged in Dulbecco's modified Eagle's medium/F12 containing 5% (v/v) fetal calf serum and 5% (v/v) calf serum. Plates of HepG2 cells were grown to approx. 80% confluency and transfected with the indicated plasmids using Superfect reagent (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's recommendations. Immediately after transfection, the cells were infected as described previously [16]. To obtain consistent levels of E1a expression, wild-type Ad5 was used at a multiplicity of infection of 100, whereas all other viruses were used at a multiplicity of infection of 200 [16]. Infected cells were incubated for 20 h before analysis.

Luciferase assays were performed on a Turner Designs TD 20/20 luminometer using the Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratories, San Diego, CA, U.S.A.) according to the supplier's directions. Transfection efficiencies were normalized by co-transfecting the cells with plasmid containing the Rous sarcoma virus long terminal repeat linked to a β-galactosidase reporter gene, and β-galactosidase levels were measured as described previously. Assays were repeated at least three times, and consistent results were obtained in all cases.

Western blot analysis of total CREB, phosphorylated CREB and C/EBPα

HepG2 cells infected and/or treated as described in the Figure legends were lysed by resuspending cell pellets in a buffer containing 20 mM Hepes, pH 7.6, 300 mM NaCl, 1.5 mM

$MgCl_2$, 1 mM EDTA, 10 % glycerol, 0.5 % Nonidet P40, 1 μg/ml leupeptin, 1 mM benzamidine, 1 μg/ml pepstatin A, 50 mM β-glycerophosphate, 2 mM sodium vanadate, 50 mM NaF, 50 μM phenylarsine oxide and 100 nM okadaic acid. The lysed cell suspension was held on ice for 30 min, during which they were subjected to brief vortexing every 5 min. Debris was removed by centrifugation at 10000 ***g*** for 5 min and the supernatant was recovered for analysis. After correcting for protein concentrations, the lysates were resolved on SDS/10 %-polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose blots were blocked with PBS containing 5 % (w/v) dried milk and 0.1 % Tween 20, and then treated with antibodies that recognize phosphorylated CREB alone, both unphosphorylated and phosphorylated forms of CREB, or C/EBPα [40]. The blots were washed and subsequently treated with goat anti-(rabbit IgG) conjugated to alkaline phosphatase. After the blots were washed, specific immune complexes were visualized with bromochloroindolyl phosphate and Nitro Blue Tetrazolium.

Labelling of cells with [^{32}P]P_i and immunoprecipitation of C/EBPα was performed as described by Wadzinski et al. [30]. Basically, HepG2 cells were grown to approx. 80 % confluency. Plates of cells (2.5×10^6/plate) were washed with PBS and the medium was replaced with 5 ml of phosphate-free Dulbecco's modified Eagle's medium containing 1 % (w/v) BSA and 1 mCi of [^{32}P]P_i. The plates were incubated for 20 h in the absence or presence of Ad5 at 37 °C and then treated with 0.3 mM dibutyryl cAMP (Bt_2cAMP) for an additional 30 min.

Following treatment, the medium was removed from the cells, which were washed once with cold PBS. The cell pellets were lysed in 1 ml of 20 mM Hepes, pH 7.9, containing 1 % SDS and 0.1 % 2-mercaptoethanol in a boiling-water bath for 2 min. The lysates were diluted with 9 ml of 20 mM Hepes, pH 7.9, containing 1 % Nonidet P40, 1 mM EDTA, 1 μg/ml leupeptin, 1 mM benzamidine, 1 μg/ml pepstatin A, 50 mM β-glycerophosphate, 2 mM sodium vanadate, 50 mM NaF, 50 μM phenylarsine oxide and 100 nM okadaic acid. After normalizing the supernatants for protein concentration, C/EBPα was recovered from the supernatants by immunoprecipitation with C/EBPα-specific antibodies covalently linked to Protein A–Sepharose beads with dimethyl pimelimidate. Immunoprecipitated material was resolved on SDS/10 %-polyacrylamide gels. The identity of the C/EBPα band and the relative amounts of C/EBPα recovered were determined by Western blotting.

