Journal of Cellular Biochemistry 57:630–640 (1995)

Activation of the C-Fos Promoter by Increased Internal pH

José R. Murguía, Luc De Vries, Lourdes Gomez-García, Axel Schönthal, and Rosario Perona

Instituto de Investigaciones Biomédicas del C.S.I.C., Madrid 28029, Spain (J.R.M., L.D.V., L.G.-G., R.P.); Department of Microbiology, University of Southern California, Los Angeles, California 90033-1054 (A.S.)

Abstract Changes in intracellular pH (pHin) take part in the mitogenic response. Their importance has been stressed by the finding that mouse fibroblasts expressing a yeast proton pumping ATPase (*PMA1*) exhibit a transformed phenotype and are tumorigenic. These cells do maintain a higher pHin, supporting the idea that elevated pHin may act as a proliferative trigger. Here we show that cells constitutively expressing *PMA1* have higher levels of the AP-1 transcription factor. The use of stable transfectants and transient transfection assays show that *PMA1* activity induces transactivation of the c-fos promoter. The activation of the promoter is mediated throughout the serum response element (SRE). The use of protein kinase C inhibitors suggests that AP-1 activation is achieved through a pathway independent of protein kinase C. © 1995 Wiley-Liss, Inc.

Key words: internal pH, transformation, c-fos, AP-1

Serum stimulation of quiescent fibroblasts induces a highly regulated series of events that results in changes in gene expression and ultimately lead to cell division [Rozengurt and Mendoza, 1985]. One of the first events that occurs after a growth factor interacts with its receptor is a change in ion fluxes across the cytoplasmic membrane [Pouysségur, 1985]. An increase in sodium influx and an efflux of protons that produces a rise in pHin of 0.15–0.3 pH units develops usually 15 seconds to 1 min after mitogen addition. This increase is maximal within 5 min and persists as long as the mitogen is maintained [Pouysségur, 1985]. The cytoplasmic alkalinization results from the activation of the Na^+/H^+ exchanger (NHE), because it is abolished in the presence or amiloride, in Na^+-free medium, or in fibroblast mutants lacking NHE activity [L'Allemain et al., 1984; Pouysségur et al., 1984]. On the other hand, the microinjection of activated H-ras [Hagag et al., 1987] or the expression of v-mos [Doppler et al., 1987] and H-ras [Kaplan and Boron, 1994] are able to induce intracellular alkalinization thorough the activation of the sodium proton exchanger. Inhibition of the activity of the NHE produced by microinjection of H-ras by using the inhibitor amiloride is able to block DNA synthesis (Hagag et al., 1987). The activation of different G-protein coupled receptors such as α-thrombin, bradikinin, vasopresin, etc., also results in an increased amiloride-sensitive Na^+/H^+ antiport. Recently it has been described that Gα13 is able to stimulate the NHE [Voyno-Yesenetskaya et al., 1994]. This activation is agonist independent when a mutant with a constitutively active GTPase (α13-Q226L) is used [Voyno-Yesenetskaya et al., 1994]. All together, this evidence suggests that increased pHin may function as a second messenger. The activation of either tyrosine kinase or G-protein coupled receptors as well as the transformation induced by some activated oncogenes leads to the activation of multiple signal transduction pathways making it difficult to evaluate the contribution of the increase in pHin to the mitogenic response. In order to investigate this point, we used a more direct approach, transfecting NIH3T3 fibroblasts with the gene of a yeast proton-pumping ATPase *PMA1* [Perona and Serrano, 1988]. These cells have pHin 0.3 units higher than the parental ones even in the presence of bicarbonate [Gillies et al., 1990] and are tumorigenic in

Received July 26, 1994; revised September 2, 1994; accepted September 6, 1994.

Address reprint requests to Rosario Perona, Instituto de Investigaciones Biomédicas del C.S.I.C., C/Arturo Duperier, 4, Madrid 28029, Spain.

nude mice and able to grow in the absence of serum [Perona and Serrano, 1988]. By using site-directed mutants of the *PMA1* gene, it has been demonstrated that pHin and the degree of transformation and tumorigenicity of fibroblasts expressing the ATPase mutants correlate with the activity of the different enzymes [Perona et al., 1990]. The expression of the yeast ATPase results in higher intracellular alkalinization than that provided by either the activation of the NHE by growth factors and oncogenes or the cooperation of the bicarbonate systems [Burns and Rozengurt, 1983; Perona et al., 1990]. This difference could explain why the large increase in proton transport and pHin produced by expressing the yeast ATPase is mitogenic, while the smaller increase produced by growth factors, oncogenes, and bicarbonate is only permissive for the growth response.

