Hormone-dependent transcriiptional regulation and cellular transformation by Fos-steroid receptor fusion proteins

(Fos-estrogen receptor fusion protein/API-dependent transcription/Fos-mediated repression/Fos target genes)

GIULIO SUPERTI-FURGA*, GABRIELE BERGERS*, DIDIER PICARD†‡, AND MEINRAd BUSSLINGER*§

*Institute of Molecular Pathology, Dr. Bohr-Gasse 7, 1030 Vienna, Austria; and †Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448

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ABSTRACT The protooncogene c-fos has been implicated in the control of proliferation and transformation of fibroblasts, and its protein product is an essential component of transcription factor API. The important target genes and, hence, the molecular mechanism of Fos function are, however, still unknown, partly due to the lack of a tightly regulated Fos-induction system. Here we show that different activities of the Fos protein can be controlled hormonally by fusing the mouse c-Fos protein to the ligand-binding domain of either the rat glucocorticoid or the human estrogen receptor. These fusion proteins stimulate API-dependent transcription and repress endogenous fos mRNA synthesis in a strictly hormone-dependent manner. Expression of these chimeric proteins in rat fibroblasts results in fast, reversible, and tightly controlled transcription in response to hormone. A Fos-estrogen receptor expressing cell line was used to isolate Fos-responsive genes by subtractive cDNA cloning. Run-on analysis of one of these genes showed that its transcription is rapidly and directly regulated by the hormone-activated Fos-estrogen receptor protein, demonstrating the potential of this induction system for identifying Fos target genes.

The protooncogene c-fos is thought to play a central role in signal transduction by coupling short-term stimulation of the cell to long-term alterations in gene expression. c-fos codes for a nuclear protein that is an important component of transcription factor API. In the API complex the Fos protein is bound to the product of another protooncogene, c-jun, via a specific leucine zipper interaction. Fos-Jun heterodimers bind API sites with high affinity in contrast to Jun homodimers and, as a consequence, efficiently stimulate API-dependent transcription (1–5). The Fos protein also can repress the transcription of some immediate early genes, including its own gene (5–7). This activity of Fos requires intact C-terminal sequences, whereas the DNA-binding domain of Fos is dispensable (6–8). The main target of Fos-mediated repression is the serum response element rather than the API-binding site (7–9). Deregulated expression of the c-fos gene transforms fibroblasts (10), which correlates with the ability of C-Fos to transactivate rather than to repress gene transcription (8).

The detailed structure–function analysis of the Fos transcription factor is in clear contrast to our present ignorance about the relevant Fos target genes involved in control of cell proliferation and transformation. Identification of such target genes would be greatly facilitated by the availability of a selective and tightly regulated Fos-induction system. Recently, the activity of certain proteins was shown to be brought under hormonal control by fusing them to the ligand-binding domain of steroid receptors (11, 12). Here we demonstrate that the transactivating, repressing, and transforming activities of c-Fos can be subjected to strict hormone-dependent regulation in Fos-steroid receptor fusion proteins. The potential of this induction system is illustrated by differential cDNA cloning of genes that are regulated by Fos in rat fibroblasts.

MATERIALS AND METHODS

Construction of fos Fusion Genes. The expression vector pX was derived from the plasmids CD8 (13) and Bluescript (Stratagene); its transcription unit consists of the cytomegalovirus enhancer/promoter, the Bluescript polylinker, the simian virus 40 small tumor antigen intron, and a polyadenylation site. Plasmid pX-cFos contains the mouse c-fos gene as a 1324 base-pair (bp) Acc I fragment of the cDNA clone pGEMfos3 (3) in the EcoRV site of pX. pX-FosGR was generated by inserting a BamHI-Sac I fragment of plasmid HE14 containing the hormone-binding domain of the human estrogen receptor (ER) (14) into the Sac I site of pX-cFos. pX-FosGR was obtained by linking the same Sac I site of pX-cFos to a Bgl II site introduced 5' of codon 512 of the rat glucocorticoid receptor (GR) gene (P. J. Godowski, personal communication). Retroviral constructs pMV-cFos, pMV- FosER, and pMV-FosGR were generated by cloning the c-fos, fos-ER gene, and fos-GR gene as EcoRI–HindIII fragments into pMV-7 (15). pSV-FosER was constructed by inserting the 970-bp Sac I fragment of pX-FosER into the Sac I site of pSV-Fos (5).

