transcription sites were displayed as yellow. The relative sizes of the corresponding areas were directly measured.

15. If we include nucleolar regions in the transcription cluster category, the area occupied by clustered replication and transcription sites will increase to about 80% of the total contoured areas.
16. Synchronization was achieved by serum deprivation followed by aphidicolin inhibition at the G_1/S border [S. Spadari *et al.*, *Drug Res.* **35**, 1108 (1985)]. Contour analysis on 3T3 mouse cells synchronized at the G_1/S border showed that 64% of the contoured area is occupied by replication and transcription site clusters.
17. Cluster analysis was performed on "Sn" number of clusters that were generated using the "random ( )" library function. Of the total number of sites, some were randomly chosen to be transcription sites (red) and the remaining were marked as replication sites (green). Computation of radii of clusters is based on a Gaussian distribution function with a standard deviation of 1.7.
18. Three-dimensional reconstruction of the contours was performed from the confocal optical sections using the drawing tool of IPlab (Signal Analytics).
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CBP: A Signal-Regulated Transcriptional Coactivator Controlled by Nuclear Calcium and CaM Kinase IV

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Recruitment of the coactivator, CREB binding protein (CBP), by signal-regulated transcription factors, such as CREB [adenosine 3′,5′-monophosphate (cAMP) response element binding protein], is critical for stimulation of gene expression. The mouse pituitary cell line AtT20 was used to show that the CBP recruitment step (CREB phosphorylation on serine-133) can be uncoupled from CREB/CBP–activated transcription. CBP was found to contain a signal-regulated transcriptional activation domain that is controlled by nuclear calcium and calcium/calmodulin–dependent (CaM) protein kinase IV and by cAMP. Cytoplasmic calcium signals that stimulate the Ras mitogen–activated protein kinase signaling cascade or expression of the activated form of Ras provided the CBP recruitment signal but did not increase CBP activity and failed to activate CREB- and CBP-mediated transcription. These results identify CBP as a signal-regulated transcriptional coactivator and define a regulatory role for nuclear calcium and cAMP in CBP-dependent gene expression.

The coactivator CBP and its close relative p300 are vital components of the cellular machinery that regulate gene expression (*1, 2*). CBP can connect sequence-specific transcriptional activators to components of the basal transcription machinery (*3, 4*) and may disrupt repressive chromatin structures through its intrinsic or associated histone acetyltransferase activity (*5*). Signals from the environment are thought to induce gene transcription by activating intracellular biochemical pathways that control the ability of transcription factors to recruit CBP to specific promoters (*3, 6–8*). In this study, we investigated the possibility that intracellular signaling mechanisms, in addition to leading to CBP recruitment, control the rate of gene transcription by regulating CBP activity. CREB phosphorylated on Ser^{133} is the prototypical CBP-interacting transcriptional activator (*1*). Using the mouse pituitary cell line AtT20, we first assessed phosphorylation of CREB on Ser^{133}, the CBP recruitment signal (*1–3, 9*), in immunocytochemical experiments with an antibody specific for CREB phosphorylated on Ser^{133} (*10*). CREB-mediated transcriptional responses were analyzed in parallel with an expression vector encoding GAL4-CREB, which was microinjected together with the reporter gene pF222ΔCREmyc. This construct contains the human c-*fos* gene including 222 base pairs (bp) of upstream regulatory sequence. It lacks the c-*fos* CRE (ΔCRE) and contains a single GAL4 site, which, upon binding of GAL4-CREB, can confer calcium (Ca^{2+}) inducibility to the reporter gene (*11*). A Myc epitope was inserted in frame into the fourth exon of the c-*fos* gene, allowing expression of the reporter gene to be detected at the single cell level by immunofluorescence with the 9E10 antibody and to be quantified by confocal laser scanning microscopy (*11*). Entry of Ca^{2+} into AtT20 cells, a mouse pituitary cell line, through L-type Ca^{2+} channels [triggered by KCl-induced membrane depolarization in the presence of the L-type Ca^{2+} channel agonist FPL 64176 (KCl-FPL treatment)] (*11*) induced phosphorylation of CREB on Ser^{133} followed by CREB-mediated transcriptional activation (Fig. 1A).

