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Regulation of c-*fos* Expression in Transgenic Mice Requires Multiple Interdependent Transcription Control Elements

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Summary

Transcription control regions of eukaryotic genes contain multiple sequence elements proposed to function independently to regulate transcription. We developed transgenic mice carrying *fos-lacZ* fusion genes with clustered point mutations in each of several distinct regulatory sequences: the *sis*-inducible element, the serum response element, the *fos* AP-1 site, and the calcium/cAMP response element. Analysis of Fos-lacZ expression in the CNS and in cultured cells demonstrated that all of the regulatory elements tested were required in concert for tissue- and stimulus-specific regulation of the c-*fos* promoter. This implies that the regulation of c-*fos* expression requires the concerted action of multiple control elements that direct the assembly of an interdependent transcription complex.

Introduction

The selective modulation of gene expression in response to temporal, spatial, and environmental cues underlies a broad range of biological processes in the central nervous system. To a large extent, the regulation of gene expression is mediated by alterations in transcription rates that occur in response to the interaction of trans-acting transcription control proteins (transcription factors) with cis-acting DNA sequence elements. In the past few years, a great number of transcription factors have been cloned and characterized in terms of their DNA-binding specificities and transcriptional activities. A paradigm has emerged in which individual DNA regulatory elements are thought to be both necessary and sufficient for transcriptional responses to particular extracellular stimuli.

The *fos* proto-oncogene (c-*fos*) has provided a useful experimental tool for the study of gene expression. In the majority of cell types, it is expressed at relatively low levels, but it can be rapidly and transiently induced by a broad range of stimuli. Thus, expression of c-*fos* has been observed in association with mitogenesis, differentiation, neuronal excitation, and even cell death (Morgan and Curran, 1991b; Smeyne et al., 1993). The protein encoded by c-*fos* (Fos) is a leucine zipper–containing transcription factor that is a prototypic cellular immediate-early protein (Curran, 1988). Fos is thought to couple short-term signals elicited by cell surface stimuli to long-term changes in cellular phenotype by regulating the expression of specific target genes. Furthermore, it can be expressed continuously in certain cell populations, as well as transiently in response to physiological and pathological signals in many tissues in vivo (Morgan and Curran, 1991a). Thus, c-*fos* can be used to investigate the molecular basis of tissue- and stimulus-specific transcription control under a variety of circumstances.

One of the features of c-*fos* that has made it attractive for the analysis of gene regulation is the rapid and dramatic induction that occurs in cultured cells in response to serum growth factors and a host of other agents (Curran, 1988). This makes it a tractable subject for the study of the molecular events involved in transcription regulation. Indeed, several specific regulatory elements have been identified in the c-*fos* promoter through studies in vitro. The serum response element (SRE) has been described as a protein-binding site required for the induction of c-*fos* expression by serum (Treisman, 1992). The SRE, together with flanking DNA sequences, serves as the site of assembly of multiprotein complexes that include SRF, p62TCF/Elk-1, and several other transcription factors (Shaw et al., 1989; Hipskind et al., 1991; Hill et al., 1993). Although the exact mechanism responsible for activation of transcription via the SRE is unclear, phosphorylation of the p62TCF/Elk-1 protein by microtubule-associated protein (MAP) kinase is believed to be a key event (Marais et al., 1993). The calcium and cyclic AMP (cAMP) response element (Ca/CRE) was defined as a DNA sequence functionally distinct from the SRE that mediates rapid gene induction by elevated intracellular calcium and cAMP (Sassone-Corsi et al., 1988a; Sheng et al., 1988; Berkowitz et al., 1989; Fisch et al., 1989). The induction of c-*fos* by calcium has been proposed to occur through phosphorylation of the cAMP response element binding protein (CREB), which binds to this site in vitro (Sheng et al., 1990). The *sis*-inducible element (SIE) was identified as the binding site for an inducible factor and was proposed to contribute to c-*fos* induction in cells exposed to platelet-derived growth factor (PDGF; Wagner et al., 1990). Recently, one of the proteins that binds to the SIE has been shown to be related to the 91 kDa component of the interferon-stimulating gene factor 3 complex, which is involved in gene induction in response to γ-interferon (Meyer et al., 1993). The c-*fos* AP-1 binding element (FAP) was initially noted as a sequence similar to AP-1 binding sites found in other genes. Many functions have been attributed to this site (Sassone-Corsi et al., 1988b; Fisch et al., 1989); however, its role in the c-*fos* promoter remains controversial.

The analysis of c-*fos* regulation in cultured cells has contributed to a model in which individual response elements are thought to act independently to regulate gene expression in response to extracellular stimuli. For example, the SRE has been proposed to be both necessary

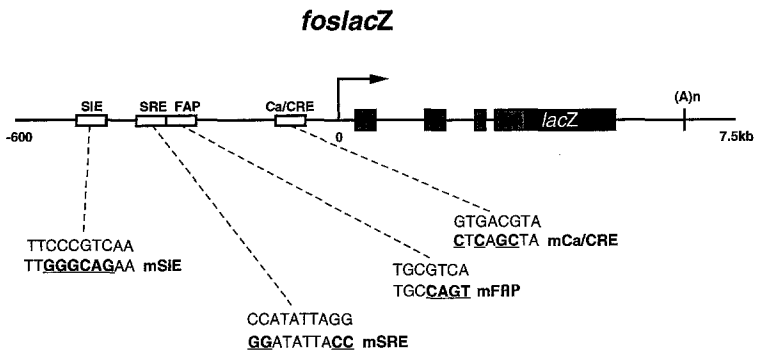


Figure 1. Mutant *fos–lacZ* Constructs

The intact *fos–lacZ* construct included 611 bp of 5′ nontranscribed sequences with all of the known c-*fos* regulatory elements, the genomic exon/intron organization, and the 3′ untranslated region (Smeyne et al., 1992b). Mutant constructs contained clustered point mutations at the SIE (mSIE), the SRE (mSRE), the FAP (mFAP), or the Ca/CRE (mCa/CRE).

and sufficient for c-*fos* induction by growth factors, but not for responses to cAMP-dependent signaling, whereas the Ca/CRE has been proposed to be sufficient for c-*fos* induction by cAMP or elevated intracellular calcium, but not for growth factor–induced expression (Gilman, 1988; Sheng et al., 1988; Berkowitz et al., 1989; Sheng et al., 1990). In some cases, several regulatory elements have been suggested to contribute at least additively to the transcriptional response to a particular signal (Berkowitz et al., 1989; Fisch et al., 1989; Wagner et al., 1990; Ginty et al., 1994). However, in the intact organism, the independent actions of individual regulatory elements are not likely to account for the wide range of stimuli that control c-*fos* expression in many different cell types. To investigate the molecular basis of c-*fos* regulation in a physiological context, we developed transgenic mouse lines carrying c-*fos–lacZ* fusion genes.