Chloramphenicol Acetyltransferase (CAT) Assays. The transactivator expression plasmid (2 μg), the CAT reporter gene (1 μg), the β-galactosidase plasmid pCH110 (2 μg), pBR322, and pSV-β-galactosidase (5 μg) were transfected into cells by the calcium phosphate coprecipitation method. Thirty-six hours later the cells were harvested, and protein extracts were separately analyzed for β-galactosidase and CAT activity as described (16).

Stable Cell Lines. The plasmids pMV-7, pMV-FosER, or pMV-FosGR were transfected by the calcium phosphate method into the packaging cell line GP+·E-86 (17) for retrovirus production. The viral supernatant of these cells was used to infect Rat-1A and FR 3T3 cells followed by G418 selection. Alternatively, Rat-1A cells were cotransfected with EcoRI-linearized pSV-FosER DNA and with the neo- mycin resistance vector p309 (18) by the calcium phosphate precipitation method.

Focus Formation and Soft Agar Assay. Rat-1A cells were seeded into four Petri dishes 24 hr after retroviral infection. One aliquot was incubated with G418 (1 mg/ml) to determine the infection efficiency. Dexamethasone (10 μM) and β-estr...
bioavailability (0.1 μM) were added to two other plates. The medium was changed every 3–4 days, and the number of foci was counted after 3 weeks. For soft agar assay, 10⁴ cells of the parental Rat-1A line and of Fos-ER-expressing cells were suspended on 35-mm Petri dishes in 4 ml of 0.35% Difco Noble agar in Dulbecco’s modified Eagle’s medium/10% newborn calf serum/1 μM β-estradiol.

S1 Nuclease, Northern (RNA) Blot, and Run-On Analysis. Fifteen to 30 μg of cytoplasmic RNA was used for S1 nuclease or Northern blot analysis, as described (19, 20). Nuclei of estrogen-treated and untreated FR 3T3 cells expressing the Fos–ER protein were isolated and nascent RNA transcripts were labeled with [α-3²P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq) as described (21).

Subtractive cDNA Cloning and Differential cDNA Hybridization. cDNA was synthesized from poly(A)⁺ RNA of estrogen-stimulated cells, was subtracted by hybridization from poly(A)⁺ RNA of untreated cells, and was cloned into Agt10 DNA as described (22). Replica filters were hybridized with radiolabeled cDNA probes as described (22).

RESULTS

Fos–Steroid Receptor Fusion Genes. The hormone-binding domain of either the human ER or the rat GR was fused to the C terminus of the mouse c-fos protein as shown in Fig. 1A. This fusion was achieved in two different ways. Either the mouse c-fos cDNA sequence was linked at its downstream Sac I site to cDNA encoding the hormone-binding domain of the two steroid receptors or these receptor cDNA fragments were inserted in-frame into the fourth exon of the mouse c-fos gene. These chimeric genes and a c-fos gene lacking most of its trailer sequences were cloned into the three different expression vectors shown in Fig. 1B.

Hormone-Dependent Transactivation of an AP1-Responsive Promoter by Fos–Steroid Receptor Fusion Proteins. The ability of the Fos–steroid receptor fusion proteins to transactivate a thymidine kinase (tk) promoter linked to five AP1-binding sites (23) was tested in HeLa cells by transient transfection and CAT assays (Fig. 2). In the absence of hormone, HeLa cells transfected with either of the Fos–steroid receptor genes show the same low-background level of CAT expression as cells transfected with the expression vector pX alone. In the presence of their specific ligand (dexamethasone or β-estradiol), both the Fos–GR and Fos–ER proteins stimulate CAT expression ~10-fold, which is similar to the extent of transactivation by the constitutively active c-Fos protein. This induction of CAT expression depends on AP1-binding sites because the thymidine kinase promoter alone in the parental CAT construct (pBLCAT2) is not stimulated at all. That the observed increase in CAT activity results from correct initiation of transcription was shown by S1 nuclease analysis (G.S.-F., unpublished data). Hormone-regulated transcription by Fos–ER was not only observed in HeLa cells but also in other cell lines of diverse origin (CV-1, NIH 3T3, F9; Fig. 2). All these experiments have, therefore, demonstrated that the transactivation function of c-Fos can be brought under hormonal control by fusion to the hormone-binding domain of steroid receptors.