Because Ca^{2+} acts in two cellular compartments, the nucleus and the cytoplasm, to activate gene expression by distinct mechanisms (*11*), we analyzed the control of CREB phosphorylation on Ser^{133} by spatially distinct Ca^{2+} signals. Selective inhibition of nuclear Ca^{2+} transients by means of nuclear microinjection of a nondiffusible Ca^{2+} chelator, BAPTA-D70 [1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid linked to a 70-kD dextran molecule] (*11*), blocked CREB-mediated transcription in response to KCl-FPL but had little inhibitory effect on CREB phosphorylation on Ser^{133} (Fig. 1A). Thus, nuclear and cytoplasmic Ca^{2+} signals have distinct roles in CREB regulation. Activation of cytoplasmic Ca^{2+} signaling pathways stimulates CREB phosphorylation on Ser^{133} but, in the absence of nuclear Ca^{2+}, CREB remains transcriptionally inactive. A second regulatory event is apparently triggered by nuclear Ca^{2+} and leads to transcriptional activation. CREB contains another phosphorylation site, Ser^{142}, that inhibits transcriptional activation by CREB (*12*). However, nuclear Ca^{2+} appears not to control CREB through phosphorylation on Ser^{142} because inhibition of nuclear Ca^{2+} signals also blocked transcriptional activation by a mutant CREB protein, GAL4-CREB(Ser^{142}Ala), which contains a serine-to-alanine mutation at position 142 (*12*).

Ca^{2+}/calmodulin-dependent (CaM) protein kinases are prime candidates for mediating Ca^{2+}-regulated gene expression (*13–15*). Inhibition of CaM kinases with KN-62 did not reduce Ca^{2+}-induced CREB phosphorylation on Ser^{133} (Fig. 1B) but did inhibit CREB-mediated transcriptional ac-

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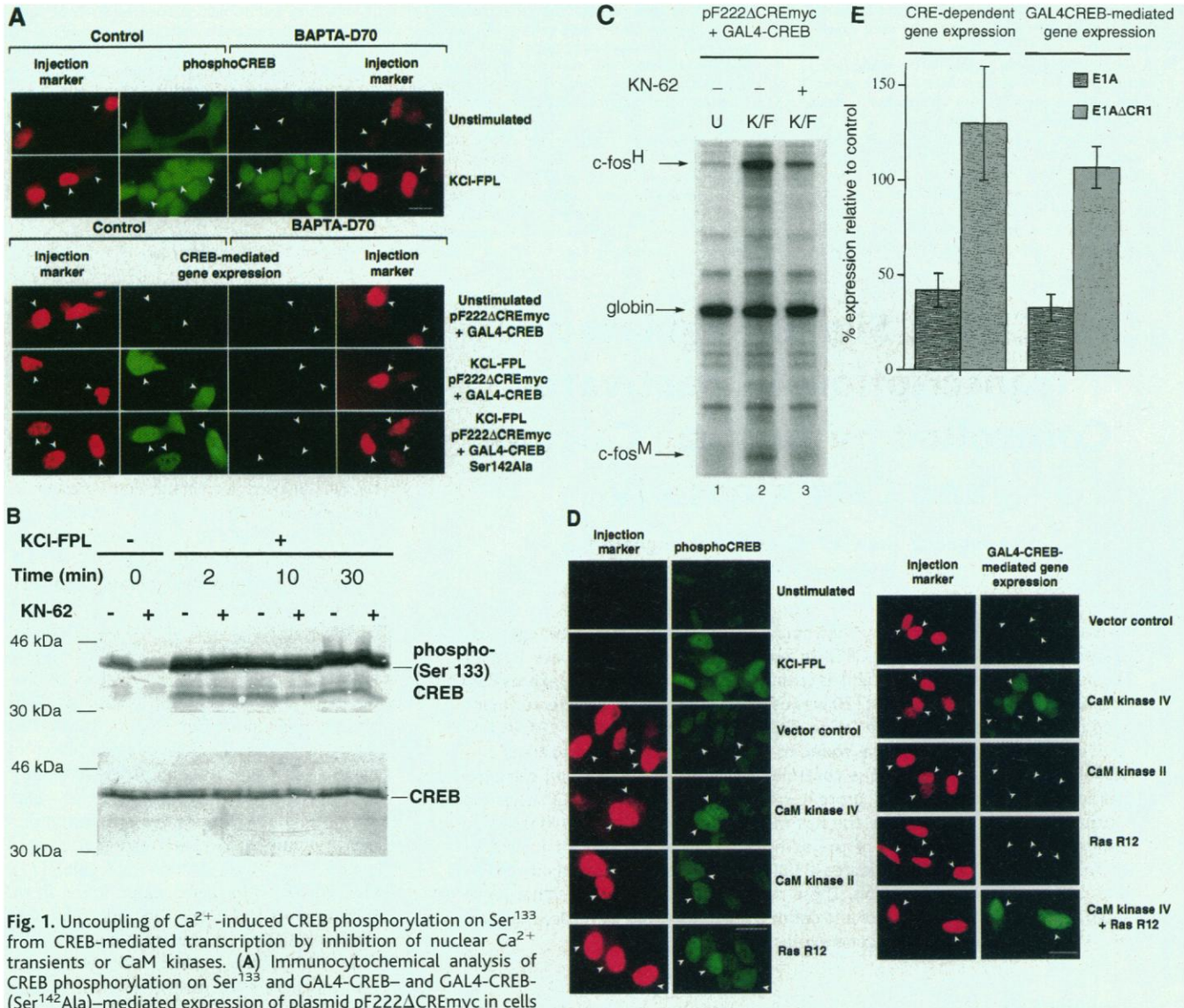

