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Induction of a Cyclic AMP-Responsive Gene in Living Cells Requires the Nuclear Factor CREB

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We constructed cell lines containing various enhancer elements cloned upstream from a marker gene. By microinjection of specific antibodies directed against transcriptional activator proteins into these cell lines, we have developed a functional assay for factors which regulate the activity of target promoters. Here we show that microinjection of a highly specific antibody to the cyclic AMP enhancer element-binding (CREB) protein diminishes gene expression in response to cyclic AMP in living fibroblasts.

Cells respond to growth factors, neurotransmitters, and hormones through a complex series of events including second-messenger production, often resulting in the activation of new transcriptional programs. Although a large number of transcription factors have been cloned, the requirement of these molecules for second-messenger-mediated gene activation in vivo has been difficult to demonstrate directly. We devised a single-cell assay system that would allow an assessment of the role of individual proteins in signal transduction events which result in transcriptional stimulation. Rat fibroblast cell lines containing the *Escherichia coli lacZ* gene under the control of different enhancer elements (2, 3, 12, 23) were constructed (16). One cell line contains cyclic AMP (cAMP) enhancer elements (CREs) linked to *lacZ* which express this gene upon activation of the cAMP-dependent protein kinase (cAPK).

The precise mechanism by which cAMP leads to the induction of genes containing CREs is unclear, although a number of molecules involved in this pathway have been identified. A 43-kDa nuclear phosphoprotein which binds to CRE elements, the CRE-binding (CREB) protein, has been isolated (17, 26). It is now clear that the CREB protein is one member of a family of related gene products including CREB 341, CRE-BP1, HB16, ATF1-ATF-8, and others (9, 10, 14). Purified CREB protein binds to and stimulates transcription from CRE-containing genes in vitro, a process which is enhanced in the presence of protein kinase A (26). To test the role of the CREB protein in the regulation of gene expression in response to cAMP, a highly specific antibody raised against a CREB peptide (8) was microinjected into mammalian fibroblasts containing a cAMP-responsive marker gene. We demonstrate that the regulated expression of this marker gene is diminished by microinjection of the CREB antibody.

Rat-2 fibroblasts were transfected with a plasmid containing five copies of a CRE cloned upstream of the *E. coli lacZ* gene under the control of the human vasoactive intestinal peptide (VIP) gene promoter (5XCRE/Z) (Fig. 1). Plasmid pSV2neo (25) was included in the transfection, and the cells were selected with G418. Individual colonies were isolated and tested for their ability to respond to 8-bromo-cAMP (8BrcAMP). The cell line exhibiting the maximal response was chosen for further analysis. Treatment of this cell line with cAMP analogs or cAMP phosphodiesterase inhibitors leads to expression of the *lacZ* gene product, β-galactosidase, which is detected by staining with the chromogenic substrate 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-Gal). Cells expressing β-galactosidase contain a dark-blue reaction product. With this immunohistochemical stain, β-galactosidase activity is first detectable at 4 h and maximal at 6 h. Typically, the maximal number of blue cells is obtained with 1 mM 8BrcAMP and 1 mM isobutylmethylxanthine (IBMX). Although logarithmically growing cells respond to this mixture, the maximal response (20 to 30% blue cells) is obtained following serum starvation for 24 h (Fig. 2). Increased concentrations, longer treatment, or combinations of drugs failed to further increase the proportion of responding cells. A similar response is seen with dibutyryl cAMP and with forskolin and following microinjection of the catalytic subunit of cAPK (15, 20). A similar