Fast and Direct Repression of the Endogenous fos Promoter by the Fos–ER Protein. The trans-repression activity of Fos–ER was analyzed in a Rat-1A fibroblast cell line that expresses a transfected fos-ER gene (pSV-FosER, Fig. 1B). As control, we used a Rat-1A cell line expressing the normal c-Fos protein from a transfected c-fos gene (pSV-Fos; ref. 5). These two cell lines and parental Rat-1A cells were serum-starved for 48 hr, and the kinetics of induction of the endogenous c-fos gene was studied by S1 nuclease analysis at 0, 30, and 120 min after serum stimulation (Fig. 3A). The following results were obtained. (i) Expression of the exogenous c-Fos protein significantly reduces the induced synthesis of endogenous c-fos mRNA, although it does not abolish it. (ii) In the absence of estrogen the endogenous c-fos gene is induced by serum to a similar level in the Fos–ER-expressing cell line as in the parental Rat-1A cell, indicating that the Fos–ER protein possesses little, if any, repression activity in the absence of estrogen. (iii) Pretreatment of

**Fig. 1.** Chimeric genes coding for Fos–steroid receptor fusion proteins. (A) Fos–steroid receptor fusion proteins. Numbers refer to amino acid positions of different proteins. The amino acids joining the two proteins are shown in the one-letter amino acid code. (B) Expression vectors. The pX-FosER plasmid was only used for transient assays (Fig. 2), whereas vectors pSV-FosER and pMV-FosER were used to generate stable rat fibroblast cell lines (Figs. 3 and 4). CMV; cytomegalovirus. MSV LTR; Moloney murine sarcoma virus long terminal repeat; SV40, simian virus 40; tk, thymidine kinase promoter.

**Fig. 2.** Hormone-dependent stimulation of transcription by Fos–steroid receptor fusion proteins. Hormone-dependent transactivation of a thymidine kinase promoter lacking (pBLCAT2; ref. 23) or containing (pAP1)₃tkCAT; ref. 23) five upstream AP1-binding sites by c-Fos, Fos–ER, and Fos–GR was analyzed by transient transfection and CAT assay in different cell lines. +, Addition of 1 μM β-estradiol or dexamethasone.
very rapidly activates the repression function of the Fos–ER protein. In a second experiment, estrogen, serum, and the protein synthesis inhibitor cycloheximide were added at the same time to serum-starved cells (Fig. 3B). Cycloheximide is known to affect fos gene expression at two different levels. It prevents transcriptional shut-off and leads to mRNA stabilization due to the block of synthesis of endogenous Fos and of a labile mRNA-destabilizing protein. The prediction for the cycloheximide experiment, therefore, is that the c-fos promoter should be repressed only in those cell lines containing preexisting exogenous Fos activity. Fig. 3B shows that a 5-fold, cycloheximide-insensitive repression is, indeed, seen in the Fos–ER-expressing cell line with, but not without, estrogen. In summary we have shown that the Fos–ER protein is rapidly activated by estrogen and that it directly represses the endogenous c-fos promoter.

**Hormone-Dependent Transformation by Fos-Steroid Receptor Fusion Proteins.** The transforming potential of the chimeric Fos proteins was analyzed in Rat-1A cells by three different criteria: focus formation, morphological transformation, and growth in soft agar (Fig. 4). The focus-formation assay was carried out by infecting Rat-1A cells with retroviruses either lacking or containing one of the three fos genes shown in Fig. 1. No foci were obtained with the parental vector pMV-7 (Fig. 4A). Neither hormone had any effect on the number of foci generated by infection with pMV-cFos. In contrast, over a 100-fold increase in focus number was seen with the two chimeric fos genes (pMV-FosGR and pMV-FosER) in the presence of the appropriate hormone.

