Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5665–5669, July 1991
Biochemistry

Regulation of a *fos–lacZ* fusion gene: A paradigm for quantitative analysis of stimulus–transcription coupling

(oncogene/signal transduction/gene regulation)

KARL SCHILLING*, DANIEL LUK†, JAMES I. MORGAN*, AND TOM CURRAN†

Departments of *Neurosciences and †Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, 340 Kingsland Street, Nutley, NJ 07110

Communicated by John J. Burns, April 4, 1991

ABSTRACT **Expression of the c-*fos* protooncogene is induced by a great variety of extracellular stimuli. A *fos–lacZ* fusion gene has been constructed that recapitulates this regulation. The *fos–lacZ* gene was introduced into B104 neuroblastoma cells for use in a quantitative assay for stimulus–transcription coupling. Both α- and β-adrenergic agonists, dibutyryl cAMP, and phorbol ester induced β-galactosidase activity in a dose-dependent manner. Thus, the interactions of receptors with agonists and antagonists, as well as intracellular second messenger-mediated signaling events, can be analyzed quantitatively. This approach represents a prototypic method for investigating stimulus–response coupling based upon gene expression.**

The c-*fos* protooncogene encodes a nuclear phosphoprotein (Fos) that functions in the regulation of gene transcription (1). Fos, as well as several related proteins encoded by the *fos* gene family, form heterodimeric complexes with proteins encoded by the *jun* gene family (2–6). Together, these proteins constitute the mammalian transcription factor AP-1 (activator protein 1) (7–10).

A characteristic feature of the *fos* and *jun* gene families is that their expression can be rapidly and transiently induced by a variety of extracellular stimuli through several second-messenger pathways (for reviews, see refs. 11–13). In fact, c-*fos* and c-*jun* belong to a larger class of genes referred to as cellular immediate-early genes (14, 15). Since the products of many (although not all) cellular immediate-early genes are transcription factors, we have proposed (15) that they should be regarded as nuclear third messengers that couple short-term stimulation events to long-term alterations in cellular phenotype by regulating expression of specific target genes.

Many stimuli, including classical pharmacological agonists and antagonists, are capable of inducing c-*fos* expression (for a review, see ref. 16). Thus, monitoring the levels of expression of c-*fos* offers a convenient method for the analysis of pharmacological interactions in living cells. The major advantages of monitoring c-*fos* expression, as opposed to measuring ligand-binding activities, is that it demands the active coupling of ligands to second-messenger systems and requires no information regarding the receptor systems under study. However, a major disadvantage of this approach is that it involves time-consuming methods for analyzing gene expression, none of which are amenable to rapid, quantitative analysis with high throughput.

Here we report a general method for quantitative analysis of c-*fos* expression based on a *fos–lacZ* fusion gene. The utility of this method is demonstrated by analysis of c-*fos* regulation in B104 cells.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture and Transfection. B104 neuroblastoma cells (17) and stably transfected derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% (vol/vol) fetal bovine serum. Cells were transfected with 10:1 mixtures of the plasmids pfos-lacZ (see below) and pVisnaNeo by the lipofection method (18). Briefly, subconfluent (40–60% confluent) monolayer cultures grown in 60-mm dishes were switched to serum-free Opti-MEM medium (GIBCO) and incubated with a Lipofectin (GIBCO)/DNA mixture (30 μl of Lipofectin, 5 μg of pfos-lacZ, and 0.5 μg of pVisnaNeo; final volume, 100 μl) for 16 hr. The transfection was stopped by replacing the medium with the standard culture medium. Twenty-four hours later, transfected cultures were split 1:10 and cultured in the presence of the neomycin analogue G418 (Sigma) at 1.2 mg/ml. After 7 days, resistant clones were isolated using cloning cylinders. Cells were further subcloned using the limiting dilution technique.

β-Galactosidase Assays. Cells were fixed for 30 min in 2% paraformaldehyde in 0.1 M Pipes buffer (pH 6.9). β-Galactosidase activity in individual cells was assayed using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), which yields an insoluble blue reaction product, as described (19). The incubation was carried out at 37°C for 12–18 hr.