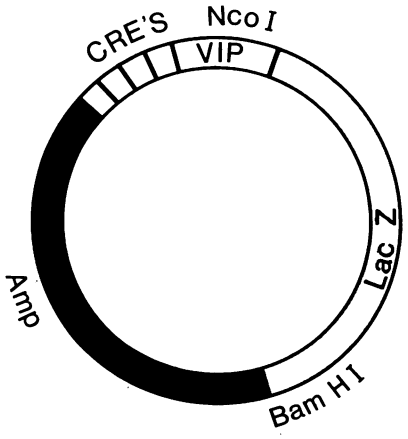

FIG. 1. Structure of the 5XCRE/Z plasmid. The 5XCRE/Z plasmid contains four copies in the sense orientation of a 25-bp synthetic CRE oligonucleotide derived from the human VIP gene (5) cloned upstream from the VIP gene promoter and the *E. coli lacZ* gene. Since the VIP promoter contains one copy of a CRE, the total number of CREs in this plasmid is five. These fragments were cloned into pBluescript KS$^+$.

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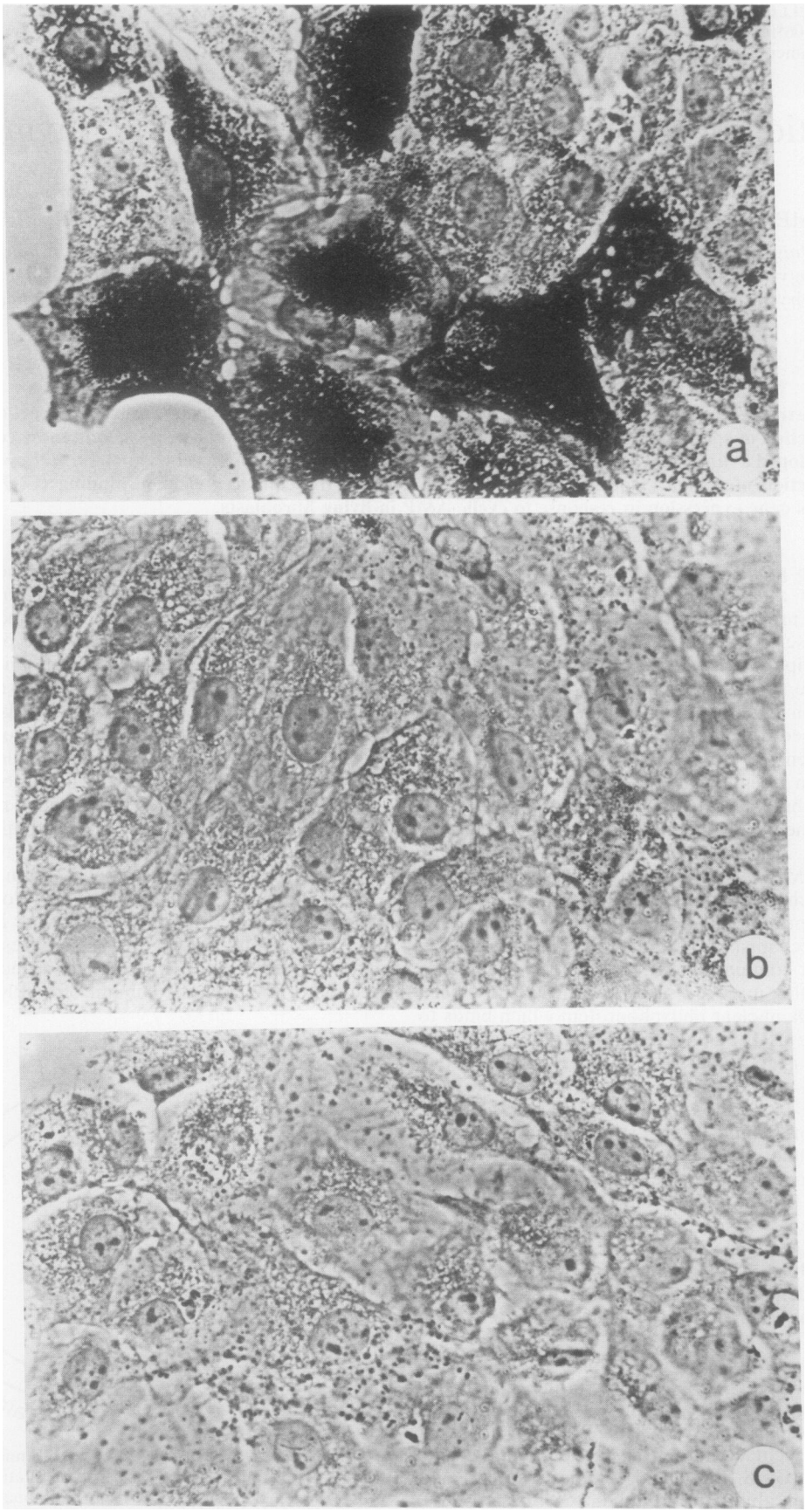