RESULTS

E1a enhances cAMP-stimulated transcription from the PEPCK gene promoter through CR3

Our interest in E1a transcriptional functions led us to investigate its effect on the well-defined PEPCK gene promoter. In a previous report [16], we demonstrated that E1a consistently stimulated basal transcription from the PEPCK gene promoter in adenovirus-infected HepG2 and H4IIE hepatoma cells, and in E1a-expressing, stably transfected 3T3 fibroblasts. This stimulation was not observed in cells infected with a mutant adenovirus expressing only 12 S E1a, or a virus expressing a CR3-deficient form of 13 S E1a. Mutations in CR1 had no effect on PEPCK gene promoter-driven transcription, but mutations in CR2 that abolish the binding of E1a to the retinoblastoma gene product further stimulated transcription by 2–3-fold. We concluded that E1a stimulates basal PEPCK gene transcription through a stimulatory mechanism involving CR3, and by attenuating a stimulatory effect of the retinoblastoma gene product via CR2. In preliminary experiments we also noted that E1a enhances the stimulation of PEPCK gene transcription by cAMP, but the mechanism(s) underlying this response was not fully explored.

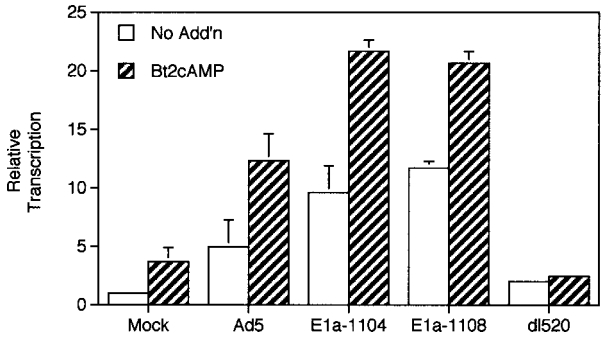

Figure 1 Superstimulation of cAMP-stimulated PEPCK gene transcription by adenoviral E1a is mediated by CR3

HepG2 cells were transfected with a plasmid containing the full-length, wild-type PEPCK gene promoter linked to a luciferase reporter gene (−490 pPCLuc). The cells were then infected with the indicated adenoviruses for 20 h. For the final 4 h of infection, duplicate plates of cells were treated with 0.3 mM Bt_2cAMP. Luciferase activity in cell lysates was measured as described in the Experimental section. Levels of transcription are shown relative to those in mock-infected cells not treated with Bt_2cAMP (= 1). E1a-1104 and E1a-1108 are Ad5 E1a CR1 and CR2 deletion mutants respectively, and dl520 expresses only the 12 S form of E1a.

To determine which of the E1a proteins and CRs were involved in the superstimulation of cAMP-stimulated PEPCK-promoter-driven transcription, we transfected HepG2 cells with a plasmid containing the full-length PEPCK gene promoter linked to a luciferase reporter gene. The cells were subsequently infected with wild-type Ad5 or mutant adenoviruses which express various forms of E1a. Mock-infected cells were used as a control. At approx. 20 h post-infection, cell lysates were prepared and luciferase activity was measured as an index of transcription from the PEPCK gene promoter. As shown in Figure 1, expression of wild-type 12 S and 13 S proteins in Ad5-infected cells increased both basal and Bt_2cAMP-stimulated transcription by 3–5-fold. These data are consistent with the results we described previously [16]. When cells were infected with adenoviruses expressing 12 S and 13 S E1a proteins with mutations in CR1 (E1a-1104; does not bind CBP/p300) or CR2 (E1a-1108; does not bind p107 or p105Rb), basal and Bt_2cAMP-stimulated transcription levels were elevated compared with levels measured in Ad5-infected cells. These data are also consistent with our previous findings [16], and suggest that interactions between E1a and p300, p107, and p105Rb may have an inhibitory effect on transcription from the PEPCK promoter. In HepG2 cells infected with a virus that expressed only a 12 S E1a protein (DL520), Bt_2cAMP-stimulated transcription was completely inhibited. This suggests that the superstimulation of cAMP-stimulated transcription from the PEPCK promoter was mediated by the 13 S E1a protein. Since the only significant difference between the 12 S and 13 S E1a proteins is the presence of CR3 in the 13 S protein, we conclude that the superstimulation is induced by CR3. Moreover, these data clearly show that, in the absence of a 13 S protein, the 12 S E1a protein is a potent inhibitor of cAMP-stimulated PEPCK gene transcription.