The *fos–lacZ* construct contains all of the known c-*fos* regulatory elements, and it recapitulates both tissue- and stimulus-specific regulation of c-*fos* expression in vivo (Schilling et al., 1991; Smeyne et al., 1992a, 1992b, 1993). Here, we have introduced clustered point mutations into individual regulatory elements within this construct to determine the respective contributions of the SIE, SRE, FAP, and Ca/CRE sequences to the control of c-*fos* expression in the context of the intact organism. The results suggest that the physiological regulation of c-*fos* expression requires the interdependent function of all the elements tested.

Results

Generation of Transgenic Mice

Clustered point mutations were introduced into the *fos–lacZ* construct at the SIE (Hayes et al., 1987), the SRE (Treisman, 1986; Treisman, 1992), the FAP (Fisch et al., 1989), and the Ca/CRE (Fisch et al., 1987; Sheng et al., 1988; Berkowitz et al., 1989) (Figure 1). These point mutations were chosen to alter the specific contact residues that mediate protein-DNA interactions at these sites. Several independent founder lines were derived for each construct. The transgene copy number varied among the lines (Table 1). However, copy number did not strictly correlate with the levels of Fos–lacZ expression observed. There was some variability in the absolute levels of *fos–lacZ* expression observed among lines containing the same construct; however, the pattern of expression was consistent.

Table 1. Independent Lines Generated for Each Construct

Transgene Construct	Line	Copy Number[a]
fos–lacZ	1	16[b]
	2	16
	3	16
	4	8
	5	8
mSIE	1	8
	2	4
	3	4
	4	2
mSRE	1	8
	2	2
mFAP	1	8
	2	2
mCa/CRE	1	2
	2	2
	3	8

[a] The number of copies of each transgene was determined by dot blot hybridization using a c-*fos* probe and normalizing the signal to nontransgenic controls.
[b] Determined by Southern blot analysis.

Analysis of Constitutive Expression

Bone tissue is a major constitutive site of c-*fos* expression (Smeyne et al., 1992b; Grigoriadis et al., 1993; Smeyne et al., 1993). Fos appears to have a critical function in bone, because mice lacking a functional c-*fos* gene develop an osteopetrotic phenotype (Johnson et al., 1992; Wang et al., 1992). Furthermore, the v-*fos* oncogene is associated exclusively with the induction of osteogenic sarcoma in mice (Curran and Teich, 1982). Together, these data suggest that Fos has an important function during bone development. Therefore, we were particularly interested in determining whether a specific regulatory element was responsible for constitutive expression of c-*fos* in bone tissue.

Longitudinal sections prepared from the tails of *fos–lacZ*, mSIE, mSRE, mFAP, and mCa/CRE transgenic mouse lines were examined for β-galactosidase activity. These sections allow simultaneous analysis of Fos–lacZ

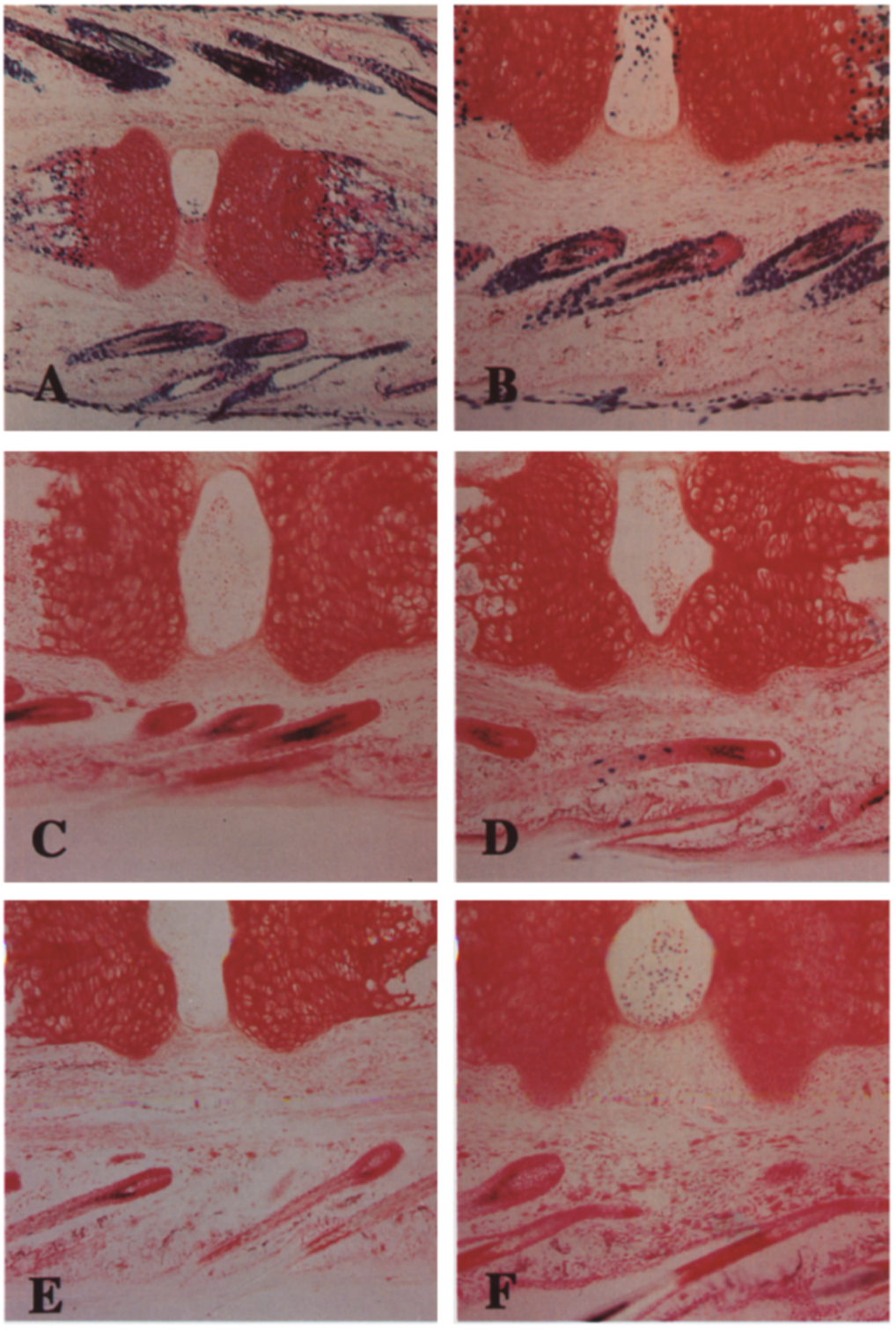


Figure 2. Constitutive Sites of Fos-LacZ Expression In Vivo

In unstimulated mice carrying the intact transgene, β-galactosidase was detected in skin, bone, and hair follicle cells (A and B). Mutations in the SIE (C), SRE (D), FAP (E), and Ca/CRE (F) virtually eliminated expression at these sites in vivo. Results shown here are representative of at least two animals from all of the mutant lines. Magnification, 100×.