Fos is a nuclear protein whose expression is induced by a variety of growth factors and oncogenes and by external agents such as inducers of oxidative stress and phorbol esters [Müller et al., 1984; Rao et al., 1993]. Furthermore, Fos protein expression is required for growth of cells in response to serum. Expression of c-fos antisense RNA [Nishikura and Murray, 1987] or the microinjection of antibodies raised against Fos into quiescent fibroblasts [Riabowol et al., 1988] has been found to block serum-stimulated reentry into the cell cycle. Fos, together with Jun, composes the AP-1 transcription factor whose activity is to propagate the mitogenic signal by controlling the expression of downstream genes that are required for proliferation [Riabowol et al., 1988; Angel and Karin, 1991]. Several oncogenes have been shown to modulate AP-1 activity. Transient expression of v-src, v-mos, polyoma middle T, Ha-ras, neu, or v-raf leads to enhanced AP-1 activity [Riabowol et al., 1988; Franza et al., 1988]. Since the *PMA1* gene, when expressed in fibroblast, behaves as an activated oncogene [Perona and Serrano, 1988], we were interested in investigating if its expression involved changes in the levels of the transcription complex AP-1. We here present evidence showing that the expression of the yeast proton pump is able to induce transactivation of the c-fos promoter that results in an increase in the levels of transcriptionally active AP-1 complex. The pathway is independent of protein kinase C activity.

MATERIALS AND METHODS

Cell Lines and Transfections

RN1a and RNT3a cell lines are two independent clones of NIH3T3 cells constitutively expressing the *PMA1* gene [Perona and Serrano, 1988]. The N-213 cell line is a clone stably transfected with the plasmid pma1-213 [Perona et al., 1990]. NIH3T3 cells and derived cell lines were maintained at 37°C in growth media containing 90% DMEM and 10% CS (GIBCO) in a humidified atmosphere of 5% CO_2. For transfection, cells were seeded into 100 mm dishes and cotransfected 24 h later by the calcium phosphate method [Chen and Okayama, 1987]. After transfection, cells were rinsed twice with PBS and incubated for 32 h in DMEM with 0.5% FCS. The agents indicated in each experiment were added for the last 6 h of incubation before collection of the cells.

Plasmids

The basic expression plasmid (pSV_hAT_5) has already been described [Perona and Serrano, 1988]. It contains the coding region of wild type yeast ATPase under the control of SV40 promoter. The pma1-213 plasmid [Perona et al., 1990] has also been described. Briefly, it was constructed by replacing the 3.8 Kilobase ClaI fragment of the pSV_hAT_5 with the equivalent fragment containing the mutation Glu233-Gln [Perona et al., 1990]. The plasmid pFC4 was kindly provided by Inder Verma and contains 400 residues from the 5′ flanking region of the human c-fos promoter linked to the CAT reporter gene [Deschamps et al., 1985]. p4XSRETK-CAT, p4XCRETK-CAT, and p4XTRETK-CAT [Schönthal et al., 1991] are plasmids derived from $pBLCAT_2$. The respective promoter elements were synthesized and inserted into the multiple cloning site in front of the thymidine kinase (TK) promoter of $pBLCAT_2$. The TRE sequence was taken from the human collagenase gene [Schönthal et al., 1991]. The CRE sequence is the one in the c-fos promoter at the position −60.

CAT and β-Galactosidase Assays

For CAT assay, cells were serum starved for 48 h and induced for 6 h with the indicated agents. Extracts were prepared as previously described [Gorman et al., 1982]. Protein content was determined by a commercial kit (Bio-Rad).

The lysates (50–100 μg of protein) were incubated in 0.2 ml of reaction mixture containing 50 mM (14C)chloramphenicol (0.5 μCi) and 2 mM acetyl-CoA in 250 mM Tris-HCl at pH 7.8 for 4 h at 37°C. CAT activity was detected and quantified by thin layer chromatography. Transfection efficiencies were standardized with the pCH110 plasmid containing the β-gal gene under the control of the simian virus 40 promoter (SV40). β-galactosidase activity was assayed in a buffer containing Na_3PO_4 (pH 7.5), 1 mM $MgCl_2$, and 200 mg of O-nitrophenyl-β-D-galactopyranoside. Samples were incubated at 37°C for 10 min and the reaction stopped by addition of Na_2CO_3. Activity was measured by absorbance at 420 nm. Mean CAT activities were calculated from at least three independent transfections and the level of induction determined by calculating the ratio of the stimulated to the control values.

Preparation of Nuclear Extracts

NIH3T3 and RN1a cells were serum-starved for 48 h and stimulated with mitogens when indicated, and nuclear extracts were prepared as described [Andrews and Faller, 1991]. Briefly, cells were scraped and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in Buffer A (10 mM Hepes-KOH (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, 0.2 mM PMSF). Samples were incubated for 5 min at 4°C and vortexed for 10 seconds. Nuclei were sedimented at 12,000 rpm for 20 seconds and resuspended in buffer C (20 mM Hepes-KOH (pH 7.9), 25% glicerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated for 20 min at 4°C. Samples were centrifuged at 6,000 rpm for 5 min, and the protein concentration was determined with a commercial BioRad kit.