Fig. 1. Uncoupling of Ca^{2+}-induced CREB phosphorylation on Ser^{133} from CREB-mediated transcription by inhibition of nuclear Ca^{2+} transients or CaM kinases. (**A**) Immunocytochemical analysis of CREB phosphorylation on Ser^{133} and GAL4-CREB– and GAL4-CREB-(Ser^{142}Ala)–mediated expression of plasmid pF222ΔCREmyc in cells microinjected into the nucleus with BAPTA-D70 (*12*) (right) or control solution (left). The Texas Red fluorescence image identifies nuclear microinjected cells (arrowheads). In cells injected into the nucleus with BAPTA-D70, the KCl-FPL–induced CREB phosphorylation on Ser^{133} was 77% ± 3% (88 cells) (mean ± SEM of independent experiments) of that obtained in control injected cells. The KCl-FPL–induced GAL4-CREB– and GAL4-CREB-(Ser^{142}Ala)–mediated responses in cells injected with BAPTA-D70 were 10% ± 0.3% (38 cells) (*11*) and 30% ± 12% (102 cells), respectively, of that obtained in cells injected with control solution. Bar = 20 μm. (**B**) Kinetics of Ca^{2+}-induced CREB phosphorylation on Ser^{133} in the presence or absence of the CaM kinase inhibitor KN-62. Cells were treated with KN-62 (10 μM) 15 to 20 min before stimulation with KCl-FPL. Protein immunoblotting was done with antibodies to CREB phosphorylated on Ser^{133} or to CREB (*10*). (**C**) Ribonuclease protection analysis (*11, 15, 28*) of GAL4-CREB–mediated expression from the transfected human c-*fos*–based reporter gene pF222ΔCREmyc (c-fos^H) and expression of the endogenous mouse c-*fos* gene (c-fos^M) in unstimulated cells (U) or in cells 50 min after stimulation with KCl-FPL (K/F). Cells were transfected with pF222ΔCREmyc, the GAL4-CREB expression plasmid, and the human α-globin gene (plasmid pSVα1) (*28*) to normalize for transfection efficiency. Cells were treated with KN-62 (10 μM) 15 to 20 min before stimulation. Relative to the increases in mRNA levels in control cells stimulated with KCl-FPL, the CaM kinase inhibitor KN-62 caused a 64% ± 4% (mean ± SEM; n = 4) inhibition of the GAL4-CREB–mediated response. (**D**) Immunocytochemical analysis of CREB phosphorylation on Ser^{133} (left) and GAL4-CREB–mediated transcriptional activation (right) in cells 3 to 4 hours after microinjection of expression vectors for constitutively active forms of CaM kinase II or CaM kinase IV or an activated form of Ras (RasR12). CREB phosphorylation on Ser^{133} (mean ± SEM of independent experiments) was 3% ± 6% (63 cells; vector control), 72% ± 2% (35 cells; CaM kinase IV), 86% ± 42% (36 cells; CaM kinase II), and 77% ± 15% (103 cells; RasR12) of the increase in CREB phosphorylation in response to KCl-FPL. Relative to cells microinjected with the vector control [Rous sarcoma virus (RSV)–β-globin] (*17*), constitutively active CaM kinase IV induced expression of GAL4-CREB 4.7 ± 1.4-fold (100 cells) (mean ± SEM of independent experiments). CaM kinase II and RasR12 failed to increase GAL4-CREB–mediated expression. In cells expressing both CaM kinase IV and RasR12, GAL4-CREB–mediated transcription was 110% ± 11% (71 cells) of that obtained in cells expressing CaM kinase IV alone. Bar = 20 μm. (**E**) Inhibition of Ca^{2+}-induced transcription mediated by the CRE and GAL4-CREB by E1A but not E1AΔCR1. Cells were microinjected with expression vectors for E1A (105 cells), E1AΔCR1 (104 cells), or RSV–β-globin (control; 258 cells) and either the CRE-dependent reporter gene pF222myc or the reporter gene pF222ΔCREmyc plus the GAL4-CREB expression vector. Between 3 and 4 hours after microinjection, cells were treated with KCl-FPL and were processed for immunocytochemistry after an additional 2 hours.