expression in bone, skin, and hair cells. In two of five *fos–lacZ* lines, β-galactosidase expression was detected throughout the vertebra in osteoblasts surrounding trabecular bone and in the proximal hypertrophic zone of the growth plate (Figure 2A and 2B; Smeyne et al., 1992b). In skin, high levels of expression were observed in the stratum corneum of parakeratotic skin. Expression was also detected in the hair follicle in cells of the root sheath, in a region above the dermal papillae. In marked contrast, little or no Fos–lacZ expression was detected in these locations in any of the mutant lines. Thus, mutation of the SIE (Figure 2C), the SRE (Figure 2D), the FAP (Figure 2E), or the Ca/CRE (Figure 2F) each eliminated virtually all of the Fos–lacZ expression at these sites. In one of the two mSRE lines, only a few scattered positive cells were observed in the hair follicle and the trabecular bone (Figure 2D). The virtual absence of the major constitutive sites of Fos–lacZ expression in skin, bone, and hair associated with mutations in the SIE, SRE, FAP, and Ca/CRE elements was consistent in all of the 11 mutant lines examined, whereas high levels of expression were observed in two of five wild-type lines (Table 1). This suggests that the c-*fos* regulatory elements analyzed here are required for constitutive expression in skin, bone, and hair follicle cells. However, the variability in the expression patterns seen in the wild-type transgenic lines indicates that flanking sequences not included in the wild-type *fos–lacZ* construct might be required for appropriate constitutive expression in these cells.

Analysis of Fos-lacZ Induction in the Nervous System

A wealth of literature is available concerning induction of c-*fos* by a variety of pharmacological and physiological stimuli in neurons in vivo (Morgan and Curran, 1991a, 1991b). In particular, the induction of seizures in experimental animals, which produces a rapid and synchronous stimulation of neuronal activity in specific brain regions, results in a concomitant widespread increase in c-*fos* expression. Therefore, induction of seizures using excitotoxic agents such as kainic acid (kainate) provides a useful model for the analysis of c-*fos* regulation in neurons in vivo.

We examined Fos–lacZ expression in the brains of control (Figures 3A, 3C, 3E, 3G, and 3I) and kainate-treated mice (Figures 3B, 3D, 3F, 3H, and 3J) from all of the transgenic lines generated. In mice carrying the intact *fos–lacZ* gene, β-galactosidase activity was detected prjmarily in the hippocampus (CA1, CA3, CA4, and dentate gyrus) and in several layers of the cerebral cortex 2 hr after kainate administration, as described previously (Smeyne et al., 1992b). Among three independently derived transgenic lines (lines 2, 3, and 5), the patterns and levels of Fos–lacZ induction were virtually identical. In the other two lines (lines 1 and 4), the same pattern of Fos–lacZ induction was observed, although the levels of expression were lower. In one of the lines (line 3), ectopic constitutive expression was also observed in several regions of the brain, including the molecular layer of the cerebellum and several lateral thalamic nuclei (data not shown).

The characteristic pattern of *fos–lacZ* gene induction was completely changed in all of the mutant lines analyzed. Mutation of the SIE (mSIE) severely diminished induced expression of Fos–lacZ in the cortex and the CA1 region of the hippocampus, although higher levels of expression remained in the CA3 and in the dentate gyrus (Figures 3C and 3D). Mutation of the serum response element (mSRE) eliminated kainate-induced expression of Fos–lacZ (Figures 3E and 3F) in all brain regions examined, with the exception of a few scattered β-galactosidase–positive cells in the cortex of one of the mSRE lines (line 2). In mFAP mice, kainate induced Fos–lacZ expression in a subset of the neurons that were positive in *fos–lacZ* mice. A low level of induction was observed in the dentate gyrus and the CA3 (Figures 3G and 3H). The expression in cortex and CA1 varied somewhat between the lines, one of which (line 1) exhibited a high level of basal expression. Mutation of the calcium/cAMP element (mCa/CRE) completely abolished basal and induced expression of Fos–lacZ in the brain (Figures 3I and 3J). The only exception was one of three mCa/CRE lines (line 3) in which ectopic constitutive expression was also observed in mitral cells of the olfactory bulb (data not shown). Thus, the SRE and the CRE were required for c-*fos* induction in response to seizures in all neurons examined, whereas the SIE and the FAP were required in a subset of the neurons.

Regulation of Fos-lacZ Expression in Primary Brain Cultures

Activation of c-*fos* expression by neuronal signaling engendered by kainate-induced seizures may involve several simultaneous stimuli. Therefore, it was not possible to determine whether the requirement for several regulatory elements to induce c-*fos* by kainate reflected the presence of multiple stimuli mediated by different elements or the involvement of multiple elements in the response to a single stimulus. To analyze the regulation of c-*fos* in response to more defined stimuli, we examined the expression of Fos–lacZ in telencephalic brain cultures prepared from two independent lines carrying the wild-type *fos–lacZ* construct (Figure 4). Unstimulated cells expressed no detectable β-galactosidase activity (Figures 4A and 4B). Treatment of the cells with increased concentrations of extracellular KCl (Figures 4C and 4D) or with kainate (data not shown) induced Fos–lacZ expression in cells that were identified as neurons by their reactivity with α-MAP2, but not with anti-glial fibrillary acidic protein (α-GFAP) antibodies. Treatment of the cultures with PDGF or 12-O-tetradecanolyphorbol 13-acetate (TPA) primarily induced Fos–lacZ expression in cells that were identified as glia by their reactivity with α-GFAP, but not with α-MAP2 antibodies (Figures 4E–4H). In addition, PDGF and TPA induced expression in cells of fibroblastic appearance that were not reactive with either α-MAP2 or α-GFAP antibodies.