Electrophoretic Mobility Shift Assays

A ^{32}P-labelled TRE oligo (5′-GATCCATCTGCGTCAGCAGGTTTG-3′) corresponding to the c-fos AP-1 binding site was used as a probe. Labelled oligo (0.2 ng) and 10 μgr of nuclear extract were incubated in a final volume of 20 μl (in 40 mM Hepes-KOH (pH 7.9), 0.5 mM DTT, 0.2 mM EDTA, 50 mM KCl, 3 μg poli(dI-dC), and 5% (v/v) glycerol and separated in a 5% polyacrylamide (TBE) gel. Specificity for AP-1 binding was confirmed by competition with a 100-fold excess of nonlabelled TRE or SRE oligos (5′-AGGATGTCCATATTAGGACATCT-3′) (indicated as TRE or SRE).

RESULTS

Transactivation of the C-Fos Promoter Is Induced by the *PMA-1* Gene

We determine if the transient expression of the *PMA1* gene was able to trigger activation of the c-fos promoter in NIH3T3 cells. The pSV2 expression vector carrying the wild type yeast ATPase gene *PMA1* was transfected [Perona and Serrano, 1988] into NIH3T3 cells together with the pFC4 plasmid containing the c-fos promoter [Deschamps et al., 1985]. As a negative control, the pma1-213 mutant that encodes an ATPase gene with a Glu233-Gln point mutation that expresses an enzyme with 10% the wild type activity [Perona et al., 1990] was also cloned in pSV2 and cotransfected with pFC4. As shown in Figure 1A, the *PMA1* gene stimulates elevenfold c-fos promoter activity when cells are incubated in low serum as compared with the transactivation produced by the pSV_2neo plasmid. The pma1-213 gene induces 2.6-fold the c-fos promoter, indicating a good correlation between transactivation and enzymatic activity of the proton pump. The stimulation obtained with the *PMA1* gene was comparable to that detected when the promoter was stimulated with 20% serum (eightfold) in three different experiments. These results suggest that the activity of the proton pump is sufficient to induce c-fos transactivation.

Altered Response of the C-Fos Promoter in *PMA1* Transfected NIH3T3 Cells

Different regulatory elements have been identified in the c-fos promoter that respond to specific mitogens. The SRE has been shown to mediate induction of the c-fos gene by serum, growth factors (such as platelet-derived growth factor or epidermal growth factor), insulin, TPA, and UV light [Sassone-Corsi et al., 1988]. In addition, the c-fos promoter contains several CREs that confere responsiveness of the gene to cAMP [Sheng et al., 1988]. We investigated if the cells transfected with the *PMA1* gene showed any alteration in the response to serum, TPA, and forskolin. Figure 1B shows the response of pFC4 activation to the inducers mentioned above. For this study we used RN1a and N-213 cells expressing, respectively, the *PMA1* and the pma1-213 allele. N-213 cells do not have a higher pHin than the parental NIH3T3 cells and are not tumorigenic. Quiescent NIH3T3 and N-213 cells show a low level of transactivation of the