CREB and C/EBPα are required for the E1a-induced enhancement of cAMP-stimulated PEPCK gene transcription

A number of *cis*-acting elements are involved in the regulation of PEPCK gene transcription by cAMP [17,22–24,26,31]. The most important of these elements are designated CRE1, P3-I, P3-II

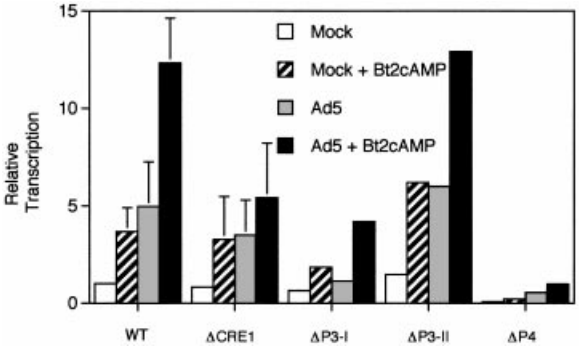

Figure 2 Superstimulation of PEPCK gene transcription by E1a and Bt_2cAMP requires binding sites for CREB and C/EBP

HepG2 cells were transfected with plasmids containing full-length PEPCK gene promoter regions linked to a luciferase reporter gene. As indicated, a wild-type (WT) PEPCK gene promoter and promoters containing site-directed mutations altering the indicated protein binding site (ΔCRE1, ΔP3-I, ΔP3-II or ΔP4) were tested. The cells were then infected with wild-type Ad5 for 20 h, with some plates receiving 0.3 mM Bt_2cAMP for the final 4 h of infection. Luciferase activity in cell lysates was measured as described in the Experimental section, and transcription levels are shown relative to levels measured in mock-infected cells not treated with Bt_2cAMP (= 1).

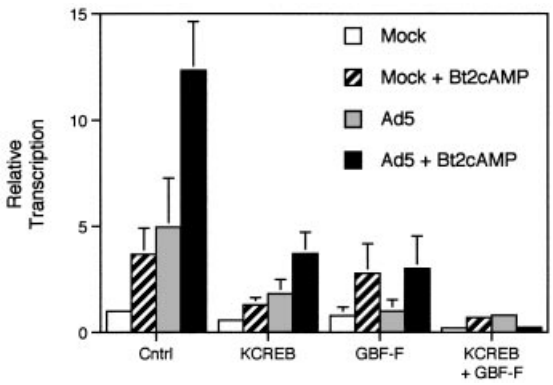

Figure 3 Superstimulation of PEPCK gene transcription by E1a and Bt_2cAMP requires CREB and C/EBP proteins

HepG2 cells were transfected with a plasmid containing the full-length, wild-type PEPCK gene promoter linked to a luciferase reporter gene. The cells were co-transfected with expression vectors for dominant-negative CREB (KCREB) and/or dominant-negative C/EBP (GBF-F). As indicated, some cells were then infected with Ad5 for 20 h, with some cells also receiving 0.3 mM Bt_2cAMP for the final 4 h of infection. Luciferase levels in cell lysates was measured as described in the Experimental section. Transcription levels are shown relative to levels measured in mock-infected cells not treated with Bt_2cAMP (= 1).