For the rapid, and quantitative, assessment of β-galactosidase expression, the soluble substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG) was used (20). Various permutations of assay conditions were tested. The protocol finally adopted was as follows: cells grown in 96-well microtiter plates were fixed as described above and permeabilized for 5 min with 0.5% Triton X-100 in phosphate-buffered saline containing 1 mM $MgCl_2$ (PBS/Mg). After two washes with PBS/Mg, they were incubated with ONPG (1 mg/ml in PBS/Mg; 125 μl per well) for 5–6 hr. The reaction was stopped 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 an Artec Multiplate reader.

Metabolic Labeling and Immunoprecipitation. Confluent cultures were serum-starved for 24 hr and switched to methionine-free DMEM 20 min before treatment with 25 nM phorbol 12-myristate 13-acetate ("tetradecanoylphorbol acetate," TPA). After stimulation, [^{35}S]methionine (final concentration, 525 μCi/ml; 1 μCi = 37 kBq) was added at the required time points and the incubation was continued for 15 min. Cells were then rinsed with PBS and lysed in RIPA buffer (21). Proteins were immunoprecipitated and analyzed by gel electrophoresis and autoradiography as described (21). The antibodies used for immunoprecipitation were directed

Abbreviations: TPA, phorbol 12-myristate 13-acetate ("tetradecanoylphorbol acetate"); X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside.

against Fos amino acids 127–152 and have been characterized previously (21, 22).

RESULTS AND DISCUSSION

Construction of the *fos–lacZ* Fusion Gene. The *fos–lacZ* fusion gene is illustrated in Fig. 1. A 5-kilobase-pair (kbp) *Hin*dIII–*Bam*HI restriction fragment containing the entire murine c-*fos* gene and including 611 bp of 5′ untranscribed sequence that encompasses all of the known regulatory elements from pc-fos (mouse)-3 (23) was ligated to a 3-kbp *Sma* I–*Sal* I fragment from pMC1871 (24) that contained the β-galactosidase gene. The β-galactosidase fragment replaced a 297-bp *Nco* I–*Sal* I fragment from the fourth exon of c-*fos* that encoded the 65 C-terminal amino acids. The *Nco* I site of c-*fos* was made flush by treatment with Klenow fragment (25). Ligation of the c-*fos Nco* I site and the *lacZ Sma* I site resulted in the in-frame fusion of the C terminus of c-Fos to the N terminus of β-galactosidase (Fig. 1). The termination codon is provided by the *lacZ* gene. Thus, the *fos–lacZ* construct produces a fusion protein containing 315 N-terminal amino acids from c-*fos* and 1015 C-terminal amino acids from *lacZ*. Importantly, the fusion construct lacks the C-terminal amino acid sequences that function as a negative regulator of c-*fos* expression (26). Thus, the fusion gene should not affect expression of the endogenous c-*fos* gene. Three amino acids (Gly-Asp-Pro) that span the c-*fos*/*lacZ* junction are encoded by a linker sequence derived from pMC1871. The ligated fragments were subcloned into the *Bam*HI and *Hin*dIII sites of pGEM-4 (Promega). The entire fusion construct can be readily excised from the vector by digestion with *Hin*dIII and *Kpn* I, resulting in a fragment of 7.75 kbp.

Characterization of Cell Lines Carrying *fos–lacZ*. The *fos–lacZ* fusion construct was introduced into B104 neuroblastoma cells by lipofection together with pVisnaNeo. Stably transformed cells were selected in the presence of G418. Several cell lines that differed with respect to basal and stimulated levels of expression of the fusion gene were obtained. Two clones, A and B, were selected for the experiments described below. Clone A was characterized by a low basal level of β-galactosidase expression and an intermediate level of expression after stimulation. Approximately 1% of nonstimulated cells of clone A were stained by X-Gal, whereas more than 80–90% of the cells expressed β-galactosidase after treatment with dibutyryl cAMP, TPA, or serum (Fig. 2 and data not shown). In both unstimulated and stimulated cells the Fos–β-galactosidase fusion protein localized to the cell nucleus, indicating that the Fos nuclear localization signal(s) can direct the much larger fusion protein to the nucleus. Basal and stimulated levels of β-galactosidase activity in clone A were too low to permit quantitative analysis. Clone B exhibited higher levels of β-galactosidase activity. Approximately 10–15% of the cells of clone B expressed β-galactosidase under basal conditions, while more than 90% of the cells showed high levels of expression after stimulation. A 6-fold increase in β-galactosidase activity was obtained after stimulation by 20 nM TPA.