G418-resistant cell lines containing the fos–ER gene were established either by infection with the retrovirus pMV-FosER or by cotransfection of plasmid pSV-FosER with a neomycin-resistance gene. Cell lines obtained by either method gave identical results in the morphology test and soft-agar-colony assay. In the absence of estrogen, the cells are flat, contact-inhibited, and indistinguishable from parental Rat-1A cells (Fig. 4B). Within 24 hr after hormone

![Fig. 3](image-url)

**Fig. 3.** Estrogen-dependent repression of the endogenous c-fos promoter by Fos-ER. (A) Parental Rat-1A cells and cell lines containing integrated fos-ER or fos genes were grown to confluence and then serum-starved for 48 hr. One micromolar β-estradiol (E2) was added to Fos-ER-expressing Rat-1A cells (lanes 7–9) during the last 24 hr of serum starvation. RNA was either directly isolated (lanes 1, 4, 7, or 10) or serum was added to a final concentration of 20% for 30 and 120 min before RNA extraction and S1 nuclease analysis by using mouse c-fos and rat glyceraldehyde phosphate dehydrogenase (gapdh) cDNA probes. (B) The experiment of Fig. 3A was repeated with the following modifications. Cycloheximide (CHX; 10 μg/ml), serum (20%), and β-estradiol (1 μM; where indicated) were simultaneously added to serum-starved cells. (C) Fos-ER-expressing Rat-1A cells were serum-starved for 48 hr. β-Estradiol (E2) (1 μM) was added 48, 24, or 2 hr before or immediately upon serum stimulation followed by RNA extraction after 30 min. endo, Endogenous; exo, exogenous.

![Fig. 4](image-url)

**Fig. 4.** Hormone-dependent transformation of rat fibroblasts by Fos-steroid receptor chimeras. (A) Focus-formation assay. Rat-1A fibroblasts were infected with retroviruses lacking (pMV-7) or containing (pMV-cFos, pMV-FosGR, and pMV-FosER) a fos oncogene. Dex, dexamethasone; E2, β-estradiol. (B) Morphological transformation. Rat-1A fibroblasts and c-Fos or Fos–ER-expressing cells were seeded at the same low density with or without 1 μM β-estradiol. Photographs were taken after 4 days. (C) Growth in soft agar. Representative colonies of parental Rat-1A cells and Fos–ER-expressing cells were photographed 20 days after seeding.
addition, the cells round up, elongate, and start to display a transformed morphology, which, after a few days, is identical with that of Fos-transformed Rat-1A cells. Moreover, transformation by Fos–ER is completely reversible, as cells incubated with estrogen for >2 weeks return to normal morphology within 2 days when reseeded in estrogen-free medium (data not shown).

Fig. 4C shows the behavior of a Fos–ER cell line in the soft-agar-colony assay. These cells grow to large colonies with estrogen, whereas their growth is inhibited to the same extent as that of the parental Rat-1A cells without hormone. Some Fos–ER cell lines were derived from soft agar colonies grown with estrogen for 3 weeks. These cell lines exhibited an untransformed phenotype in estrogen-free medium and, if retested, grew in soft agar only with hormone. This further demonstrates that hormone-dependent transformation by Fos–ER is tightly regulated and that continuous Fos activity is required for maintaining the transformed phenotype. We therefore conclude that Fos–steroid receptor fusion proteins induce reversible hormone-dependent transformation of Rat-1A fibroblasts as defined by three different criteria.

Isolation of Fos-Responsive Genes by Subtractive cDNA Cloning. By S1 nuclease analysis we could not detect any endogenous ER mRNA in two rat fibroblast cell lines, Rat-1A and FR 3T3, confirming our observation that estrogen is an inert signal for these cells (Fig. 4 and G.S.-F., unpublished data). As a consequence, estrogen very specifically activates the exogenous Fos–ER protein in our stable cell lines, which leads to reprogramming of gene expression as manifested by the observed phenotypic changes in response to hormone. Fos–ER-expressing cell lines should, therefore, be useful for the identification of Fos-regulated genes by subtractive cDNA cloning techniques. For this we have chosen an FR 3T3 cell line infected with the retrovirus pMV-FosER. A subtracted cDNA library was constructed in λgt10 from poly(A)+ RNA of estrogen-stimulated cells (24 hr) by using RNA of untreated cells for cDNA subtraction, according to Rhyner et al. (22). Thirty thousand plaques were screened with radiolabeled cDNA probes synthesized from either stimulated or unstimulated RNA. Three rounds of differential cDNA hybridization and subsequent Northern blot analysis led to the identification of three different phases with 600- to