Telencephalic cultures were also prepared from two independent lines containing each mutant construct. As observed in the brains of intact animals, mutation of any one of the c-*fos* regulatory elements reduced the number of neurons induced to express Fos–lacZ by elevated KCl or kainate (Table 2). Mutation of the SRE or the Ca/CRE

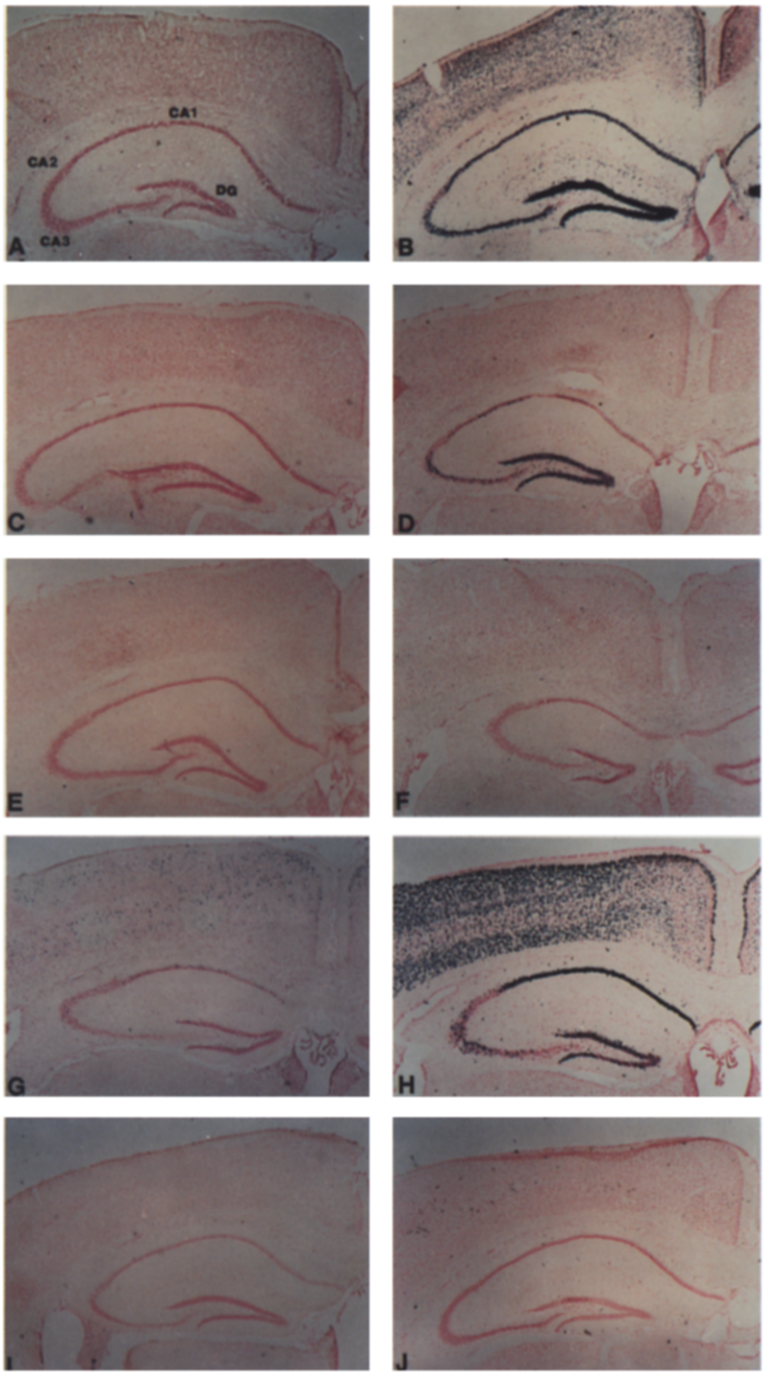


Figure 3. Regulation of Fos–lacZ Expression *In Vivo*

Fos–lacZ expression was examined in the brains of control (A, C, E, G, and I) and kainate-treated mice (B, D, F, H, and J) from all the lines generated: *fos–lacZ* (A and B); mSIE (C and D); mSRE (E and F); mFAP (G and H); mCa/CRE (I and J). Results shown here are representative of all the animals examined, with exceptions discussed in the text. None of the mutant lines exhibited higher levels of induction than shown here. Magnification, 40×.

inhibited expression in more neurons than mutations in the SIE or the FAP. Mutation of any one of the c-*fos* regulatory elements also reduced Fos–lacZ expression in glia (Table 2). In this case, mutation of the SRE and the FAP had the greatest effects. Thus, different cell types vary both in their responsiveness to distinct signals and in the spectrum of regulatory elements required to mediate such signals. However, in the majority of circumstances, both in neurons and in glia, all of the c-*fos* regulatory elements were required in concert for complete responses to single, defined stimuli.

Regulation of Fos–lacZ Expression in Cultured Fibroblasts

The preceding results challenge some of the conclusions derived from studies of c-*fos* regulation in transfected fibroblasts. Therefore, to assess the respective roles of the SIE, SRE, FAP, and Ca/CRE elements in the regulation