FIG. 2. Response of the 5XCRE cell line to drug treatment. 5XCRE cells were plated in growth medium for 24 h. Following starvation in 0.1% fetal calf serum for 24 h, the cells were treated with 1 mM 8BrcAMP and 1 mM IBMX in 0.1% fetal calf serum (a), 10% fetal calf serum (b), or 200 ng of TPA per ml in 0.1% fetal calf serum (c). Cells containing β-galactosidase contain a dark-blue reaction product.

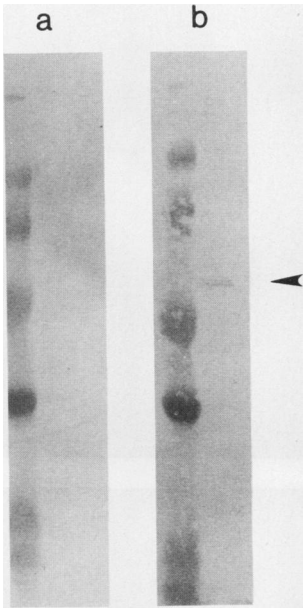

FIG. 3. The W39 anti-CREB antiserum recognizes, at most, two proteins in 5XCRE cells. Whole-cell extracts were prepared from 5XCRE cells, separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and incubated with nonimmune rabbit IgG (a) or the W39 anti-CREB antiserum (b). The W39 antiserum recognized a protein doublet of the size expected for the CREB protein (arrows in panel b).

proportion of responding cells was found in three independent cell lines containing similar constructs: C6 glioma, mouse L cells, and B/c 3T3 fibroblasts (15, 20). Moreover, the proportion of responding cells in each of these cell lines declines upon continuous propagation even in the presence of G418. The 5XCRE cell line does not express β-galactosidase in response to serum, phorbol esters (e.g., 12-*O*-tetradecanoylphorbol-13-acetate [TPA]) (Fig. 2), or platelet-derived growth factor (data not shown). Thus, *lacZ* gene expression in this cell line appears specific for agents which act through the second messenger, cAMP.

A cell line expressing the *lacZ* gene under the control of TPA-responsive enhancer elements (AP-1 elements) and a fragment of the Rous sarcoma virus promoter was also constructed (16). This cell line, 4XTRE, expresses β-galactosidase in response to treatment with serum or TPA (see Fig. 4e) but not in response to cAMP or microinjection of the cAPK catalytic subunit (data not shown). The specificity of this cell line for TPA and that of the 5XCRE cell line for agents acting through cAMP suggest that the 5XCRE cell line is a useful reagent for analyzing proteins which function in signaling pathways mediated by cAMP. To test this hypothesis, we analyzed the role of the CREB protein in transcriptional activation in living cells.