The Fos–β-galactosidase fusion protein expressed in clone B was immunoprecipitated by anti-Fos antibodies. In SDS gels, it displayed a molecular mass of ≈140 kDa (Fig. 3*A*). This protein was not detected in nontransfected B104 cells (Fig. 3*A*). Immunoprecipitation of Fos and the Fos–β-galactosidase fusion protein from pulse-labeled, TPA-stimulated cultures showed that the synthesis of the two proteins followed essentially the same time course (Fig. 3*B*). Increased synthesis of both Fos and the fusion protein was detected 15 min after stimulation. Maximal incorporation of radioactivity in both proteins occurred between 30 and 45 min. After 120 min, incorporation of radioactivity into Fos as well as the fusion protein had declined to basal levels. Synthesis of the Fos–β-galactosidase fusion protein was detected at lower levels in nonstimulated cells.

Quantitative Assay for *fos–lacZ* Expression. Clone B was used to establish a simple, quantitative screening procedure for Fos–β-galactosidase induction, using the soluble substrate ONPG. The amount of β-galactosidase activity detected following treatment with TPA followed a classic dose–response relationship (Fig. 4). After stimulation, maximal β-galactosidase activity was reached within 3 hr. Enzymatic activity declined slowly and after 24 hr was still above baseline levels (Fig. 5).

Stimulus–Transcription Coupling. Treatment of cells with norepinephrine, an adrenergic agonist that activates α and β receptors, resulted in the induction of β-galactosidase activity. Both α- and β-adrenergic receptors mediated this induction. Induction of β-galactosidase activity by 6-fluoronorepinephrine, a selective activator of α_1-adrenergic receptors, was blocked by prazosin, a selective antagonist of α receptors (Fig. 6), but not by propranolol, a selective blocker of β-adrenergic receptors. Stimulation of β receptors with isoproterenol also induced β-galactosidase activity. This action could be blocked by propranolol (Fig. 6) but not by prazosin.

The *fos–lacZ* fusion gene can be induced via several second-messenger pathways, including those involving protein kinase A and protein kinase C, as demonstrated by the direct activation of the fusion gene by dibutyryl cAMP and phorbol ester in a time- and dose-dependent fashion (Figs. 4 and 5 and unpublished data). Therefore, this cell line can be used to investigate pharmacological interactions involving agents that impact either cAMP- or protein kinase C-mediated signaling events. Indeed, β-adrenergic receptors are

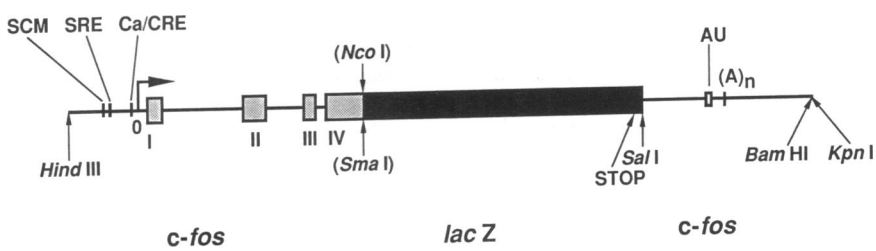

FIG. 1. Structure of the *fos–lacZ* fusion gene drawn approximately to scale. The restriction sites used for construction of the fusion gene are indicated. The *Kpn* I site was derived from the vector and, together with the *Hin*dIII site, was used for excision of the construct from the vector. Sites shown in parentheses were destroyed during ligation of the fragments. Several features of the fusion gene are illustrated. Lightly shaded boxes represent the four exons of c-*fos* (I, II, III, and IV), while the black box indicates the region containing *lacZ* sequence. The transcriptional start site (0) is indicated by a bent arrow and the position of the stop codon in *lacZ* is shown (STOP). In the 5′ untranslated region of c-*fos*, the positions of the transcriptional regulatory sequences, including the *sis*-conditioned medium (SCM) element, the serum response element (SRE), and the calcium/cAMP response element (Ca/CRE) are illustrated. The A+U-rich region (AU) and polyadenylylation signal [$(A)_n$] in the 3′ untranslated region of c-*fos* are also indicated.