tivation measured by DNA transfection and ribonuclease protection analysis (Fig. 1C). Thus, blockade of CaM kinases also leads to uncoupling of CREB phosphorylation on Ser^{133} from transcriptional activation. Because CaM kinase IV is localized to the nucleus (*16*) it may be critical for transcription activated by nuclear Ca^{2+}. Indeed, specific inhibition of CaM kinase IV by expression of a negative interfering mutant inhibited Ca^{2+}-activated but not cAMP-activated transcription mediated by the CRE (*17*), indicating that CaM kinase IV controls CREB-dependent gene expression. Expression of the interfering mutant of CaM kinase IV did not affect Ca^{2+}-induced phosphorylation of CREB on Ser^{133} (*17*). Thus, cytoplasmic Ca^{2+} signals cause CREB phosphorylation by a mechanism that is independent of CaM kinases and fails to activate transcription.

To directly test the role of CaM kinases and the mitogen-activated protein (MAP) kinase pathway that is activated by Ca^{2+} signals (*18*), in controlling CREB function, we microinjected cells with expression vectors for constitutively active forms of CaM kinase II and CaM kinase IV (*12*) or the activated form of Ras (RasR12) (*19*) and analyzed CREB phosphorylation on Ser^{133} and CREB-mediated transcription. Expression of each of these proteins induced phosphorylation of CREB on Ser^{133}, but only CaM kinase IV stimulated CREB-mediated transcription (Fig. 1D). The failure of the Ras/MAP kinase signaling cascade to activate CREB-mediated transcription might reflect activation of the protein kinase $pp90^{rsk}$, which, upon activation of MAP kinases, can form a complex with CBP and block transcriptional induction (*20*). However, expression of RasR12 did not inhibit activation of CREB-mediated transcription by CaM kinase IV (Fig. 1D), indicating that the absence of a transcription activating signal was a more likely cause of the lack of transcriptional induction by RasR12. These results indicate that at least three distinct Ca^{2+}-activated signaling pathways result in a modification of CREB that allows CBP recruitment, but only nuclear Ca^{2+} and CaM kinase IV trigger an additional regulatory event required for transcriptional activation. We investigated whether disruption of CBP function interfered with Ca^{2+}-activated, CRE/CREB–mediated transcription. Expression of the adenovirus E1A protein, which blocks CBP function by binding to a COOH-terminal domain of CBP (*2, 21*), inhibited Ca^{2+}-activated gene expression mediated by either the CRE or GAL4-CREB (Fig. 1E). In contrast, CRE- or CREB-mediated transcriptional activation was unaffected by the expression of a mutant form of E1A (E1AΔCR1) (*8*) that contains a deletion in the NH_2-terminal conserved region 1 that abolishes interaction of E1A with CBP (Fig. 1E). These results indicate that CBP may participate in Ca^{2+}-activated or CRE- or CREB-mediated transcription.