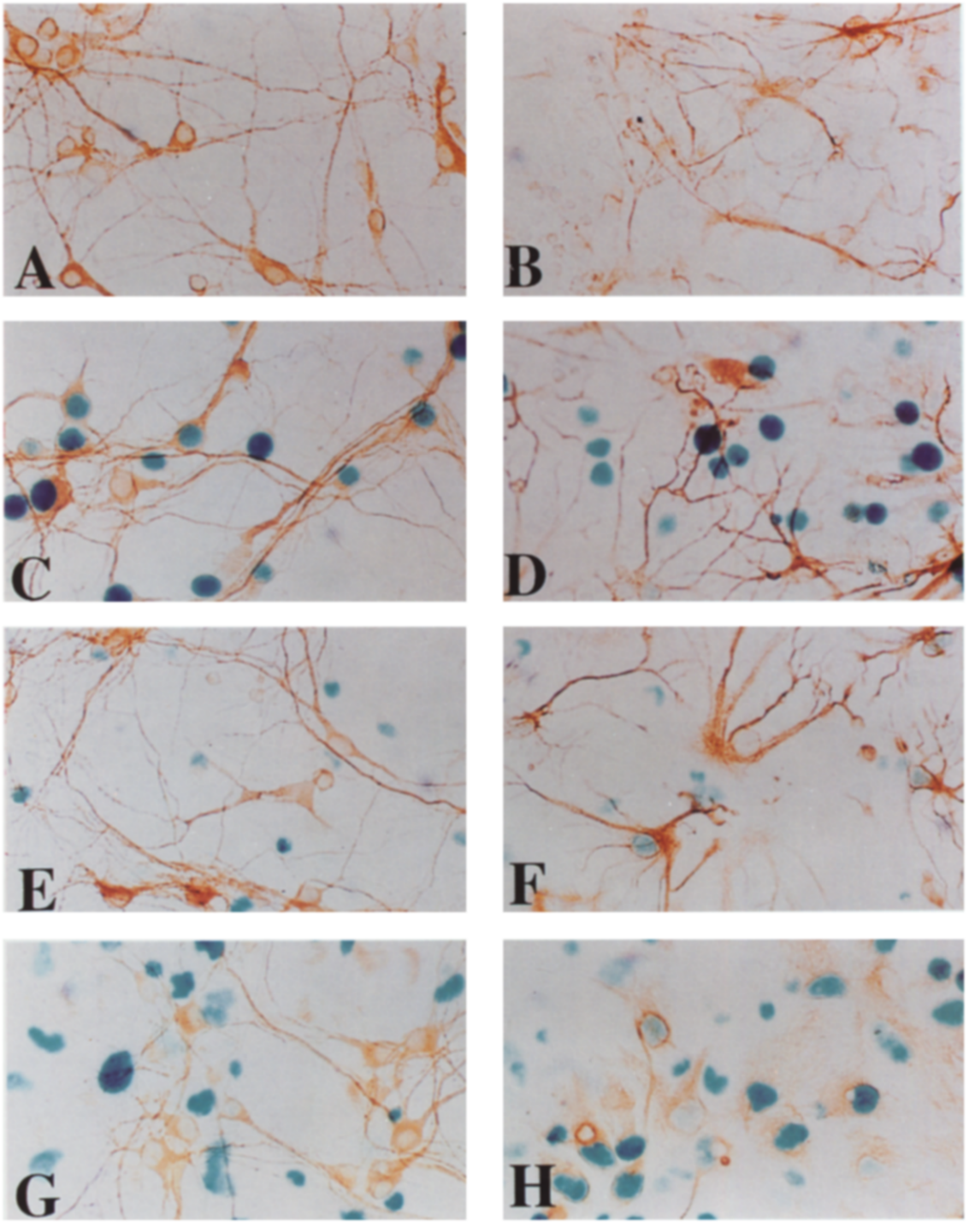


Figure 4. Regulation of Fos–lacZ Expression in Telencephalic Brain Cultures

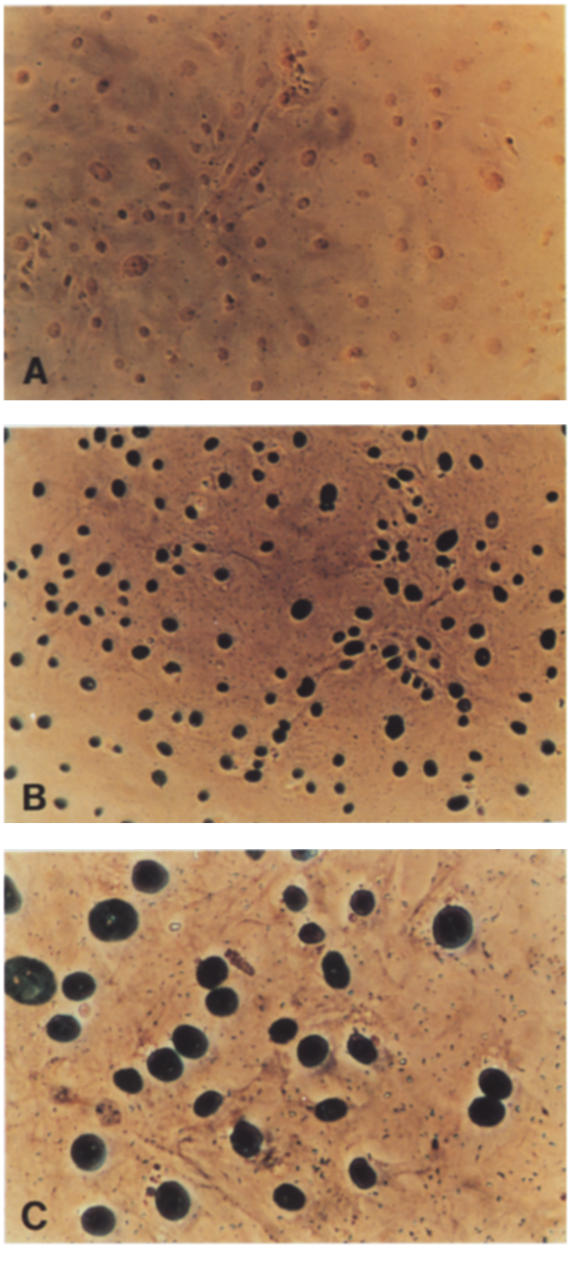
The cells were exposed to a variety of stimuli, including 26 mM KCl (C and D), 10 ng/ml PDGF (E and F), 20 nM TPA (G and H), and 50 μM kainic acid (data not shown). Control cultures (A and B) were treated with an equal volume of carrier. Subsequently, cultures were immunostained for MAP2 (A, C, E, and G) or GFAP (B, D, F, and H). Cultures prepared from independent transgenic founder lines 2 and 3 gave indistinguishable results. Magnification, 400× (A–H).

of c-*fos* expression in this cell type, we prepared embryo fibroblasts from at least one representative line for each of the constructs. Previously, we found that the intact *fos–lacZ* construct, in 3T3 fibroblasts derived from *fos–lacZ* mice (Smeyne et al., 1993) or stably integrated into B104 neuroblastoma cells (Schilling et al., 1991), could respond to a range of stimuli. Therefore, we investigated the effects of serum, TPA, PDGF, and dibutyryl-cAMP (dbcAMP) on Fos–lacZ expression in embryo fibroblasts derived from the different transgenic mouse lines.

Table 2. Regulation of Fos-lacZ Expression in Telencephalic Brain Cultures

	Neurons (MAP2 Immunoreactive Cells)					Glia (GFAP Immunoreactive Cells)				
Mutant	Control	KCl (26 mM)	PDGF (10 ng/ml)	TPA (20 nM)	Kainic Acid (50 μM)	Control	KCl (26 mM)	PDGF (10 ng/ml)	TPA (20 nM)	Kainic Acid (50 μM)
fos-lacZ	−	+++++	−	+	+++	−	−	+	+++++	+
mSIE	−	++	−	−	+	−	−	−	+	−
mSRE	−	+	−	−	−	−	−	−	−	−
mFAP	−	++	−	−	+	−	−	−	−	−
mCa/CRE	−	+	−	−	−	−	−	−	++	−

Percentage of MAP2 or GFAP immunoreactive cells that express Fos–lacZ. +++++, >45%; ++++, 36%–45%; +++, 26%–35%; ++, 16%–25%; +, 6%–15%; −, <6%. Values were determined by visually counting at least ten microscopic fields. Approximately 200 cells were counted per condition. The same range of values was obtained by analyzing cell cultures from independent transgenic lines for each construct. In addition, similar results were observed in at least two independent experiments.