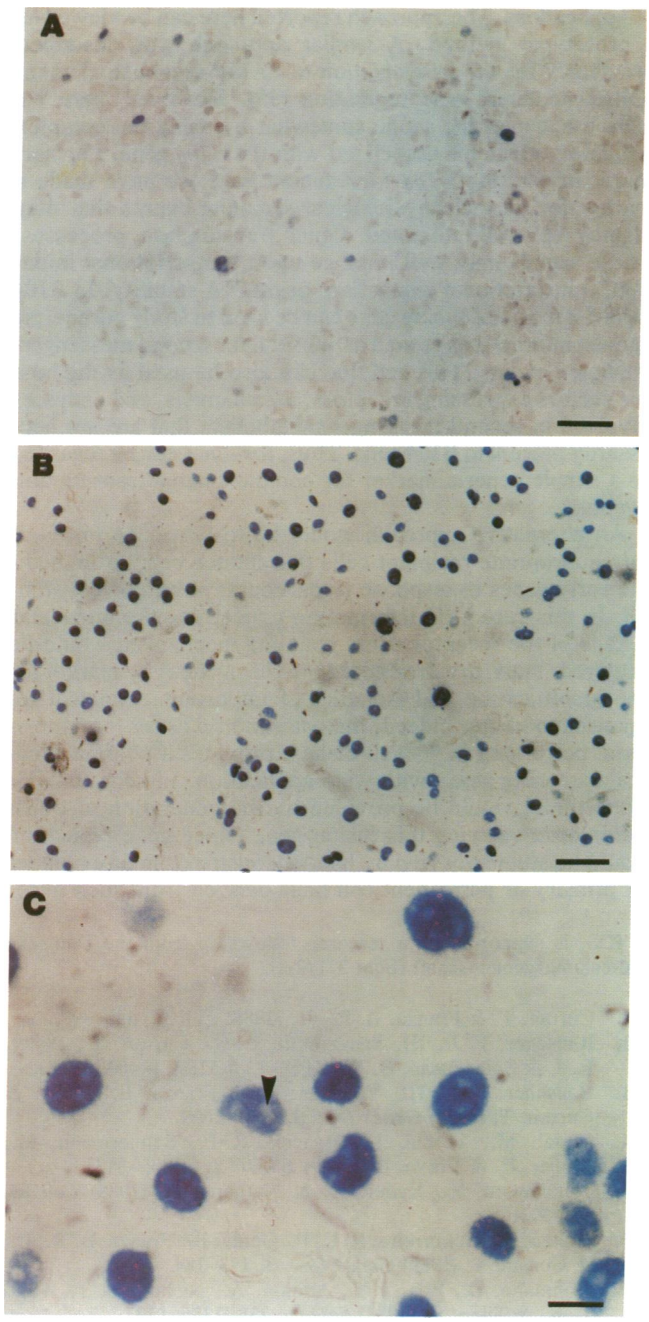

FIG. 2. B104 cells (clone A) stably transfected with the gene construct depicted in Fig. 1 are shown before (*A*) and after (*B* and *C*) stimulation with 8 nM TPA. Cells were stained with X-Gal to visualize β-galactosidase activity. Note single cells expressing the fusion gene even without stimulation (*A*). β-Galactosidase activity is confined to the cell nucleus and shows a distinctive nuclear staining pattern with the nucleoli remaining unstained (arrow in *C*). (Bar = 50 μm in *A* and *B* and 10 μm in *C*.)

normally coupled to the cAMP cascade whereas α_1 receptors are coupled to phosphatidylinositol turnover (28).

While previous studies of c-*fos* regulation using fusion genes have allowed mapping of several of the important regulatory elements, these have relied on mRNA detection methods that are not applicable to mass screening (13, 29, 30). Attempts to use constructs containing the c-*fos* promoter fused to the chloramphenicol acetyltransferase gene have not been successful because of the high basal levels of expression in unstimulated cells (ref. 31 and T.C., unpublished data). Indeed, this phenomenon led some investigators to conclude