and P4, and have been shown to be bound by CREB and C/EBP family members. To determine which, if any, of these sites is important in the superstimulation of cAMP-dependent transcription from the PEPCK gene promoter by E1a, we tested the ability of E1a and/or Bt_2cAMP to regulate transcription from full-length fragments of the PEPCK gene promoter containing individual mutations in each of these elements that block nuclear protein binding [41]. We found that mutation of the P3-II site had no significant effect on Bt_2cAMP-, E1a- or E1a + Bt_2cAMP-stimulated transcription levels as compared with the wild-type promoter fragment (Figure 2). However, mutations in CRE1, P3-I or P4 all decreased the superstimulation of transcription by E1a plus Bt_2cAMP. The CRE1 mutation had little effect on either Bt_2cAMP- or E1a-stimulated transcription levels, but reduced the superstimulation by E1a and Bt_2cAMP together by 50–60 %. The mutation in P3-I decreased the responsiveness of the PEPCK promoters to both Bt_2cAMP and E1a, and decreased the E1a + Bt_2cAMP-induced superstimulation by 60–70 % compared with levels with the wild-type PEPCK gene promoter. Mutation of the P4 site severely reduced transcription levels in response to all stimuli by 80–90 %. These data clearly indicate that multiple *cis*-acting elements in the PEPCK gene promoter are required for responsiveness to cAMP and E1a, and suggest that CREB and members of the C/EBP family of transcription factors are involved in these processes.

To demonstrate that CREB and C/EBP proteins are involved in the response of the PEPCK gene promoter to cAMP and E1a, the effects of dominant-negative CREB and C/EBP proteins on Bt_2cAMP-, E1a- and Bt_2cAMP + E1a-stimulated transcription from the full-length, wild-type PEPCK gene promoter were measured. KCREB [42], which forms heterodimers with endogenous CREB and inhibits DNA binding, was employed as the dominant-negative CREB. The chimaeric GBF-F protein was used as the dominant-negative C/EBP; this protein is composed of the DNA-binding region of the plant GBF-1 protein linked to a modified leucine zipper domain, and preferentially forms heterodimers with all members of the C/EBP family [43]. Since GBF-F lacks a transactivation domain, heterodimers with C/EBP proteins are transcriptionally inactive. Ectopic expression of KCREB inhibited the responsiveness of the PEPCK gene promoter to Bt_2cAMP and/or E1a, as well as basal transcription levels (Figure 3). In spite of this overall inhibitory effect, the general pattern of induction by Bt_2cAMP or E1a, and superstimulation by the Bt_2cAMP + E1a combination, was still observed. This response differed from that measured in cells expressing GBF-F. The dominant-negative C/EBP protein also inhibited the stimulation of transcription from the PEPCK gene promoter in response to Bt_2cAMP and/or E1a. However, GBF-F appeared to completely block the effect of E1a, whereas a modest response to cAMP remained. Introduction of KCREB and GBF-F together resulted in global inhibition of basal transcription levels and of responsiveness to Bt_2cAMP and/or E1a. These data indicated that both CREB and C/EBP proteins are required for E1a and/or cAMP to stimulate PEPCK gene promoter-driven transcription.

To begin to define the role of CREB and C/EBP proteins in E1a/cAMP-regulated transcription, we attempted to reconstitute this response using a plasmid (pGal4TKLuc) containing an enhancerless promoter linked to four copies of the Gal4 response element (Figure 4B). Since HepG2 cells lack endogenous Gal4-binding proteins, this system permitted the introduction of chimaeric proteins composed of the Gal4 DNA-binding domain linked to the transactivation regions of CREB, C/EBPα and C/EBPβ (Figure 4A). As shown in Figure 4, transcription from the Gal4-responsive promoter was virtually undetectable in the absence of the chimaeric transcription factors. When cells were co-transfected with the Gal4–CREB expression vector, basal transcription from the Gal4TK promoter was increased to measurable levels; in addition, Bt_2cAMP stimulated transcription by approx. 3.5-fold and E1a produced a 5–6-fold increase in luciferase expression. While the presence of Gal4–CREB was sufficient to mediate separate responses to cAMP and E1a, no superstimulation was noted when these factors were tested in combination. Expression of Gal4–C/EBPα also increased basal transcription to a level comparable with that noted with Gal4–CREB, and also supported a 5-fold stimulation in the presence of E1a. However, no stimulation of transcription was noted with