We tested the ability of CBP artificially recruited to the promoter by means of fusion to the GAL4 DNA binding domain, to confer Ca^{2+} inducibility to the reporter gene construct pF222ΔCREmyc. The COOH-terminal amino acids 1892 to 2441 of CBP fused to GAL4 (construct GAL4-CBP_c) (*22*) were analyzed because they contain a putative glutamine-rich transcriptional activation domain (*1*). Expression of pF222ΔCREmyc was virtually undetectable in cells transfected with the vector control and increased in cells expressing GAL4-CBP_c (Fig. 2), indicating that artificially tethering CBP to the promoter increases the basal rate of transcription. Upon activation of Ca^{2+} influx through L-type

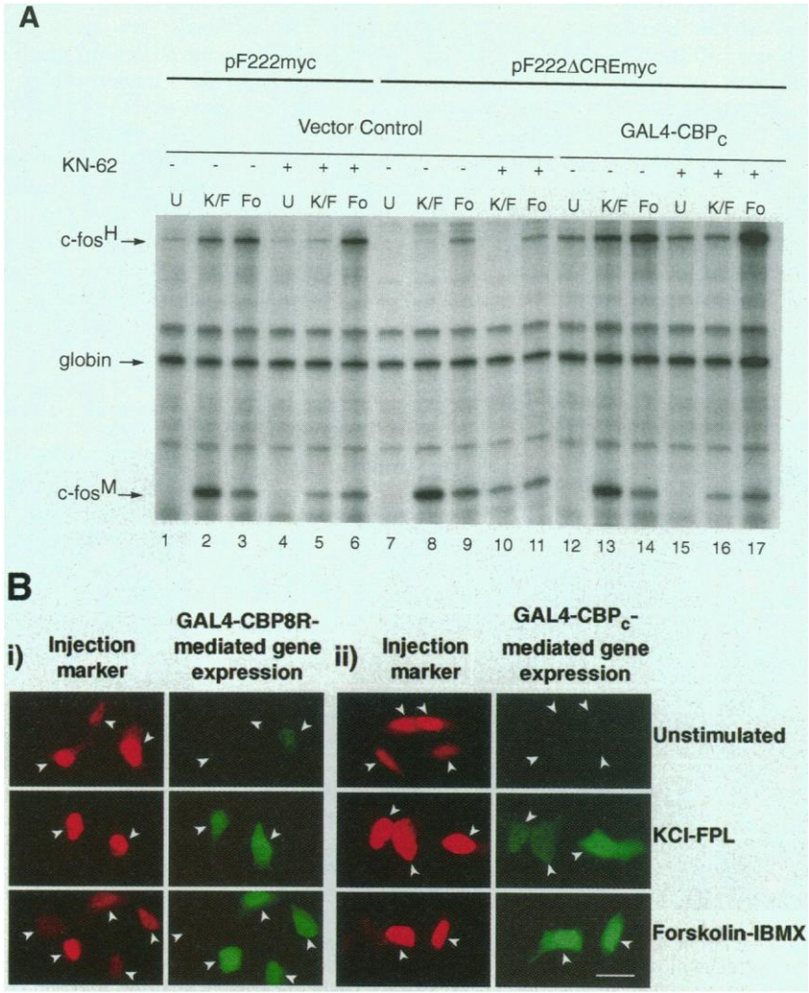

Fig. 2. Ca^{2+}-induced transcription mediated by CBP. (**A**) Ribonuclease protection analysis (*11, 15, 28*) was used to measure expression of the endogenous mouse c-*fos* gene (c-fos^M), expression of the transfected human α-globin gene (plasmid pSVα1) to normalize for transfection efficiency, and expression of the transfected human c-*fos* gene constructs pF222myc (lanes 1 to 6) and pF222ΔCREmyc (lanes 7 to 17) in unstimulated cells (U) or in cells 50 min after stimulation with KCl-FPL (K/F) or forskolin (Fo) to increase the intracellular concentration of cAMP. Cells were also transfected with a GAL4-CBP_c expression plasmid (*22*) (lanes 12 to 17) or the vector control (*23*) (pHKG; lanes 1 to 11). Expression of GAL4-CBP_c restored the KCl-FPL–induced and forskolin-induced transcriptional responses of plasmid pF222ΔCREmyc to 107% ± 11% (n = 3) and 125% ± 25% (n = 3) (mean ± SEM), respectively, of that obtained with plasmid pF222myc. Relative to the increases in mRNA levels in control cells stimulated with KCl-FPL, the CaM kinase inhibitor KN-62 [cells were treated with KN-62 (10 μM) 15 to 20 min before stimulation] caused a (mean ± SEM) 77% ± 7% (pF222myc; n = 5) and a 90% ± 23% (GAL4-CBP_c–mediated response; n = 2) inhibition of the response. KN-62 did not affect cAMP-induced expression. (**B**) Immunocytochemical analysis of Ca^{2+}- and cAMP-induced, GAL4-CBP8R– and GAL4-CBP_c–mediated gene expression (*22*). In cells treated with KCl-FPL, transcription was increased (mean ± SEM of independent experiments) 6 ± 2-fold (GAL4-CBP8R; 53 cells) and 5 ± 1-fold (GAL4-CBP_c; 98 cells). Forskolin-IBMX increased transcription 5 ± 1-fold (GAL4-CBP8R; 38 cells) and 15 ± 5-fold (GAL4-CBP_c; 53 cells).