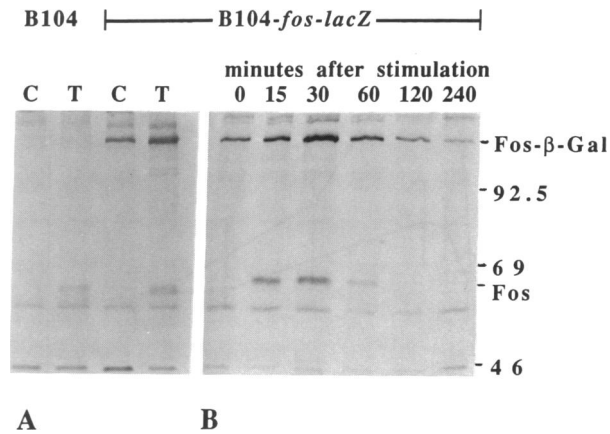

FIG. 3. (*A*) Identification of the Fos–β-galactosidase fusion protein in immunoprecipitates. B104 cells and B104 cells transfected with the *fos–lacZ* fusion gene (B104-*fos-lacZ*; clone B) were treated with vehicle (control, lanes C) or 25 nM TPA (lanes T). [^{35}S]Methionine was added 15 min later, and after another 30 min of incubation the cells were lysed and treated with anti-Fos antibodies. The Fos–β-galactosidase fusion protein (Fos-β-Gal) has an apparent molecular mass of about 140 kDa. It is absent from nontransfected B104 cells. (*B*) Time course of synthesis of Fos and the Fos-β-galactosidase fusion protein. Cultures (clone B) were stimulated with 25 nM TPA, and [^{35}S]methionine was added at the times indicated. The incubation was stopped 15 min after the addition of radioactivity, and Fos and the Fos-β-galactosidase fusion protein were immunoprecipitated. Size markers (92.5, 69, and 46 kDa) are indicated at right.

erroneously that the sequences required for induction of c-*fos* were located outside of the region studied here (31). When such fusion genes were used, long periods of stimulation were required to cause increases in chloramphenicol acetyltransferase in response to serum stimulation. Thus, it is not possible to use these promoter-fusion genes to study the rapid and dramatic fluctuations that occur in c-*fos* expression in response to physiological signals. Here we show that full gene fusions can be used to study the modulation of c-*fos* expression by physiological signals. This approach represents a departure from the routine reductionist methodolo-

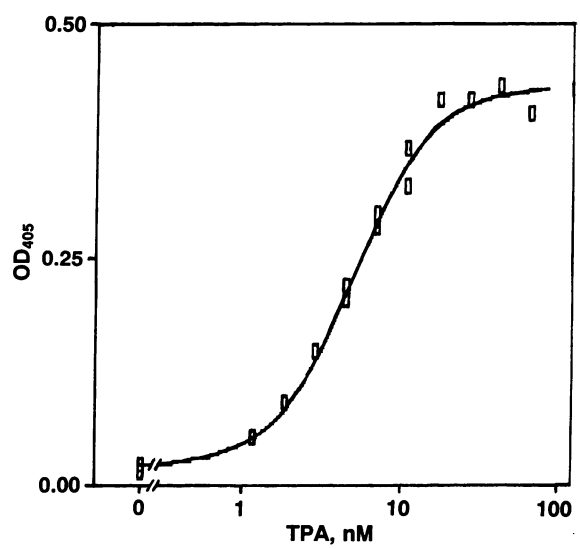

FIG. 4. β-Galactosidase activity following stimulation with increasing doses of TPA. Activity was measured by monitoring the conversion of ONPG at 405 nm. Each data point is the mean of eight independent measurements with SD < 10% of the mean. Values were fitted to a sigmoidal curve by using a four-parameter logistic model (27). The EC_{50} calculated from this curve is 3.53 ± 0.34 nM TPA.

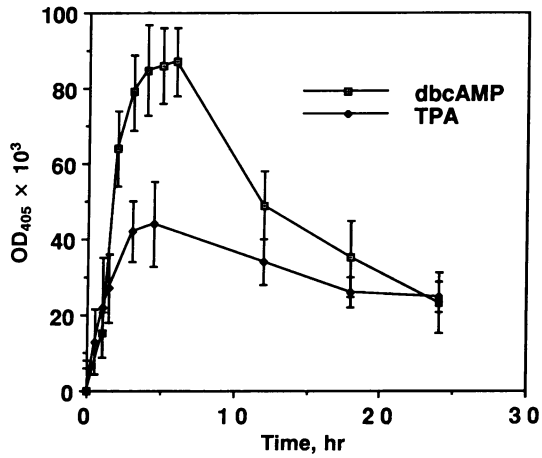

FIG. 5. Time course of expression of β-galactosidase activity after stimulation with TPA (4 nM) or dibutyryl cAMP (dbcAMP, 1 mM). Subconfluent cells (clone B) were stimulated for various times and β-galactosidase activity was measured using the soluble substrate ONPG. Activity is expressed in OD units with the level in nonstimulated controls set to 0.