A highly specific antibody was previously generated against a tryptic peptide of the CREB protein (8). The W39 antibody was affinity purified against peptide, exhibits nuclear fluorescence in rat brain, and immunoprecipitates a single 43-kDa phosphoprotein from PC12 cells (8). Treatment of nuclear extracts with the W39 antibody removes CRE-binding activity when assayed by DNase I protection of the somatostatin gene promoter (8). These characteristics demonstrate that anti-W39 is a highly specific reagent capable of recognizing the CREB protein both in vitro and in vivo. To determine its specificity in the 5XCRE cell line, we prepared whole-cell extracts and subjected them to Western immunoblot analysis. The W39 antibody recognized two proteins of the expected size for the CREB protein in 5XCRE cells (Fig. 3). Since the two proteins are of very similar size, it is most likely that they represent alternative forms of the CREB protein which differ in the presence or absence of the α peptide (27) or in the extent of phosphorylation. It is unlikely that the W39 antiserum recognizes other related members of the CREB protein family, since of the CREB variant proteins described, only ATF1 is homologous to CREB in the region recognized by the W39 antiserum (16a). Furthermore, since a single-amino-acid change abolishes the ability of the W39 antiserum to recognize the CREB protein and since ATF1 and CREB are not completely homologous in this region, it is unlikely that either of the proteins detected in this experiment represents ATF1.

The W39 antiserum was microinjected into 5XCRE cells to determine whether it could diminish the ability of this cell line to respond to cAMP, presumably by blocking CREB protein function. Previous experiments in which it was found that microinjected anti-*fos* antibodies blocked serum-stimulated DNA synthesis in fibroblasts suggested that this approach might be capable of being used in blocking other nuclear transactivator proteins (21). As a control, protein A-purified nonimmune rabbit immunoglobulin G (IgG) was injected. Antibodies were injected at 0.5 to 2.0 mg/ml in 10 mM $NaPO_4$–100 mM KCl. This concentration of IgG results in the injection of about 10^6 molecules of IgG per cell. Since there are fewer than 10^4 CREB molecules per cell (16a), this represents at least a 100-fold excess of antibody over CREB protein. Following injection, the cells were incubated for 14 to 21 h before stimulation with 8BrcAMP and IBMX. These time intervals were chosen to maximize the binding of injected antibody to newly synthesized cytoplasmic CREB protein and were based on the observations that the half-life

TABLE 1. Microinjection of W39 anti-CREB antibody decreases the ability of the 5XCRE cell line to respond to 8BrcAMP and IBMX

Expt	Cell line	β-Galactosidase expression with:						
		W39 anti-CREB			Nonimmune rabbit IgG			
		No. of blue cells	No. of white cells	% of blue cells	No. of blue cells	No. of white cells	% of blue cells	P^a
1	5XCRE	12	117	9.3	39	137	22.2	0.005
2	5XCRE	0	27	0.0	20	46	30.3	0.003
3	5XCRE	32	398	7.4	29	141	17.1	0.001
4	4XTRE	35	24	59.3	33	46	41.8	0.062

[a] *P* values calculated by a 2 × 2 contingency table chi-square analysis with Yates correction.