Ca^{2+} channels with KCl-FPL, and in response to elevated intracellular levels of cAMP, transcription from pF222ΔCREmyc is increased in GAL4-CBP_c transfected cells (*23*). The quantities of Ca^{2+}-induced and cAMP-induced expression of pF222ΔCREmyc mediated by GAL4-CBP_c are similar to those obtained with the CRE-dependent reporter gene construct pF222myc (*11*) (Fig. 2A). This reporter gene is identical to pF222ΔCREmyc except that it lacks the GAL4 binding site and contains the c-*fos* CRE at nucleotide position −60, which can recruit endogenous CBP via CRE-bound CREB phosphorylated on Ser^{133}. Treatment of cells with the CaM kinase inhibitor KN-62 blocked GAL4-CBP_c–mediated and CRE-mediated Ca^{2+}-activated transcription but left cAMP-induced transcription unaffected (Fig. 2A). This suggests a role for CaM kinases in the control of CBP activity. We also tested the ability of GAL4-CBP8R, which contains full-length CBP fused to the GAL4 DNA binding domain (*22*), to mediate Ca^{2+}-activated transcription. In cells microinjected with the expression vector for GAL4-CBP8R and, for comparison, GAL4-CBP_c, expression of pF222ΔCREmyc was low before stimulation and strongly induced upon Ca^{2+} entry into the cells or increasing intracellular concentrations of cAMP (Fig. 2B). These results demonstrate that the activity of CBP is regulated by Ca^{2+} and cAMP signals.

To test whether CBP-mediated transcription is stimulated by cytoplasmic or nuclear Ca^{2+} signals, we microinjected BAPTA-D70 into the nucleus to specifically inhibit nuclear Ca^{2+} transients (*11*). Nuclear microinjection of BAPTA-D70 reduced GAL4-CBP8R– and GAL4-CBP_c–mediated Ca^{2+}-activated transcription, whereas there was little effect on transcription activated by cAMP (Fig. 3A). Thus, the activity of CBP is differentially controlled by spatially distinct Ca^{2+} signals and CBP is a nuclear Ca^{2+}-regulated transcriptional coactivator.

To directly test the role of CaM kinases and the MAP kinase signaling cascade in controlling CBP activity, we microinjected cells with expression vectors encoding constitutively active forms of CaM kinase II and CaM kinase IV (*12*) or the RasR12 expression vector (*19*) together with expression vectors for either GAL4-CBP8R or GAL4-CBP_c. Constitutively active CaM kinase IV stimulated transcription mediated by GAL4-CBP8R and GAL4-CBP_c (Fig. 3B). In contrast, constitutively active CaM kinase II increased the activity of GAL4-CBP8R only slightly and did not stimulate GAL4-CBP_c–mediated transcription. Transcription from a different c-*fos*–based reporter gene, pF711myc (*11*), that contains two Ca^{2+} responsive elements, the CRE and the serum response element (*11, 15*), was increased by constitutively active CaM kinase II and also by constitutively active CaM kinase IV. Expression of RasR12 failed to activate GAL4-CBP8R– or GAL4-