![Fig. 5. Estrogen (E2)-induced Fit-1 mRNA synthesis in Fos-ER-expressing cells. (A) Inducible Fit-1 gene expression in Fos-ER-expressing FR 3T3 cells. Cytoplasmic RNA (30 μg) was used for Northern blot hybridization first with radiolabeled Fit-1 cDNA and then with a rat gapdh probe. (B) Time course of Fit-1 mRNA accumulation in Fos-ER-expressing FR 3T3 cells in response to estrogen (1 μM). The same filter was hybridized simultaneously with the Fit-1 and gapdh cDNA probes. (C) Time course of Fit-1 mRNA accumulation in a Fos-ER-expressing Rat-1A cell line in response to estrogen (1 μM).](image)

Fig. 6. Direct transcriptional activation of the Fit-1 gene by Fos–ER. Fos–ER-expressing FR 3T3 cells were exposed to estrogen (1 μM) for the indicated time before nuclei preparation and labeling of nascent RNA by run-on transcription. The different RNA probes were hybridized to immobilized cDNA of the Fit-1, rat gapdh, and mouse actin genes as well as to pSP64 DNA. Where indicated, α-aminopterin (2 μg/ml; α-am.) was added to the nuclei before labeling of nascent transcripts.

900-bp-long cDNA inserts that hybridized to estrogen-induced transcripts. For further analysis we have chosen one of these cDNA clones, which we refer to as Fos-induced transcript 1 or Fit-1 gene.

**RAPID TRANScriptional Aктивation of the Fit-1 Gene by Fos-ER.** The Fit-1 cDNA probe detects a mRNA of ~2600 nucleotides (Fig. 5A). In the Fos–ER-expressing FR 3T3 cells the mRNA level increases ~10-fold in response to estrogen, reaching a level identical to that of the Fos-transformed FR 3T3 cell line. The Fit-1 mRNA level is, however, very low in the parental FR 3T3 cells, even after 24-hr treatment with estrogen. This fact clearly indicates that expression of the Fit-1 gene is regulated by the exogenous Fos protein rather than by an endogenous estrogen-induced activity.

The kinetics of Fit-1 mRNA synthesis in response to estrogen was studied in Fos–ER-expressing FR 3T3 and Rat-1A cells (Fig. 5B and C). In both cases Fit-1 gene transcripts start to accumulate after 1 hr of estrogen stimulation and reach a maximal level between 3 and 8 hr. The relatively fast kinetics of Fit-1 mRNA accumulation suggests direct transcriptional regulation of this gene by Fos–ER. This hypothesis was tested by nuclear run-on analysis. Nascent RNA transcripts were labeled in nuclei isolated from Fos–ER cells stimulated with estrogen for 0, 1, 3, 8, and 24 hr and were then hybridized to cDNA of the Fit-1, gapdh, and actin genes and to pSP64 plasmid DNA. Fig. 5 shows that the transcription rate of the Fit-1 gene increases to maximum during the first 3 hr and then decreases slightly the next 20 hr. Maximal transcriptional activation of the Fit-1 gene clearly precedes maximal accumulation of its transcript. We conclude, therefore, that the Fit-1 gene is rapidly and directly regulated at the transcriptional level by the hormone-activated Fos–ER protein.

**DISCUSSION**

**Hormone-Dependent Activity of Fos–Steroid Receptor Fusion Proteins.** Here we report that three well-characterized activities of the c-Fos protein—i.e., AP1-dependent transcription, repression of its own promoter, and cellular transformation—can be brought under hormonal control by fusing Fos to the ligand-binding domain of the ER or GR. Previously, the activities of two other nuclear oncogenes, E1A and Myc, have been subjected to hormonal regulation in exactly the same way (11, 12). All three induction systems take advantage of a general “protein inactivation” function that is part of the ligand-binding domain of steroid receptors and which, in the absence of hormone, can repress other activities present on the same polypeptide chain (11, 24). It has been postulated that the interaction of the abundant heat shock protein hsp90 with the unliganded hormone-binding domain may cause this repression function (11). In agreement, recent in vivo experiments have shown an important role of hsp90 in signal transduction by steroid receptors (25). The hormone-binding region of steroid receptors possesses, in addition to the “protein inactivation” function, a hormone-inducible dimerization and a potent transactivation domain as
well as a nuclear localization signal in the case of the GR (for review, see ref. 26). Grafting these functions onto the Fos protein might, therefore, modify Fos activity in the fusion protein. However, so far we have not seen any significant qualitative or quantitative differences between Fos and Fos–
steroid receptor fusion proteins in transactivation, repression, and transformation assays.