Unstimulated fibroblasts containing the intact *fos–lacZ* construct expressed very low levels of β-galactosidase activity (Figure 5A). However, exposure to 20% serum for 3 hr caused a dramatic increase in nuclear β-galactosidase activity (Figures 5B and 5C). The time course and magnitude of the induction were similar to those observed for the endogenous c-*fos* gene. Virtually all cells responded to the stimulus, permitting quantitative analysis of the magnitude of the response (Figure 6). In addition to serum, treatment with TPA, PDGF, or dbcAMP increased β-galactosidase activity in a dose-dependent manner. These agents were chosen based on their reported regulatory targets in the c-*fos* promoter. High serum (Treisman, 1986) and TPA (Fisch et al., 1987) are thought to affect transcription of c-*fos* through the SRE; PDGF has been reported to function, at least in part, through the SIE (Wagner et al., 1990); and dbcAMP has been proposed to regulate transcription through the Ca/CRE (Sassone-Corsi et al., 1988b; Berkowitz et al., 1989). Therefore, based on these observations, mutations in these promoter elements were predicted to affect the response to a given agonist specifically. Fibroblast cultures from transgenic animals carrying a mutated Ca/CRE exhibited this predicted response. β-galactosidase activity was increased in response to 20% serum, TPA, and PDGF, but not in response to dbcAMP (Figure 6). However, fibroblast cultures from transgenic mSIE, mSRE, and mFAP mice exhibited little or no response to any of the agonists tested. This was a surprising contrast to the effect of the Ca/CRE mutation, but it was consistent with the results from telencephalic cultures, in which all of the c-*fos* regulatory elements were required in the majority of circumstances. In

Figure 5. Regulation of Fos–lacZ Expression in Fibroblast Cultures Derived from Transgenic Animals Containing the Intact *fos–lacZ* Construct

(A) Unstimulated transgenic fibroblast cultures exhibited no blue reaction product.

(B) Serum stimulation resulted in a high level of Fos–lacZ expression.

(C) Expression was localized to the nucleus. Fibroblasts prepared from independent transgenic founder lines 2 and 3 gave identical results. Magnification, 200× (A and B); 400× (C).

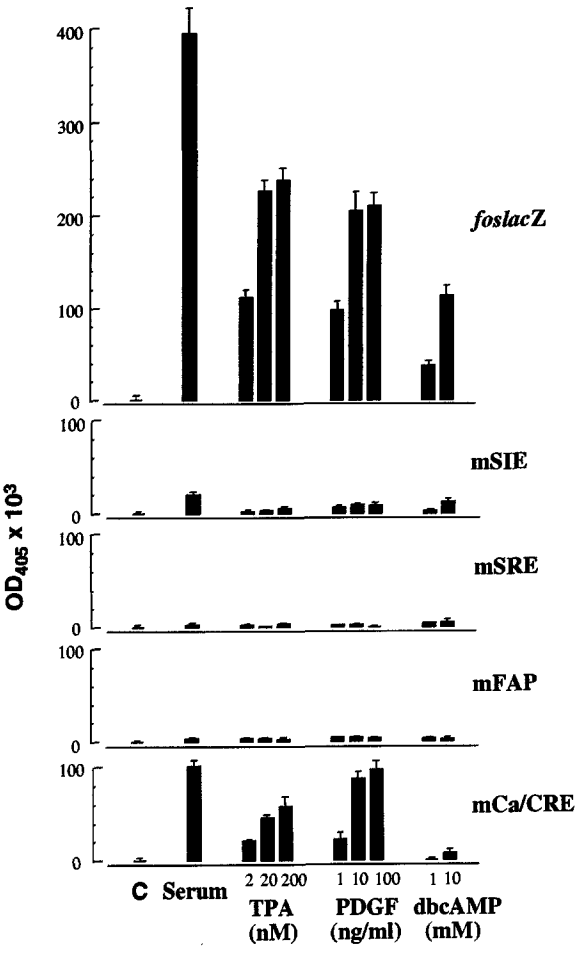


Figure 6. Quantitative Analysis of Fos–lacZ Expression in Cultured Fibroblasts from Transgenic Animals

Transgenic fibroblasts were prepared from a representative line for each construct (line 2, *fos–lacZ*; line 1, mSIE; line 2, mSRE; line 1, mFAP; line 3, mCa/CRE). β-galactosidase activity was determined after 3 hr stimulation with a variety of agonists, including 20% serum, TPA, PDGF, or dbcAMP. Control cultures were treated with an equal volume of carrier. Activity is expressed in optical density units with the level in nonstimulated controls set to 0. Error bars indicate SD (n = 6–8 replicate cultures). Similar results were obtained in several independent experiments.

addition, although the mFAP construct exhibited some Fos–lacZ expression in response to neuronal excitation in vivo and to elevated KCl in cultured neurons, it did not respond to any of the agonists tested in cultured fibroblasts. Therefore, even in a defined in vitro experimental system such as cultured embryo fibroblasts, a combination of regulatory elements is required for the activation of c-*fos* expression by extracellular stimuli.

Discussion

The c-*fos* gene has been widely used as a model system to study the molecular mechanisms involved in the activation of transcription in response to extracellular signals. Its promoter region contains several transcription factor–binding sites that have been proposed to act as independent transcriptional regulatory elements. The analysis reported here demonstrates that four major regulatory elements, the SIE, SRE, FAP, and Ca/CRE, are essential for the normal function of the c-*fos* promoter in vivo. Mutation of any single element resulted in a profound loss of tissue-specific and stimulus-evoked gene expression. This was true even in the case of the FAP, which appears to play little or no role in c-*fos* regulation in transient transfection assays. These results demonstrate that the transcription factor–binding sites studied function as components of an interdependent transcription control system in the c-*fos* gene. Indeed, since the *fos–lacZ* construct used is a genomic fusion, it is possible that additional regulatory elements are present either in the 5′ nontranscribed region, in intragenic sites, or in 3′ sequences. Thus, multiple sequence elements are required in concert for the physiological regulation of c-*fos* expression.

In addition to transcription factors, regulatory mechanisms involving higher order chromatin structure or nuclear organization could potentially influence *fos–lacZ* expression. In some genes, regions located at some distance from the promoter are required for position-independent expression in transgenic animals (Grosveld et al., 1987; Bonifer et al., 1990; Palmiter et al., 1993), whereas in other genes, such distal regions are not required (Chamberlain et al., 1991). Our experiments do not address directly the role of such distal regions in c-*fos* expression. We do find differences in the levels of Fos–lacZ in mouse lines carrying the same construct, for example, in the mFAP lines. These differences do not generally correlate with the transgene copy number, indicating that the *fos–lacZ* construct does not contain regulatory elements that confer copy number–dependent expression regardless of the insertion site. In our studies of the expression of wild-type and mutant *fos–lacZ* transgenes in brain, we found a high degree of consistency in the patterns of expression that we observed among several independent founder lines carrying the same construct. This relative lack of position-dependent variation in the patterns of expression suggests that the *fos–lacZ* construct encompasses most or all of the regulatory elements that are required for induction of c-*fos* expression in the brain. In contrast, when we examined constitutive expression of *fos–lacZ* at several sites in the mouse tail, more variation was observed. Of five wild-type *fos–lacZ* transgenic founder lines analyzed, two exhibited expression in bone, skin, and hair follicles. One line exhibited weaker expression only in hair follicles, and the remaining two lines exhibited no detectable expression. Of the transgenic lines carrying mutant constructs, 10 of 11 exhibited no detectable expression in these tissues. One of the mSRE lines (line 2) had low levels of expression in some hair follicle cells. This suggests that the c-*fos* regulatory elements analyzed here are also required for constitutive expression in skin, bone, and hair follicle cells. However, because of the marked insertion site effect on the expression of the wild-type *fos–lacZ* gene in these cells, it is likely that additional regulatory elements located outside of the region included in the *fos–lacZ* construct are also required. These regulatory elements may

be functionally related to the locus control regions identified in other genes that are thought to influence nuclear organization or higher order chromatin structure (Grosveld et al., 1987; Bonifer et al., 1990; Palmiter et al., 1993).