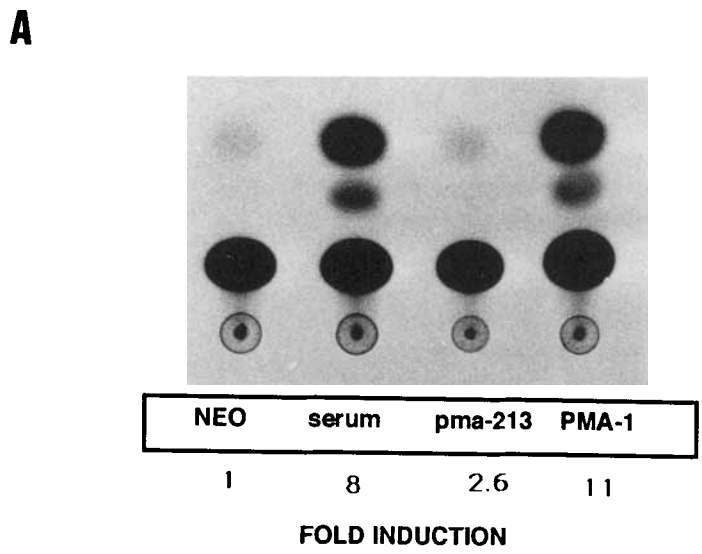


Fig. 1. Transactivation of pFC4 by the yeast H^+-ATPase gene. **A:** The *PMA1* gene transiently expressed transactivates pFC4. NIH3T3 cells were cotransfected with the pFC4 plasmid and either the *PMA1,* the pma1-213, or the pSV2neo plasmid. *Serum* indicates cells transfected with the pSV2neo plasmid (NEO) and stimulated with 20% FCS. **B:** Transfection of pFC4 in stable cell lines expressing the wild type (RN1a) or the pma1-213 (N-213) mutant ATPase genes in control NIH3T3 cells. Thirty-two hours after transfection the cells were either nontreated (quiescent) or induced for 6 h with the following agents: FCS (20%), TPA (200 nm), or forskolin (10 μM). The data shown were obtained in a representative experiment, but essentially the same results were obtained in three independent experiments with less than 10% variation between experiments.

c-fos promoter, while RN1a cells are able to constitutively activate the pFC4 sevenfold higher than the parental cells. These results are very similar to those obtained using transient assays on NIH3T3 cells (Fig. 1A). In relation with the inducibility of the c-fos promoter, while NIH3T3 and N-213 cells are responsive to serum and TPA, (six- and fivefold induction, respectively), RN1a cells show a very low response to serum (1.2-fold) and TPA (1.3-fold). When forskolin was used, the three cell lines showed quite similar responses. The results presented above suggest that the transcription factors involved in the transactivation of the SRE are already in-

duced in the absence of serum in RN1a cells and can only be induced slightly by serum and phorbol esters. On the other hand the factors involved in CRE transactivation are not constitutively induced in the absence of stimulation.

Activity of the *PMA1* Gene Induces Transactivation of the SRE and TRE

The transfection of the *PMA1* gene is sufficient to activate the c-fos promoter (Fig. 1A). In order to study the signal transduction pathway activated by the *PMA1* gene, we analyzed defined cis-acting elements of the c-fos promoter and their responsiveness to expression of the yeast proton pump. To do these experiments we used reporter plasmids containing four tandem repeats of the SRE or CRE element fused to a herpes simplex thymidine kinase-CAT gene construct [Schönthal et al., 1991]. NIH3T3 cells were transiently cotransfected with the reporter plasmids and either the *PMA1* or pma1-213 expression plasmids. As shown in Figure 2, the *PMA1* gene is able to induce activation of the SRE (fivefold), while serum was able to induce this element thirtyfold. This situation is similar to the one described for oncogenes such as abl, src, or raf [Angel and Karin, 1991; Hori et al., 1990; Kaibuchi et al., 1989]. On the contrary, the CRE is not transactivated by the *PMA1* gene, while forskolin was able to induce the activity of this promoter element. The responses to cotransfection with the pma1-213 plasmid are similar to those obtained with pSV_2neo. A construct that contained four copies of the gene of the human collagenase TRE (4XTRE) was induced by the expression of the *PMA1* gene (Fig. 2). This indicates that activation of transcription of c-fos might result in active Fos/Jun (AP-1) complexes.

In order to compare if the activation of the c-fos promoter induced in NIH3T3 cells by the *PMA1* gene was similar to the one observed in stable transfectants, we performed experiments in RN1a and N-213 cells. The activity of the SRE was constitutively high only in the RN1a cells. The CRE was not activated in any cell line (Fig. 3). We also detected constitutive activation of the TRE in the RN1a cells, indicating the presence of transcriptionally active AP-1 complexes. These results are in complete agreement with those obtained in NIH3T3 cells cotransfected with the *PMA1* gene and the reporter plasmids. The results presented above indicate that the change in pHin induced by the yeast proton pump was able to activate signal transduction