The c-Fos protein stimulates AP1-dependent transcription by binding to AP1 recognition sequences as part of a hetero-

meric activation complex with Jun proteins (1–5). Transcriptional

activation by Fos–steroid receptor fusion proteins also de-

pends on AP1-binding sites and is lowest in F9 cell, where the endogenous Jun levels are very low (Fig. 2). Furthermore, in vitro-translated Fos–ER protein can bind to AP1 sites only in the presence of Jun protein (G.S.-F. and M.B., unpublished data). All this evidence indicates that Fos–steroid receptor fusion proteins also require complex formation with Jun proteins for transcriptional activity. The activated GR has recently been shown to repress AP1 activity (27), and yet our Fos–GR fusion protein stimulates AP1-dependent transcription (Fig. 2), suggesting that the hormone-binding domain alone is not sufficient to interfere with AP1 activity.

Transcriptional shut-off of the c-fos promoter depends on nuclear translocation, and the c-fos gene product itself is thought to act as a repressor by interfering with activity of the serum response element (5–9). It is, however, still unclear whether Fos is directly involved in repression by interacting with factors binding to the serum response element or whether it affects expression of another nuclear protein that then controls c-fos transcription. To address this question, we have taken advantage of stable rat fibroblast cell lines that constitutively express Fos–ER protein. This exogenous protein can repress serum-induced transcription from endogenous c-fos promoter in a hormone-dependent manner (Fig. 3). Its repression function is immediately activated upon hormone addition and does not require ongoing protein synthesis, indicating that the Fos protein plays a direct role in down-regulation of its own promoter.

Hormone-dependent transformation of rat fibroblasts by the Fos–ER protein is fast and reversible, indicating that continuous Fos activity is required for maintaining the transformed phenotype. Most importantly, transformation is tightly controlled by estrogen. In this context it is relevant to note that the ER used in this study contains an amino acid substitution in the hormone-binding domain (Gly400→Val), which reduces the affinity for estrogen and, hence, conve-
niently prevents activation by trace amounts of hormone (28).

Thus, estrogen does not morphologically transform Fos–ER cell lines at 0.1 nM. However, 1 nM estrogen results in slow transformation after a week, whereas fast transformation within a day is observed at concentrations of 10 nM and above (data not shown). Transcriptional activation of estrogen-responsive genes by the mutant ER (HEO) is maximal between 1 nM and 0.1 nM estrogen (28) and, therefore, parallels cellular transformation by Fos–ER in its concentration dependence.

Identification of Fos-Regulated Genes. The Fos–ER induc-

tion system in combination with subtractive cDNA cloning was used to identify an endogenous Fos-responsive gene referred to as Fit-1. Northern blot and nuclear run-on analyses revealed that in fibroblasts the Fit-1 gene is rapidly and directly regulated at the transcriptional level by the Fos–ER protein, resulting in a 10-fold accumulation of Fit-1 mRNA (Figs. 5 and 6). Fit-1 gene transcripts are equally abundant in c-Fos-transformed fibroblasts. Their expression is, however, low in the parental fibroblast cell line, where it is inducible by serum stimulation, as expected for a Fos-responsive gene (G.B., unpublished data). In the absence of estrogen, an elevated basal level of Fit-1 mRNA synthesis is seen in Fos–ER-expressing cells. The slight leakiness of hormone-

dependent transactivation by Fos–ER suggests that the unliganded hormone-binding domain of the ER cannot com-

pletely suppress all Fos activity in the fusion protein. This leaky regulation of Fit-1 gene expression sharply contrasts with the tight control of the transcriptional phenotype by Fos–ER. Only high-expression levels of the c-Fos protein are known to cause transformation of rodent fibroblasts (10, 29). All Fos–ER cell lines used have been selected for hormone-

dependent transformation and, hence, for high Fos–ER expression. In the absence of estrogen, the Fos activity in these cell lines is apparently below a critical threshold level for transformation and yet is already high enough to partially activate the Fit-1 gene. Despite this caveat, the Fos–ER induction system has already proved useful for cloning of Fos-regulated genes and consequently has opened the way to a molecular analysis of the role of Fos in control of cell proliferation and transformation.

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