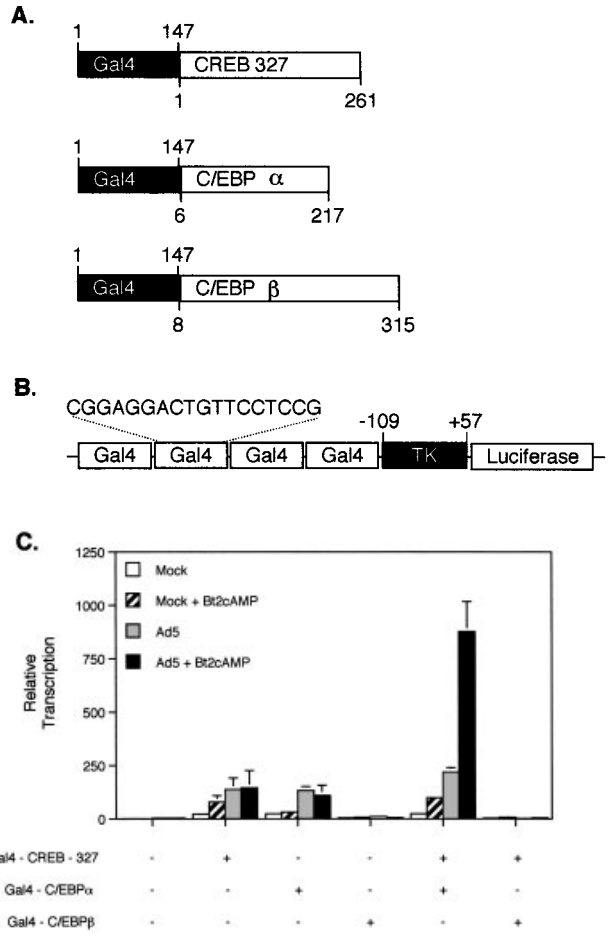

Figure 4 Reconstitution of superstimulation of transcription by E1a and Bt_2cAMP on a Gal4-responsive promoter

(**A**) Diagrammatic representation of chimaeric Gal4–CREB 327, Gal4–C/EBPα and Gal4–C/EBPβ proteins. Numbers above each protein designate amino acids of the Gal4 DNA-binding domain, and numbers below each protein designate amino acids of the transactivation regions for CREB 327, C/EBPα and C/EBPβ. (**B**) Diagrammatic representation of the region of the luciferase reporter plasmid, pGal4TKLuc, containing the four copies of the Gal4 DNA-binding sequence linked to the enhancerless TK promoter. The sequence of the Gal4 binding sites is shown above one of the domains, and the numbers above the TK promoter indicate base positions with respect to the transcription start site. (**C**) HepG2 cells were transfected with a plasmid containing an enhancerless TK promoter linked to four copies of the Gal4 DNA-binding sequence upstream of a luciferase reporter gene (pGal4TKLuc). As indicated, some cells were also co-transfected with plasmids from which chimaeric Gal4–CREB, Gal4–C/EBPα or Gal4–C/EBPβ proteins were expressed. Following transfection, cells were infected with Ad5 for 20 h, with some cells receiving 0.3 mM Bt_2cAMP for the final 4 h of infection. Luciferase levels in cell lysates were measured as described in the Experimental section, and transcription levels are shown relative to levels measured in mock-infected cells transfected with pGal4TKLuc alone and not treated with Bt_2cAMP.