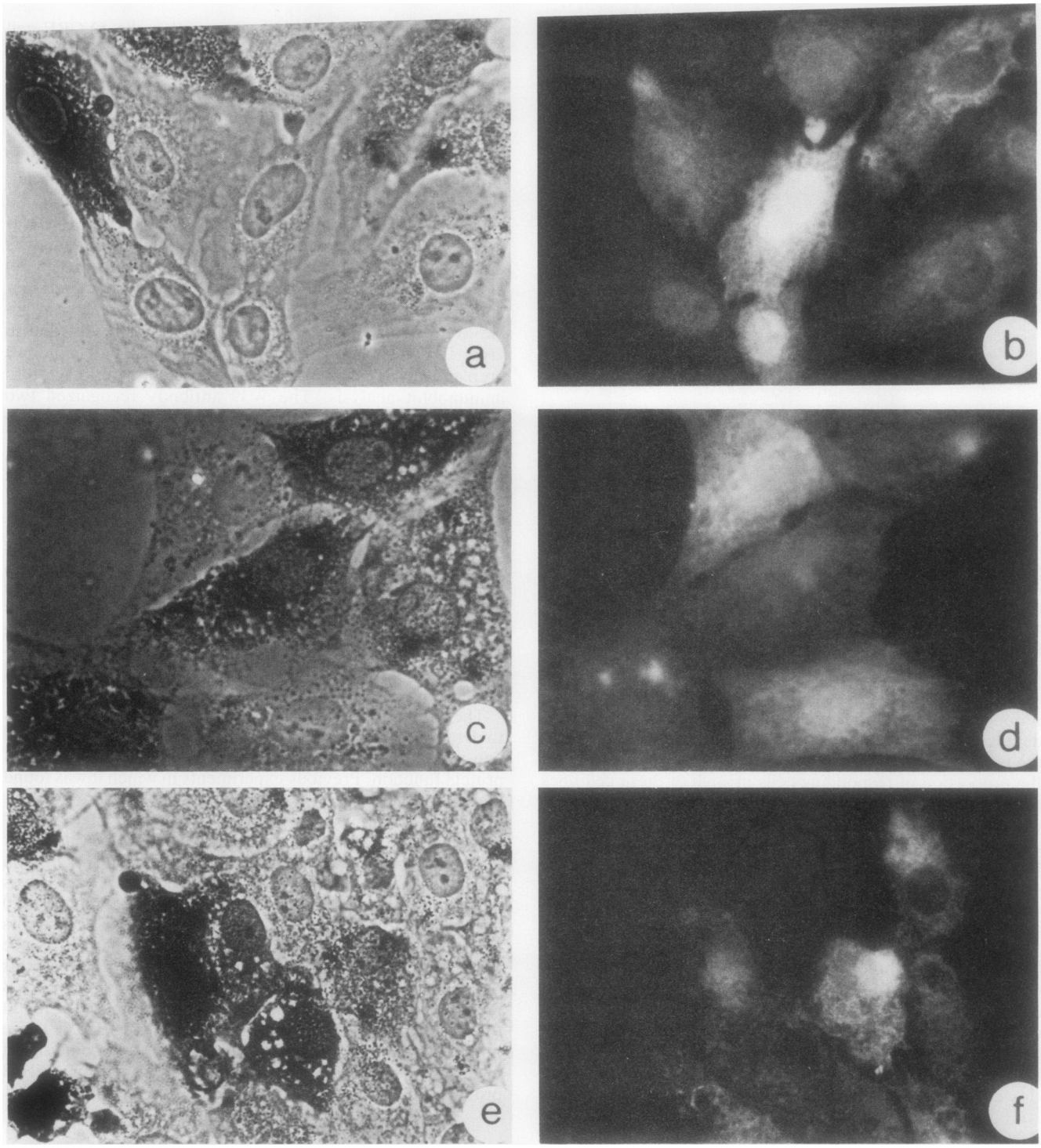

FIG. 4. Microinjection of anti-CREB diminishes *lacZ* gene expression in the 5XCRE cell line, but not in the 4XTRE cell line. For injection, cells were transferred to 0.1% fetal calf serum-supplemented medium, injected with W39 or control antibodies at 1 mg/ml, and incubated for 14 to 20 h at 37°C. The cells were then induced with either 8BrcAMP and IBMX (5XCRE) (a and c) or TPA (4XTRE) (e) in 0.1% fetal calf serum-supplemented medium for 6 h, fixed, and stained with X-Gal. Following color development for β-galactosidase (a, c, and e), the cells were stained sequentially with anti-rabbit biotinylated IgG and streptavidin Texas red (b, d, and f) to detect microinjected cells. Fluorescent cells were scored for the presence or absence of a dark-blue reaction product. Panels a and b show that 33% of the cells in this field injected with the W39 anti-CREB antiserum expressed β-galactosidase, whereas panels c and d show that 60% of cells injected with nonimmune rabbit IgG expressed β-galactosidase, after 8BrcAMP-IBMX treatment. Although these values are higher than found routinely following 8BrcAMP-IBMX treatment, this is due to the small number of cells and the heterogeneity of β-galactosidase expression in this cell line. The fields displayed were chosen to contain as many blue cells as possible in order to demonstrate that the injected population was capable of expressing β-galactosidase. It should be noted that the seemingly reduced fluorescence seen in panel d is due to the intense blue staining of these cells and not to a smaller amount of antibody injected.