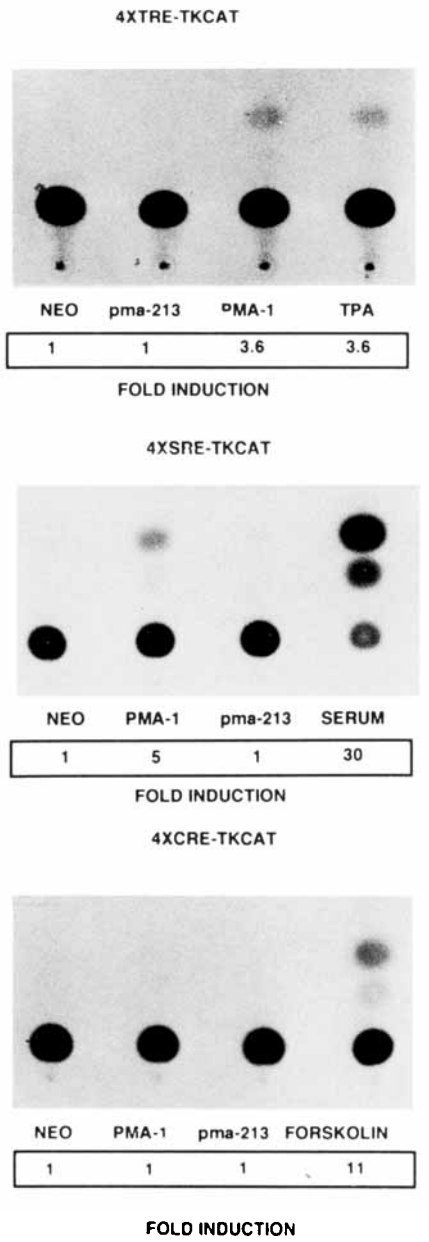


Fig. 2. Transactivation of single promoter elements by the *PMA1* gene. NIH3T3 cells were seeded and transfected with reporter plasmids containing four tandem repeats of the SRE, CRE, or TRE regulatory elements linked to the minimal thymidine kinase gene promoter and the CAT reporter gene and the *PMA1*, pma-213, or pSV2neo expression vectors. Some of the cells transfected with pSV2neo were induced with serum (4XSRE-TKCAT reporter), forskolin (4XCRE-TKCAT reporter), or TPA (4XTRE-TKCAT reporter) under the same conditions described in the legend of Fig. 1. The data shown were obtained in a representative experiment, but essentially the same results with less than 10% variation between each were obtained in three independent experiments.

pathways commonly activated by growth factors and oncogenes and that transactivation of c-fos promoter should be an intermediary step in the transformation induced by the *PMA1* gene.

Expression of *PMA1* induces AP-1 Activity

Transactivation of the TRE element has been shown to be mediated by AP-1 complexes [Franza et al., 1988]. To determine if the AP-1 transcription factor was involved in the transactivation of the TRE element in RN1a cells, we performed band shift assays on RN1a cells and NIH3T3 cells using an oligonucleotide containing the AP-1 site of the c-fos promoter. We observed low levels of complex formation on the TRE site when quiescent NIH3T3 cells were used (Fig. 4A). A stronger retarded band appeared after treatment of the cells with TPA. This band was efficiently compited with an excess of cold TRE oligo but not with an oligo containing the SRE. RN1a cells constitutively show similar levels of the retarded band as NIH3T3 cells induced with TPA. The levels of this band do not increase any further after treatment of the cells with TPA (data not shown).

The expression of the *PMA1* gene is sufficient to induce the formation of AP-1 complexes in quiescent NIH3T3 cells. We performed transient transfection followed by band shift assays using an oligonucleotide containing the AP-1 site of the c-fos promoter. The cells were transfected with increasing amounts of the *PMA1* expression vector pSV_hAT_5 and nuclear extracts prepared and tested for AP-1 activity by band shift assays. The plasmid pSV2neo was used as a negative control and to complete the amount of DNA used in each transfection up to 20 μg. The formation of the complex was dependent on the amount of *PMA1* gene used to transfect the cells (Fig. 4B). A maximal amount of complex was obtained with 10 μg of *PMA1*. A complex with similar movility was obtained when the cells were stimulated with serum (Fig. 4B) or TPA (not shown). The results shown above suggest that *PMA1* expression induces AP-1 transcription factor activity. Further support of this hypothesis was obtained by manipulating the expression of one of the AP-1 components, c-fos, in these cells. NIH3T3 or RN1a cells were transfected with either pSVfos (expressing fos) or pSVsof (expressing an antisense of fos) vectors [Guo and Cole, 1989] together with the TRE reporter construct. As previously described, TRE-mediated transcriptional activity increases sevenfold upon transfection of NIH3T3 cells with pSVfos (Fig. 5) [Schönthal et al., 1988]. This activity was abolished when the fos antisense vector was cotransfected together with pSVfos. In the case of RN1a cells, the constitutive TRE-mediated transcriptional activity was