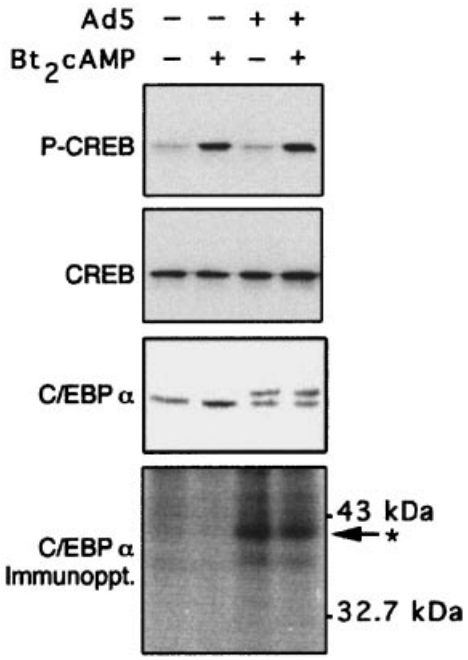

Figure 5 Changes in CREB and C/EBPα phosphorylation in HepG2 cells with adenovirus infection and/or Bt_2cAMP treatment

HepG2 cells were infected with Ad5 for 20 h and/or treated with 0.3 mM Bt_2cAMP for 30 min, as indicated above the gels. The top three panels show representative Western blots of cell lysate proteins performed with antibodies to CREB phosphorylated on serine-133 (P-CREB), total CREB (CREB) or C/EBPα. For the bottom panel, cells were incubated in medium containing 1 mCi of [^{32}P]P_i during the 20 h infection period. C/EBPα was immunoprecipitated from cell lysates with C/EBPα-specific antibodies linked to Protein A-conjugated Sepharose beads. The immunoprecipitated material was separated on an SDS/10%-polyacrylamide gel. The panel shows a representative autoradiogram of the resulting gel.

Bt_2cAMP, nor was superstimulation observed in the Gal4–C/EBPα-expressing cells. However, when Gal4–CREB and Gal4–C/EBPα expression vectors were co-transfected into HepG2 cells, a pattern of regulation by Bt_2cAMP and E1a similar to that observed for the PEPCK gene promoter was noted. The combination of these two proteins supported a 4-fold increase in transcription with Bt_2cAMP, a 10–11-fold stimulation in the presence of E1a, and an approx. 40-fold superstimulation with Bt_2cAMP and E1a together. The ability to reconstitute this response with the Gal4TK promoter by expressing both Gal4–CREB and Gal4–C/EBPα suggests that these two factors play the same roles in the PEPCK gene promoter system. No stimulation of basal or Bt_2cAMP- and/or E1a-regulated transcription was measured in Gal4–C/EBPβ-expressing cells. Furthermore, when Gal4–C/EBPβ was expressed in combination with Gal4–CREB, no increases in basal or stimulated levels of transcription were observed. Thus the C/EBPβ transactivation region not only fails to support responses to cAMP and E1a, but appears to inhibit the ability of CREB to participate in these functions as well.

The ability of E1a to stimulate transcription from the PEPCK gene promoter is correlated with increased C/EBPα phosphorylation

The stimulation of gene transcription by cAMP generally requires the phosphorylation of CREB at a specific serine residue (Ser-133). As anticipated, we found that the amount of CREB phosphorylated at this site (Figure 5, P-CREB panel), but not total CREB content (Figure 5, CREB panel), was increased in HepG2 cells following treatment with Bt_2cAMP. Unexpectedly, we found that phosphorylation of C/EBPα was elevated in E1a-expressing cells. Phosphorylated C/EBPα was recovered from [^{32}P]P_i-labelled cytosolic and nuclear fractions from Ad5-infected cells (E1a expressing), but not from mock-infected cells (Figure 5, C/EBPα Immunoppt. panel). This phosphorylation appeared to be independent of Bt_2cAMP treatment. Likewise, Western

blots of nuclear proteins from mock-infected cells exhibited a single C/EBPα band, whereas two bands were detected in extracts from E1a-expressing cells (Figure 5, C/EBPα panel). The upper band of the doublet seen in extracts from E1a-expressing cells appears to represent the phosphorylated C/EBPα protein displaying a differential electrophoretic mobility from the unphosphorylated form. No interactions between CREB, C/EBPα and/or E1a proteins were detected in co-immunoprecipitation reactions (results not shown).