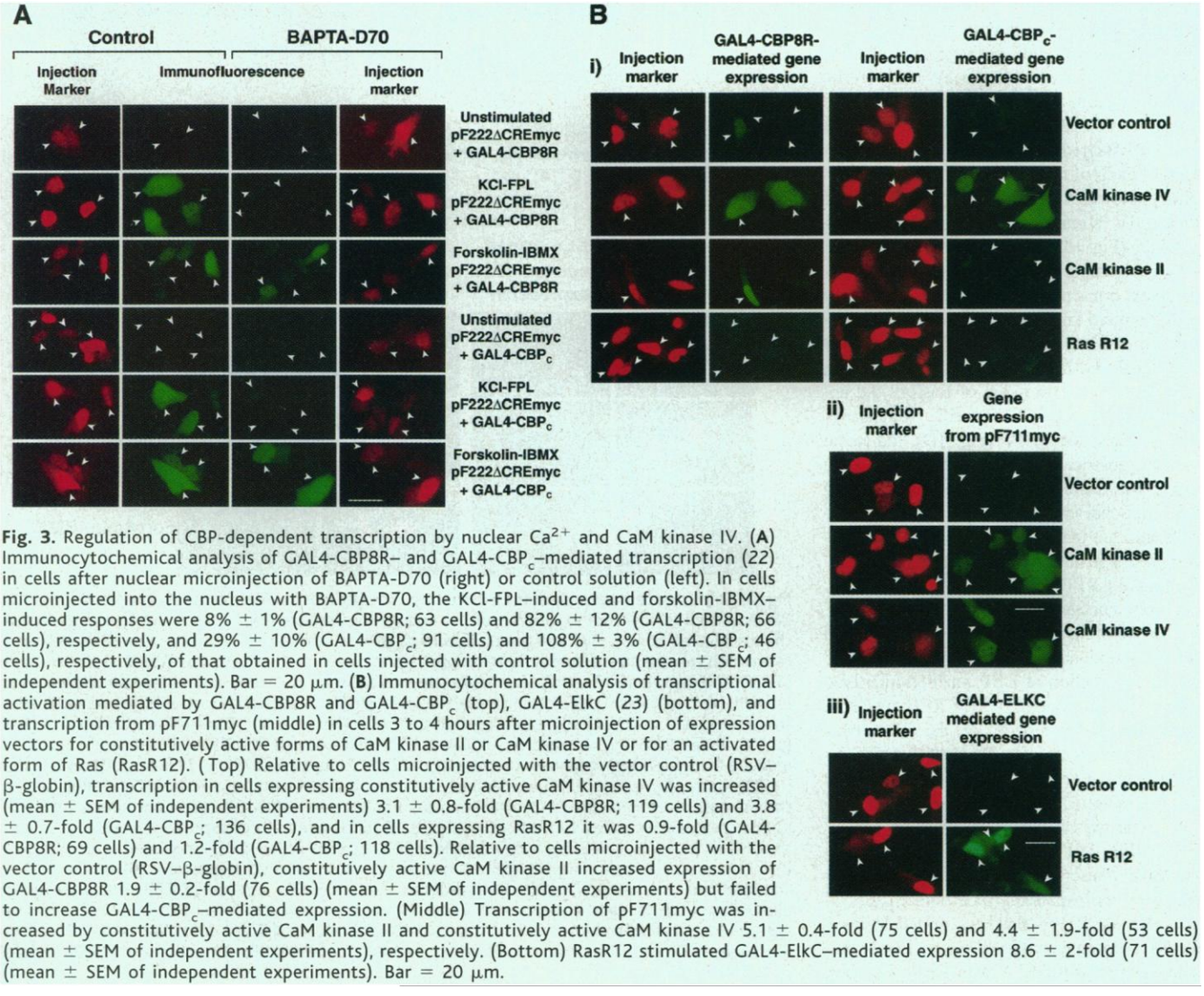

Fig. 3. Regulation of CBP-dependent transcription by nuclear Ca^{2+} and CaM kinase IV. (**A**) Immunocytochemical analysis of GAL4-CBP8R– and GAL4-CBP_c–mediated transcription (*22*) in cells after nuclear microinjection of BAPTA-D70 (right) or control solution (left). In cells microinjected into the nucleus with BAPTA-D70, the KCl-FPL–induced and forskolin-IBMX–induced responses were 8% ± 1% (GAL4-CBP8R; 63 cells) and 82% ± 12% (GAL4-CBP8R; 66 cells), respectively, and 29% ± 10% (GAL4-CBP_c; 91 cells) and 108% ± 3% (GAL4-CBP_c; 46 cells), respectively, of that obtained in cells injected with control solution (mean ± SEM of independent experiments). Bar = 20 μm. (**B**) Immunocytochemical analysis of transcriptional activation mediated by GAL4-CBP8R and GAL4-CBP_c (top), GAL4-ElkC (*23*) (bottom), and transcription from pF711myc (middle) in cells 3 to 4 hours after microinjection of expression vectors for constitutively active forms of CaM kinase II or CaM kinase IV or for an activated form of Ras (RasR12). (Top) Relative to cells microinjected with the vector control (RSV–β-globin), transcription in cells expressing constitutively active CaM kinase IV was increased (mean ± SEM of independent experiments) 3.1 ± 0.8-fold (GAL4-CBP8R; 119 cells) and 3.8 ± 0.7-fold (GAL4-CBP_c; 136 cells), and in cells expressing RasR12 it was 0.9-fold (GAL4-CBP8R; 69 cells) and 1.2-fold (GAL4-CBP_c; 118 cells). Relative to cells microinjected with the vector control (RSV–β-globin), constitutively active CaM kinase II increased expression of GAL4-CBP8R 1.9 ± 0.2-fold (76 cells) (mean ± SEM of independent experiments) but failed to increase GAL4-CBP_c–mediated expression. (Middle) Transcription of pF711myc was increased by constitutively active CaM kinase II and constitutively active CaM kinase IV 5.1 ± 0.4-fold (75 cells) and 4.4 ± 1.9-fold (53 cells) (mean ± SEM of independent experiments), respectively. (Bottom) RasR12 stimulated GAL4-ElkC–mediated expression 8.6 ± 2-fold (71 cells) (mean ± SEM of independent experiments). Bar = 20 μm.

CBP_c–mediated transcription but activated transcription mediated by the prototypical transcription factor target of the MAP kinase cascade, Elk-1 (*24, 25*). These results demonstrate that CBP is a nuclear Ca^{2+}/CaM kinase IV–regulated coactivator.