The demonstration that the regulatory elements in c-*fos* function interdependently is inconsistent with gene regulation models that are based on the independent action of individual transcription factors. Transcription factors are thought to activate gene expression through interactions with components of the general initiation complex. Cooperativity among transcription factors has been interpreted to occur through independent contacts between each transcription factor and one or more of the general initiation factors. However, this mechanism of transcriptional cooperativity does not explain our observation that mutation of any one of four separate elements abolishes *fos–lacZ* transcription in many cell types. Each element was found to exhibit a stimulatory effect of several hundred-fold in the presence of the others, but any combination of three elements failed to activate transcription to a detectable level in several cell types. This absolute interdependency among distinct regulatory elements compels us to propose a new model for transcription factor cooperativity. We suggest that the transcription factors that bind to these regulatory elements form a nucleation site that directs the assembly of an interdependent transcription complex (ITC). The ITC may include, in addition to transcription factors bound to regulatory elements, components of the general initiation complex and their associated factors (i.e., TAFs) as well as other proteins recruited to the promoter through interactions with the transcription complex. The assembly of the ITC may be highly cooperative by virtue of multiple interactions among components of the complex. Thus, the absence of any single component could result in either destabilization or inactivation of the ITC.

The proteins that form the ITC may differ among various tissues and cell types, and the requirements for its assembly may not be the same for different promoters. Indeed, we find that in some cells, c-*fos* expression does not require all of the regulatory elements examined here. Therefore, the ITC may comprise a different structure in different cell types. This is reminiscent of the elastase I enhancer, in which any two of three regulatory elements can direct pancreatic acinar cell–specific expression in transgenic mice (Swift et al., 1989). Thus, a subset of regulatory elements in the ITC can direct the assembly of an active complex at certain promoters. However, such complexes may not be able to respond to the full range of regulatory signals in vivo.

A prediction of the ITC hypothesis is that the c-*fos* regulatory elements would be occupied by proteins in most cells, regardless of the stimulus involved in transcription activation. In fact, in vivo footprinting analysis of the c-*fos* promoter has demonstrated that all c-*fos* regulatory elements examined here are occupied following exposure to an inducing stimulus such as epidermal growth factor (Herrera et al., 1989; Dey et al., 1991). In addition, the SRE, FAP, and Ca/CRE sites have been shown to be occupied prior to the addition of an activating stimulus. Thus, the ITC at the c-*fos* promoter may be partially assembled, even in the absence of an activating stimulus.

Another prediction of the model is that ITC assembly would depend on a network of transcription factor interactions. Indeed, several cases in which transcription factor interactions mediate cooperative binding to individual regulatory elements have been described (Stern et al., 1989; Jain et al., 1993; McCaffrey et al., 1993). Among these is the SRF-p62TCF/Elk-1 complex that binds to the c-*fos* SRE (Shaw et al., 1989; Hipskind et al., 1991; Shaw, 1992; Hill et al., 1993). These cooperative interactions with DNA may represent the first level of ITC assembly. Higher levels of ITC assembly could occur subsequent to DNA binding by all of the transcription factors. ITC assembly would also require extensive remodeling of the DNA structure to permit interactions among transcription factors bound to separate sites on DNA. This process could be facilitated by protein-induced DNA bends or other DNA structural changes. Indeed, several eukaryotic transcription factors have now been shown to induce DNA bending, which could serve to promote interactions among transcription factors bound to separate DNA sequence elements.

Traditional approaches to promoter analysis, using in vitro transcription and transient transfection assays with chimeric promoter constructs, have been useful in the identification of DNA elements that are required for transcriptional activation. However, the demonstration that a given transcription control element is both necessary and sufficient to generate a response to a specific signal in cultured cells does not address the question of how this element functions in the context of a natural promoter in the intact organism. In some cases, such as in the myogenin promoter, elements that are dispensable in cell culture are required for appropriate regulation in vivo (Edmondson et al., 1992). In contrast, in the elastase I enhancer, elements that are essential in cultured cells have apparently redundant functions in transgenic mice (Swift et al., 1989). Consequently, a combination of approaches is necessary to determine the array of regulatory elements required for the physiological regulation of gene expression.

In part, the discrepancies between results obtained in transient transfection assays and transgenic mice may arise from differences in chromatin organization between transfected and chromosomal DNA. It is important to note that the published transfection studies have utilized either artificial c-*fos* promoter constructs or truncated constructs to analyze the respective contributions of each of the transcription factor–binding sites studied here. Therefore, the results reported here are not directly comparable to these studies. In addition, we have been unable to obtain consistently high levels of induction in transient transfection assays performed using our natural promoter constructs.

The control of c-*fos* expression in neurons by calcium provides an example of transcriptional specificity in vitro. Transient transfection experiments in cultured neurons have suggested that induction of c-*fos* expression by elevated intracellular calcium resulting from activation of N-methyl-D-aspartate receptors occurs through the SRE, whereas activation of L-type calcium channels with high

KCl can efficiently stimulate transcription through the Ca/CRE, even in the absence of the SRE (Bading et al., 1993). Our results in transgenic neuronal cultures confirms this important role of the Ca/CRE in mediating KCl-stimulated c-*fos* transcription. However, in our hands, mutation of the SRE had the same negative effect as mutation of the Ca/CRE on KCl induction of Fos–lacZ expression. In addition, mutation of the SIE and the FAP severely reduced expression in a large number of neurons. This suggests that multiple elements are necessary and none are sufficient for complete activation of gene expression by KCl. A similar result was observed in *vivo*. The induction of c-*fos* expression in response to kainate-induced seizures may involve several signaling pathways, including calcium flux through voltage-gated calcium channels. However, we found that point mutations in either the SRE or the Ca/CRE completely abolished kainate-induced Fos–lacZ expression in the majority of neurons. Furthermore, mutation of the SIE caused a dramatic loss of Fos–lacZ induction in many neurons, although both the SRE and the Ca/CRE remained intact. Therefore, our experiments demonstrate that, within the context of the intact c-*fos* promoter, the Ca/CRE, SRE, SIE, and FAP are required in combination for induction of c-*fos* expression in many neurons both in vitro and in vivo. Thus, rather than viewing signal-activated gene transcription as a linear pathway that ultimately impinges on a single response element, we suggest that physiological signals in the CNS may be transduced by interdependent networks of transcription factors that require multiple regulatory elements to function appropriately.