DISCUSSION

The results presented in this paper show that adenovirus E1a protein enhances cAMP-stimulated transcription from the PEPCK gene promoter. This effect appears to be mediated by the 13 S form of E1a through CR3, since the 12 S protein did not support this response and even produced an inhibition of cAMP-stimulated transcription levels. E1a proteins with mutations in CR1 or CR2 evoked higher levels of basal and cAMP-stimulated transcription than wild-type E1a proteins, suggesting that these regions exert slightly inhibitory effects on the PEPCK gene promoter system. We have reported these negative effects, particularly on basal transcription levels, in a previous report [16]. Overall, these results are consistent with other reports demonstrating that the 13 S form of E1a participates in transcriptional activation via CR3, whereas 12 S acts as a transcriptional repressor through CR1- and CR2-mediated interactions.

An interesting aspect of the present results was the requirement for both CREB and C/EBPα for the superstimulation of transcription from the PEPCK gene promoter by cAMP and E1a. The relative roles of CREB and C/EBPs α and β in mediating the response to cAMP of the PEPCK gene promoter have been debated extensively in the literature. Our data indicate that dominant-negative inhibitors of either CREB or C/EBP proteins inhibit transcription from this promoter in response to cAMP and/or E1a. In addition, reconstitution of this process with a Gal4-responsive promoter clearly required both CREB and C/EBPα, but not C/EBPβ. Based on these data, we conclude that CREB and C/EBPα participate in the superstimulation of PEPCK gene transcription by cAMP and E1a. In particular, the results presented in Figure 4 suggest that CREB mediates an increase in transcription in response to cAMP, whereas C/EBPα supports a transcriptional stimulation in response to E1a. When expressed together, these two transcription factors generate a superstimulation of transcription in response to cAMP plus E1a.

Another interesting result from the present studies was the correlation between E1a-stimulated transcription from the PEPCK gene promoter and increases in C/EBPα phosphorylation in Ad5-infected cells. Phosphorylation appears to produce both stimulatory and inhibitory effects on C/EBPα. Phosphorylation of serine-299 of C/EBPα, which lies within the basic region of the DNA-binding domain, inhibits C/EBPα DNA-binding activity and thereby decreases the protein transactivating function [44]. Alternatively, MacDougald and colleagues [45,46] have shown that C/EBPα is normally phosphorylated in 3T3-L1 adipocytes. Dephosphorylation of C/EBPα in response to insulin or insulin-like growth factor 1 is correlated with a decrease in C/EBPα transcriptional activity, suggesting that phosphorylation potentiates C/EBPα transcriptional activity. Our data indicate that C/EBPα undergoes an E1a-induced phosphorylation(s), concomitant with increased transcriptional activity. Thus E1a may induce C/EBPα transcriptional activity in a manner analogous to the stimulation of CREB activity by phosphorylation via the cAMP/PKA signalling system. We have undertaken studies to identify the sites within C/EBPα that are phosphorylated in response to E1a, as well as the kinase(s) involved in this process. Protein kinase CKII is a potential candidate for this role, since its activity is stimulated by interactions with CR2 of E1a, although its participation in this system remains unclear.