The identification of a nuclear Ca^{2+}/CaM kinase IV and cAMP-regulated transcriptional activation domain in CBP suggests a two-step model for CREB/CBP-mediated gene expression. In this model the CBP recruitment signal, which renders the CREB/CBP complex transcriptionally competent, has to coincide with a CBP activating signal to stimulate transcription. The mechanism by which nuclear Ca^{2+} and cAMP regulate CBP activity may involve phosphorylation of CBP. CBP is a phosphoprotein but we were unable to detect a change in the overall phosphorylation of CBP upon activation of Ca^{2+} signaling pathways, and mutations of putative CaM kinase phosphorylation sites in the COOH-terminal activation domain did not affect CBP-mediated transcriptional activation (*26*). Although CBP activity is differentially controlled by spatially distinct Ca^{2+} signals, CREB phosphorylation on Ser^{133} is a common end point for many signaling pathways. Thus, CREB functions as a sensor for cell activation and may, in the absence of CBP activating signals, serve a general supportive role in transcriptional responses that are primarily mediated by other activators such as Elk-1 or the serum response factor (*25, 27*). The importance of nuclear Ca^{2+} and cAMP in stimulating CBP activity may explain why growth factor receptor tyrosine kinases, which do not elicit Ca^{2+} transients or cause increases in the intracellular concentration of cAMP, are poor activators of CRE/CREB–dependent transcription (*25, 27, 28*), despite their ability to efficiently induce CREB phosphorylation on Ser^{133} via the MAP kinase pathway (*27, 29*). Given that many transcriptional activators can recruit CBP to a promoter, control of CBP function by nuclear Ca^{2+} and cAMP may prove to be of general importance for signal-regulated transcription.

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Identification of c-*MYC* as a Target of the APC Pathway

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The adenomatous polyposis coli gene (*APC*) is a tumor suppressor gene that is inactivated in most colorectal cancers. Mutations of *APC* cause aberrant accumulation of β-catenin, which then binds T cell factor–4 (Tcf-4), causing increased transcriptional activation of unknown genes. Here, the c-*MYC* oncogene is identified as a target gene in this signaling pathway. Expression of c-*MYC* was shown to be repressed by wild-type APC and activated by β-catenin, and these effects were mediated through Tcf-4 binding sites in the c-*MYC* promoter. These results provide a molecular framework for understanding the previously enigmatic overexpression of c-MYC in colorectal cancers.

Most human colorectal tumors are initiated by inactivation of the *APC* tumor suppressor gene, located on chromosome 5q21 (*1*). APC is a cytoplasmic protein that can bind to and promote the degradation of β-catenin (*2*). Among β-catenin functions is the ability to bind members of the Tcf family of transcription factors and activate gene transcription (*3*). Accordingly, human colorectal tumors with APC or β-catenin mutations