The *fos* proto-oncogene (c-*fos*) has provided a general experimental model for the study of transcription regulation. Control elements, first described in the c-*fos* promoter, have been identified subsequently in regulatory regions of many other genes. Therefore, the implications of our results extend beyond the confines of c-*fos* regulation. For example, sequence elements closely related to the c-*fos* SRE have been reported to function in the regulation of other genes (Treisman, 1992). If the SRE does not function independently in c-*fos*, it may not do so in other promoters. Similarly, AP-1 and CRE sites are found in transcription control regions of many genes. These elements were proposed to be targets of TPA- and cAMP-activated signal transduction pathways. However, since these sequences are not sufficient for the activation of c-*fos* expression in the context of the natural promoter, they may not function independently in the regulation of other genes. Recent studies have demonstrated that the SIE, which is functionally related to regulatory elements found in genes that are responsive to γ-interferon, may also function in the response of c-*fos* to growth factors and ciliary neurotrophic factor (Bonni et al., 1993; Meyer et al., 1993; Sadowski et al., 1993). Therefore, the same sequence element may function in coupling many different extracellular signals to changes in gene expression. Consequently, these elements are likely to operate in conjunction with other regulatory sequences in the promoter regions of specific target genes. Thus, despite the strong interdependence of the regulatory elements in the c-*fos* promoter, it is unlikely that they are constrained to function only in the combination found in c-*fos*. Indeed, the functions of these elements may depend entirely on their context in a particular ITC. This contradicts the concept of a simple transcription factor response element, and it suggests that tissue- and stimulus-specific gene regulation occurs through the modulation of interdependent transcription complexes.

Experimental Procedures

Design of Transgenic Animals

A bacterial β-galactosidase (*lacZ*) gene was fused, in frame, into the carboxyl-terminal region of c-*fos* (Schilling et al., 1991; Smeyne et al., 1993). Clustered point mutations were introduced into the 5′ regulatory region of the *fos–lacZ* construct at the SIE (Hayes et al., 1987), the SRE (Treisman, 1986; Treisman, 1992), the FAP (Fisch et al., 1989), and the Ca/CRE (Sheng et al., 1988; Berkowitz et al., 1989; Fisch et al., 1989) using an overlapping oligonucleotide polymerase chain reaction (PCR) strategy. The identity of the cloned PCR products was confirmed by sequence analysis. Purified DNA fragments corresponding to the entire *fos–lacZ* gene containing the respective mutations were then introduced into B6C3F1 mice by microinjection of 1–2 cell embryos as previously described (Smeyne et al., 1992b). Animals containing the transgenes were identified by Southern analysis and/or PCR (Smeyne et al., 1992b). Several founder lines were derived for each construct. The transgene copy number in each line was determined by quantitative dot blot analysis using the endogenous c-*fos* gene as a standard. All animals used in this study were heterozygous transgenics generated by breeding transgenic males to B6C3F1 females.

Seizure Paradigm

Seizure was induced by intraperitoneal injection of kainic acid (20 mg/kg) as described previously (Smeyne et al., 1992b). Seizures usually began 15–30 min after drug administration. At 2 hr after injection, animals were given an overdose of Avertin (0.02 milliliters per gram of body weight), and they were transcardially perfused with 2% paraformaldehyde in 0.1 M PIPES buffer (pH 6.9). The uninduced levels of Fos–lacZ were determined in mice perfused immediately after removal from the home cage. Animals were maintained on a 12 hr light/12 hr dark cycle, with lights on at 6:00, and they were examined between the hours of 11:00 and 12:00.

Histology

Tissues were dissected, postfixed in fresh fixative for 1 hr, and cryoprotected for 24 hr in 30% sucrose in phosphate-buffered saline with 1 mM $MgCl_2$ (PBS + Mg; Smeyne et al., 1992b). Cryostat sections of 20 μm were cut and thaw mounted on gelatin coated or Superfrost Plus slides (Fisher). Sections were air dried for 24–48 hr and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase (X-Gal; Boehringer Mannheim) to determine β-galactosidase activity (Oberdick et al., 1990). The reactions were incubated for 16–20 hr at 37°C in the dark. Sections were counterstained with neutral red to facilitate visualization of cells.

Cell Culture

Primary neuronal cultures were established from telencephalic tissue of embryonic day 18 mice from animals containing the intact *fos–lacZ* transgene, as previously described (Huettner and Baughman, 1986; Smeyne et al., 1992). Embryos were generated by crossing a transgenic male and a normal B6C3F1 female. Therefore, approximately 50% of the cells in the cultures prepared from an entire litter were expected to contain the transgene. After 6–8 days in culture, cells were exposed to a variety of stimuli, including 26 mM KCl, 10 ng/ml PDGF, 20 nM TPA, and 50 μM kainic acid. Agonists were added from 100 × stocks, and KCl was used under isotonic conditions. Control cultures were treated with an equal volume of carrier. After 3 hr of stimulation, the cells were fixed and processed for β-galactosidase activity and immunostained for MAP2 or GFAP as described previously (Smeyne et al., 1992b).

Fibroblast cultures were prepared from 16 day transgenic embryos

from one representative line of each of the transgenic constructs. Cells were grown to confluence and passaged once before analyzing Fos–lacZ expression. For each experiment, cells were plated in multi-well plates and grown to confluence (24–48 hr) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Subsequently, the culture medium was replaced with DMEM containing 0.5% fetal bovine serum. After serum starvation for 48 hr, the cell cultures were treated with 20% fetal bovine serum, TPA, PDGF, or dbcAMP for 3 hr and assayed for β-galactosidase activity. Control cultures were treated with an equal volume of carrier.

β-Galactosidase Assay

The methods used for the analysis of β-galactosidase activity in cultured cells have been described previously (Schilling et al., 1991). Prior to the assays, the cells were fixed for 30 min in 2% paraformaldehyde in 0.1 M PIPES buffer. β-galactosidase in individual cells was detected using X-Gal as a substrate. The reactions were incubated for 6–12 hr at 37°C in the dark. For quantitation of β-galactosidase activity, cells were grown in 96 well microtiter plates, fixed, and permeabilized for 5 min in PBS + Mg with 0.5% Triton X-100. Following permeabilization, they were incubated with o-nitrophenyl β-D-galactopyranoside (1 mg/ml in PBS+Mg; 125 μl per well). The reaction was stopped after 6 hr by the addition of glycine/NaOH buffer (100 mM glycine [pH 10.3]; 75 μl per well), and the absorption was measured at 405 nm using a Molecular Devices Multiplate reader.

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