gies generally applied to the study of gene expression. We contend that regulation of gene expression is a complex process that involves several regulatory mechanisms acting in concert. In the case of c-*fos*, two intragenic modes of gene regulation have been identified in addition to the many 5′ control elements (for review, see ref. 32). One acts upon 3′ and intragenic target sequences in mRNA that influence the rate of mRNA turnover (29, 33). The other affects the rate of transcriptional progression through an intragenic regulatory sequence (34). Indeed, it is possible that other c-*fos* intragenic regulatory elements are still to be discovered. In the many and varied situations in which c-*fos* is expressed, each of these elements could play a major role. Therefore, all of the potential regulatory elements should be retained in gene fusions designed to monitor physiologically relevant changes in c-*fos* expression. Recently, this approach has been vindicated by the successful recapitulation of constitutive and inducible levels of c-*fos* expression in transgenic mice, using the *fos–lacZ* fusion gene described here (R. J. Smeyne, K.S., L. M. Robertson, D.L., J. Oberdick, T.C., and J.I.M., unpublished data).

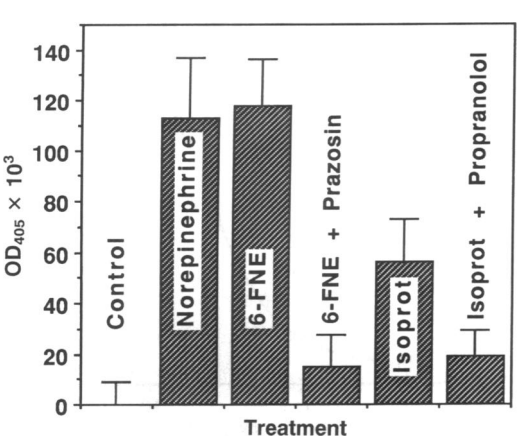

FIG. 6. Induction of β-galactosidase activity by adrenergic drugs. β-Galactosidase activity is induced by norepinephrine, 6-fluoronorepinephrine (6-FNE), and isoproterenol (Isoprot). Induction by 6-fluoronorepinephrine is attenuated by prazosin and induction by isoproterenol is suppressed by propranolol. Bars show means and 1 SD.

Applications. The approach reported here can be viewed as a prototypic method. A similar approach was described previously for the investigation of cAMP response element (CRE)-mediated gene regulation (35). However, here we have broadened the scope somewhat by using the complex regulatory elements associated with the c-*fos* gene. Furthermore, in creating a *fos–lacZ* fusion gene we have made a marker for normal physiological signaling events that may employ several integrated signal transduction processes. These signals may well impinge upon transcriptional initiation, transcriptional elongation, or mRNA stability. In B104 cells the *fos–lacZ* fusion gene can be used to study adrenergic agonist interactions as well as subsequent second messenger-mediated events. This cell line can also be used as the host for vectors expressing receptors, ion channels, and enzymes involved in second-messenger metabolism that are not necessarily present in B104 cells. Thus, *fos–lacZ* can be regarded as a simple generic marker for complex signal transduction events.

An alternative approach would be to introduce *fos–lacZ* into appropriate recipient cells that induce cellular immediate-early genes in response to particular physiological stimuli. In this case little information concerning the molecular details of the receptor or its coupling mechanism would be required. Thus, *fos–lacZ* could provide an activity marker for the identification and isolation of important biological response modifiers. Indeed, the colorimetric assay for expression, performed in 96-well dishes, provides a pharmacological screening assay with wide application. Finally, a major opportunity would be provided by the generation of transgenic mice carrying this fusion gene, since gene regulation could be studied *in vivo* by histochemistry or in any number of primary or permanent cell lines derived from such mice.

K.S. is supported by a research fellowship from the Deutsche Forschungsgemeinschaft (Schi 271/2-2).