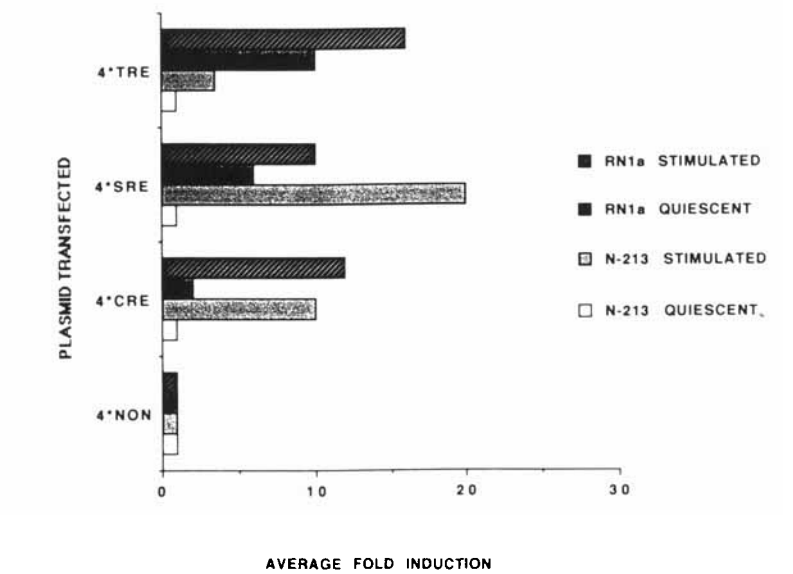


Fig. 3. Transfection of stable cell lines expressing the proton pump genes with single promoter elements. NIH3T3, RN1a, or N-213 cells were seeded and transfected with reporter plasmids 4XTRETK-CAT and 4XSRETK-CAT or 4XCRETK-CAT. Transfected cells were either noninduced (quiescent) or induced with TPA (4XTRE transfected cells), serum (4XSRE transfected cells), or forskolin (4XCRE transfected cells) as described in the legend of Fig. 1. Essentially the same results were obtained in three independent experiments with less than 10% variation between each experiment.

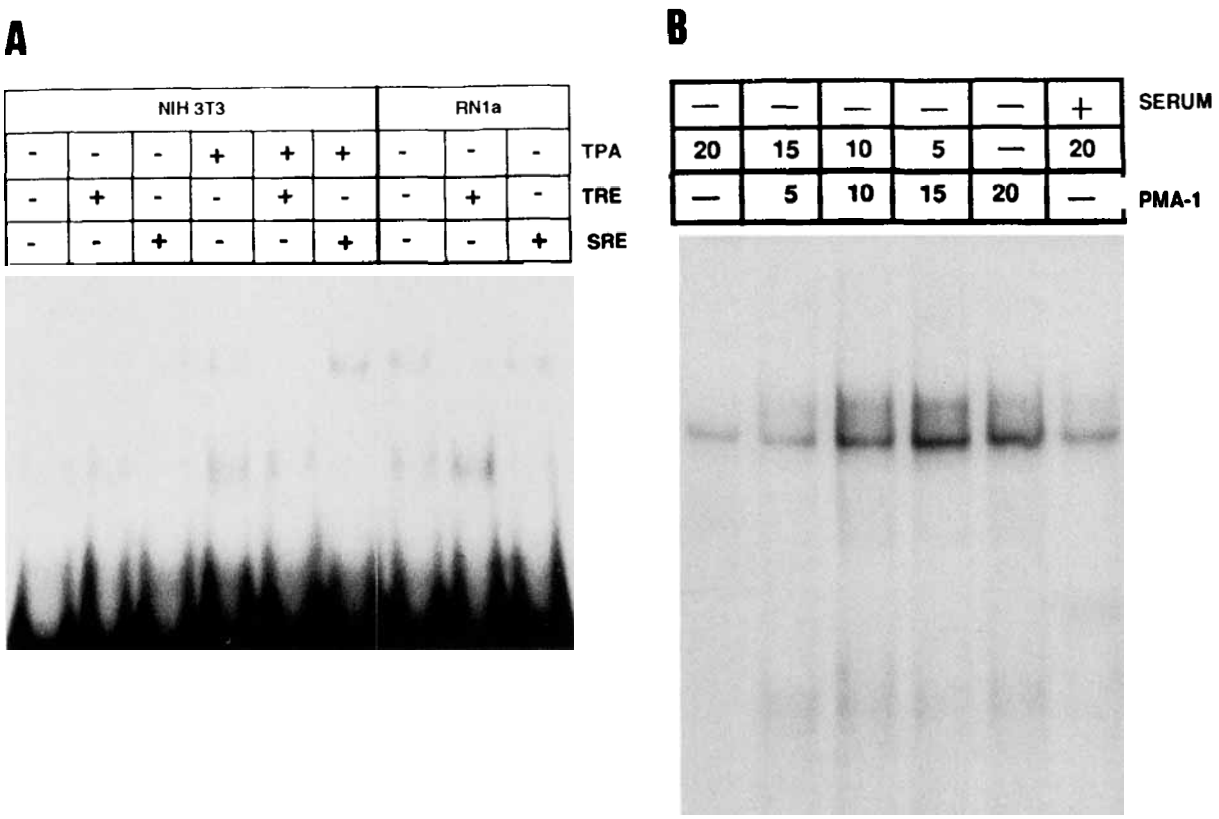
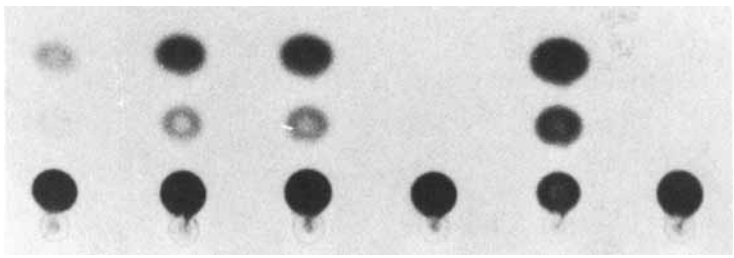