of the CREB protein is about 2.5 h and that IgG molecules are detectable for as long as 36 h following injection (5). In three separate experiments, microinjection of W39 anti-CREB antibody, but not of nonimmune rabbit IgG, significantly reduced β-galactosidase expression induced by 8BrcAMP and IBMX (Table 1). The reduction in β-galactosidase expression was greater than twofold in all experiments. 5XCRE cells injected with nonimmune rabbit IgG expressed the *lacZ* gene in response to 8BcAMP and IBMX at the same level as did uninjected cells that were similarly treated. Representative examples of microinjected cells are displayed in Fig. 4. It is interesting that approximately 11% of the anti-CREB antibody-injected cells exhibited fluorescent nuclei, whereas labeled nuclei were found only in 1.5% of the nonimmune rabbit IgG-injected cells. These results may suggest nuclear transport of an antibody directed against a nuclear antigen, as described previously (1). In contrast to the results obtained with the 5XCRE cell line, microinjection of anti-CREB antibody did not diminish TPA-induced β-galactosidase expression in the 4XTRE cell line. Therefore, the diminished β-galactosidase expression observed in 5XCRE cells is not due to a toxic effect of the anti-CREB antibody. In 4XTRE cells, slightly higher levels of β-galactosidase expression were found following injection of W39 anti-CREB antibody than were found following injection with nonimmune rabbit IgG (Table 1). It is unclear whether this higher level of *lacZ* gene expression is biologically significant. However, given the ability of the CREB protein to repress the c-*jun* promoter via an AP-1 element (13), it is possible that the higher level of β-galactosidase expression in anti-CREB antibody-injected 4XTRE cells represents a relief of repression imposed by the CREB protein.

These experiments demonstrate that the CREB protein is required in rat fibroblasts in vivo for maximum expression of a gene containing cAMP-inducible promoter elements. Because the antibodies to CREB did not completely block cAMP-induced β-galactosidase expression, it is possible that other factors, such as other members of the CREB family or the AP-2 protein (11), also contribute to cAMP regulation of gene expression in these cells. Alternatively, it is possible that the concentration or affinity of antibody in the injected cell is insufficient to completely block CREB protein function. Our results complement previous studies that demonstrated that the CREB protein binds specifically to CRE elements (4, 18) and stimulates transcription of CRE-containing genes in vitro (26). An earlier study (20) showed that microinjection of the cAPK catalytic subunit activated gene expression in vivo from both transfected and endogenous genes containing CREs. We now extend these observations by demonstrating that both the CREB protein and the cAPK catalytic subunit are involved in the activation of cAMP-inducible genes in living cells. These results are consistent with the activation of the CREB protein by protein kinase A in vivo. In fact, recent evidence demonstrates that both the CREB protein and cAPK are required for the transcriptional activation of a cAMP-responsive reporter gene (7). It remains to be determined whether protein kinase C or other protein kinases regulate the activity of the CREB protein in vivo, since a role for both protein kinase C (26) and casein kinase II (8) has been suggested from in vitro studies. A recent study indicates that a Ca^{2+}/calmodulin kinase may also regulate CREB activity by phosphorylation (22). The cell lines described here provide the framework for an analysis of proteins involved in signal transduction pathways from the cell surface to the nucleus. By using needle microinjection to introduce purified proteins or antisera into the appropriate indicator cell line, it may be possible to dissect the protein components of these signaling networks. The experiments described above suggest that these cell lines should provide valuable insight into signaling pathways in mammalian cells.

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