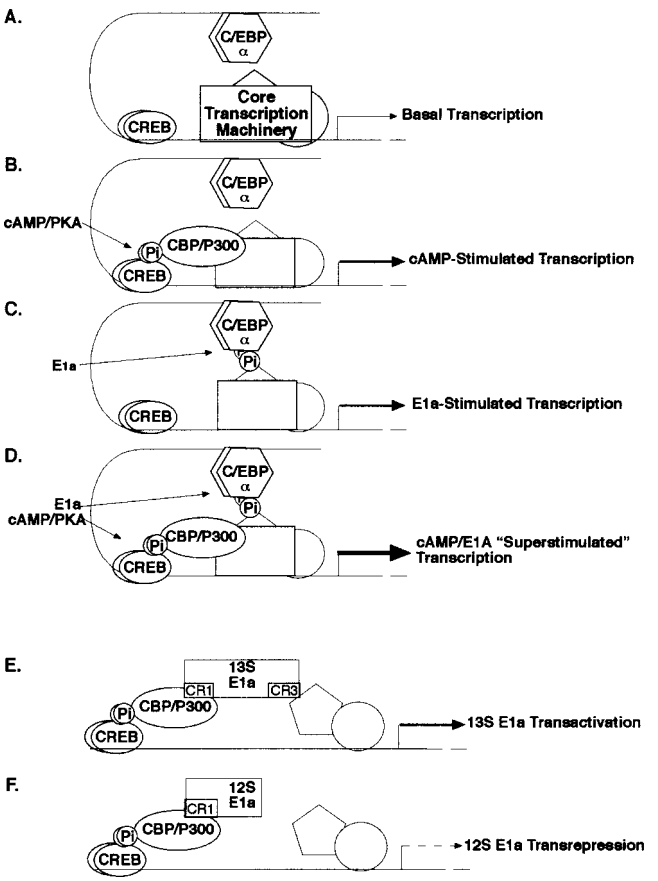

Figure 6 Proposed mechanism by which E1a and cAMP superstimulate PEPCK gene promoter-driven transcription

Panels (**A**)–(**D**) indicate potential mechanisms by which cAMP or E1a individually stimulate PEPCK gene transcription, and together produce a superstimulated transcriptional response. Panels (**E**) and (**F**) show a potential mechanism by which 13 S E1a may stimulate gene transcription, whereas 12 S E1a would inhibit transcription. Levels of transcription are indicated by the thickness of the arrows.

How, then, do E1a and cAMP generate a superstimulation of transcription from the PEPCK (or Gal4-responsive) promoter? We propose the models shown in Figure 6, which take into consideration data presented in this paper and information from other laboratories. These models focus on CREB and C/EBPα for the sake of simplicity, but multiple factors are clearly involved in the overall transcriptional response of the PEPCK gene promoter. In untreated cells, CREB and C/EBPα are 'unphosphorylated', and core transcription factors are recruited to the transcription start site at a low rate, producing the 'basal' level of transcription (Figure 6A). When cells are treated with a cAMP agonist, CREB is phosphorylated by PKA, which enhances its interaction with the transcriptional co-activators, CBP and p300 (Figure 6B). CBP and/or p300 potentiates the rate of core transcription factor recruitment/assembly, producing a 'cAMP-stimulated' level of transcription. Similarly, E1a expression in Ad5-infected cells results in the phosphorylation of C/EBPα (Figure 6C). This phosphorylation event may increase the ability

of C/EBPα to interact directly with components of the core transcription machinery, thereby producing an 'E1a-stimulated' level of transcription. When E1a-expressing cells are treated with a cAMP agonist, each of these stimulatory mechanisms is activated, and in concert produce a 'superstimulated' level of transcription (Figure 6D). The ability of 13 S E1a to stimulate, and of 12 S E1a to repress, transcription is indicated in Figures 6(E) and 6(F) respectively. We propose that 13 S E1a binds to the CREB–CBP complex through interactions between CR1 of E1a and CBP. CR3 of 13 S E1a can then interact with components of the core transcriptional machinery, enhancing the rate of transcription. Alternatively, 12 S E1a may interact with the CREB–CBP complex through CR1, like the 13 S protein. However, since 12 S E1a lacks CR3, no interaction with the core transcription machinery is possible, and transcription is repressed.

The question remains as to how a particular transcriptional response (either positive or negative) is generated when a transcriptional activator (13 S E1a) and a transcriptional repressor (12 S E1a) are expressed in the same cell following adenovirus infection. In many cases a particular response probably reflects the differential ability of 13 S and 12 S E1a proteins to interact with certain transcription factors, which is supported by studies demonstrating direct interactions between 13 S E1a and ATF2, TFIID and AP-1 [3–7]. However, both 12 S and 13 S E1a appear to bind to CBP/p300, and one would expect the effect of one form of E1a to counteract the effect of the other protein in such situations. Perhaps one form of E1a is expressed at a higher level than the other in certain cell types or under specific conditions. The particular transcriptional response would then reflect the effect of the E1a protein expressed in greater abundance. Studies designed to address these possibilities should provide important information not only on the function of E1a proteins, but also on the transcriptional systems they modulate.

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