Fig. 4. Expression of the *PMA1* gene induces AP-1 complex formation. **A:** Band shift assay of extracts from cell lines expressing the *PMA1* gene with the AP-1 binding site. NIH3T3 and RN1a cells were serum-starved for 48 h. Half of the 3T3 plates were stimulated with 200 nM TPA, and nuclear extracts were prepared as described [Hori et al., 1990]. A ^{32}P-labelled TRE oligo corresponding to the c-fos AP-1 binding site was used as a probe. Competitors are nonlabelled TRE or SRE (100-fold excess) oligos indicated as TRE or SRE. **B:** Analysis of AP-1 complex formation in transient transfection assays. NIH3T3 cells were transfected with the indicated amounts of the *PMA1* expression (PSV_hAT_5) plasmid or the control plasmid pSV_2neo (NEO) and serum-starved for 48 h. Cells transfected with pSV_2neo alone were induced with 20% FCS for 1 h (SERUM). Essentially the same results were obtained in three independent experiments.



NIH	RN1a	NIH	NIH	RN1a	RN1a	CELL LINE
-	-	pSVfos	pSVfos pSVsof	pSVfos	pSVsof	CO-PLASMID
1	5	7	<1	30	<1	FOLD INDUCTION

Fig. 5. Antisense fos RNA inhibits CAT expression from the 4XTRETK-CAT reporter construct. NIH3T3 and RN1a cells were transfected with 5 μg of the reporter construct 4XTRETK-CAT and 10 μg of either pSVfos and/or pSVsof plasmids (CO-PLASMID). After transfection, cells were serum-starved for 48 h, and protein extracts were prepared and tested for CAT activity as described above. Fold induction was calculated over the CAT activity of untreated NIH3T3 cells. Essentially the same results, with less than 10% variation, were obtained in three independent experiments.

fivefold higher than that of the control cells, as described above (Fig. 3). This activity increased dramatically upon transfection of the pSVfos plasmid, suggesting that the amount of fos is limiting in these cells and that other components of the AP-1 complex, probably one of the members of the Jun family [Angel and Karin, 1991], might also be overexpressed. The constitutive transcriptional activity of RN1a cells is abolished upon pSVsof transfection, indicating that the fos protein is one of the components of the complex whose basal activity is high in RN1a cells. These results were confirmed using band shift assays. NIH3T3 cells transiently transfected with the *PMA1* gene (Fig. 6) show high levels of the AP-1 complex that decrease when cells are cotransfected with the pSVsof plasmid. We are not able to compete totally AP-1 binding, maybe because c-fos expression is not totally abolished by the pSVsof plasmid in the conditions used in the experiment (data not shown). These results strongly support the hypothesis that Fos protein is one of the members of AP-1 complexes induced by the *PMA1* gene.

Increase in AP-1 Activity Is Independent of Protein Kinase C (PKC) and A (PKA)

It has been shown that the TRE can be stimulated by PKC-dependent and PKC-independent pathways [Hori et al., 1990; Kaibuchi et al., 1989]. Depletion of PKC activity in RN1a cells by TPA pretreatment [Kaibuchi et al., 1990] causes only a small decrease in the constitutive transactivation of the TRE element (Fig. 7A). On the other hand, staurosporine, an inhibitor of PKC, does not have any effect on the basal transactivation observed on the SRE in RN1a cells (data not shown). Even more, no change was observed in the constitutive levels of AP-1 complex in RN1a cells after pretreatment of the cells with TPA (not shown) or with the inhibitor H-7 (Fig. 7B), an inhibitor of PKA and PKC [Hidaka et al., 1991]. On the contrary, when NIH3T3 cells where stimulated either with forskolin or TPA, a decrease in the level of AP-1 complex was observed in the presence of the inhibitor. These results strongly suggest that the *PMA1* gene activates AP-1 through a PKC- and PKA-independent signal transduction pathway.

DISCUSSION

The role of internal pH in proliferation and transformation has been elusive for a long time.