1. Curran, T. & Franza, B. R., Jr. (1988) *Cell* **55**, 395–397.
2. Rauscher, F. J., III, Sambucetti, L. C., Curran, T., Distel, R. J. & Spiegelman, B. M. (1988) *Cell* **52**, 471–480.
3. Rauscher, F. J., III, Voulalas, P. J., Franza, B. R., Jr., & Curran, T. (1988) *Genes Dev.* **2**, 1687–1699.
4. Zerial, M., Toschi, L., Ryseck, R. P., Schuermann, M., Müller, R. & Bravo, R. (1989) *EMBO J.* **8**, 805–813.
5. Nakabeppu, Y., Ryder, K. & Nathans, D. (1988) *Cell* **55**, 907–915.
6. Cohen, D. R., Ferreira, P. C. P., Gentz, R., Franza, B. R., Jr., & Curran, T. (1989) *Genes Dev.* **3**, 173–184.
7. Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K. & Tjian, R. (1987) *Science* **238**, 1386–1392.
8. Bohmann, D. & Tjian, R. (1989) *Cell* **69**, 709–717.
9. Rauscher, F. J., III, Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R. & Franza, B. R., Jr. (1988) *Science* **240**, 1010–1016.
10. Abate, C., Rauscher, F. J., III, Gentz, R. & Curran, T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1032–1036.
11. Curran, T. (1988) in *The Oncogene Handbook*, eds. Reddy, E. P., Skalka, A. M. & Curran, T. (Elsevier, New York), pp. 307–325.
12. Morgan, J. I. & Curran, T. (1989) *Trends Neurosci.* **12**, 459–462.
13. Sheng, M. & Greenberg, M. E. (1990) *Neuron* **4**, 477–485.
14. Lau, L. F. & Nathans, D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1182–1186.
15. Curran, T. & Morgan, J. I. (1987) *BioEssays* **7**, 255–258.
16. Morgan, J. I. & Curran, T. (1991) *Annu. Rev. Neurosci.* **14**, 421–451.
17. Schubert, D., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinbach, J. H., Culp, W. & Brandt, B. L. (1974) *Nature (London)* **249**, 224–227.
18. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. &

Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7413–7417.
19. Oberdick, J., Smeyne, R. J., Mann, J. R., Zackson, S. & Morgan, J. I. (1990) *Science* **248,** 223–226.
20. Craven, G. R., Steers, E., Jr., & Anfinsen, C. B. (1965) *J. Biol. Chem.* **240,** 2468–2477.
21. Curran, T., Van Beveren, C., Ling, N. & Verma, I. M. (1985) *Mol. Cell. Biol.* **5,** 167–172.
22. Franza, B. R., Jr., Sambucetti, L. C., Cohen, D. R. & Curran, T. (1987) *Oncogene* **1,** 213–221.
23. Curran, T., MacConell, W. P., Van Straaten, F. & Verma, I. M. (1983) *Mol. Cell. Biol.* **3,** 914–921.
24. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. & Chou, J. (1983) *Methods Enzymol.* **100,** 293–308.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
26. Gius, D., Cao, X., Rauscher, F. J., III, Cohen, D. R., Curran, T. & Sukhatme, V. P. (1990) *Mol. Cell. Biol.* **10,** 4243–4255.
27. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
28. Lefkowitz, R. J. & Caron, M. G. (1988) *J. Biol. Chem.* **263,** 4993–4996.
29. Treisman, R. (1985) *Cell* **42,** 889–902.
30. Treisman, R. (1990) in *Seminars in Cancer Biology, Transcription Factors, Differentiation and Cancer*, ed. Jones, N. C. (Saunders, London), Vol. 1, pp. 47–58.
31. Deschamps, J., Meijlink, F. & Verma, I. M. (1985) *Science* **230,** 1174–1177.
32. Curran, T. (1991) in *Molecular Aspects of Cellular Regulation*, eds. Cohen, P. & Foulkes, J. G. (Elsevier, New York), Vol. 6, pp. 371–384.
33. Shyu, A.-B., Belasco, J. G. & Greenberg, M. E. (1991) *Genes Dev.* **5,** 221–231.
34. Lamb, N. J. C., Fernandez, A., Tourkine, N., Jeanteur, P. & Blanchard, J.-M. (1990) *Cell* **61,** 485–496.
35. Riabowol, K. T., Fink, J. S., Gilman, M. Z., Walsh, D. A., Goodman, R. H. & Feramisco, J. R. (1988) *Nature (London)* **336,** 83–86.