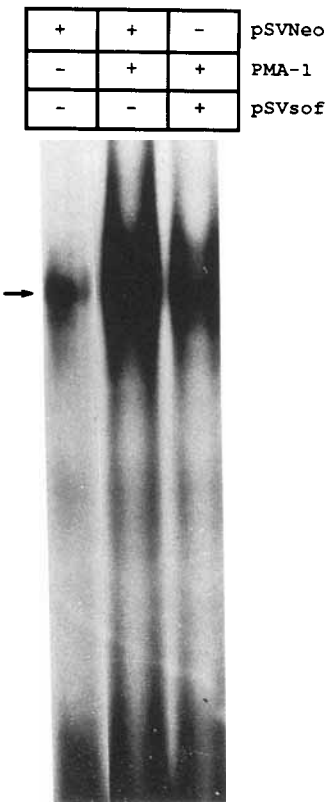


Fig. 6. Inhibition of AP-1 complex formation induced by the *PMA1* gene by fos antisense RNA. NIH3T3 cells were transiently transfected with 20 μg of pSV2neo, 10 μg of *PMA1* and 10 μg pSV2neo, or 10 μg of *PMA1* plus 10 μg of pSVsof. Cells were serum-starved and nuclear extracts prepared and incubated with an oligonucleotide containing the AP-1 binding site. The arrow shows the possition of the AP-1 complex. Essentially the same results were obtained in three independent experiments.

We have demonstrated that NIH3T3 cells expressing a yeast proton pump have constitutively higher pHin than control cells [Perona and Serrano, 1988]. Increased pHin correlates in these cells with tumorigenicity and serum independence [Perona et al., 1990]. Experiments described in this paper show that transfection of NIH3T3 cells with the *PMA1* gene produces changes in gene expression. In particular, we have studied the c-fos promoter.

Since c-fos plays an important role in the cells' proliferative responses and is one of the earliest genes induced after cell activation by growth factors, we have tried to examine the mechanisms underlying its transcriptional activation in *PMA1*-transfected cells. The expression of the *PMA1* gene is able to induce the c-fos promoter. The magnitude of the transactivation produced is similar to the one produced by serum. Furthermore, the proton pump activity of the yeast enzyme is necessary for the physiological alter-

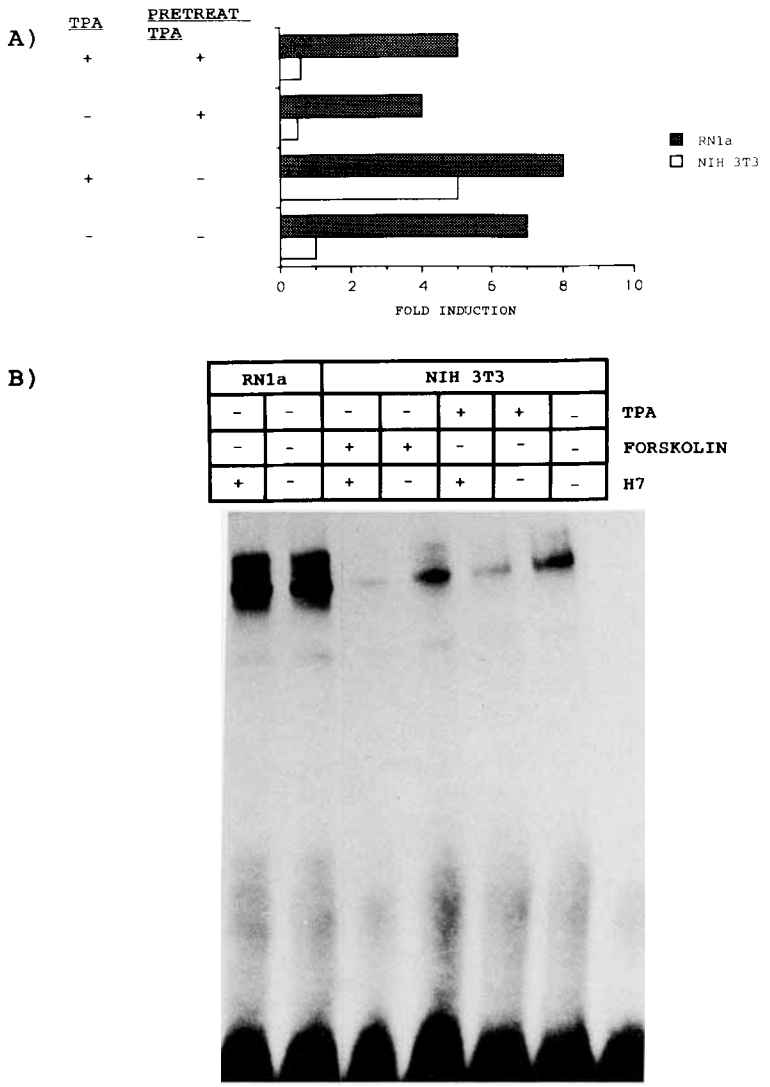


Fig. 7. Transactivation of c-fos by *PMA1* is independent of PKC and PKA activities. **A:** NIH3T3 and RN1a cells were transfected with the 4XTRETK-CAT reporter plasmid. Cells were incubated in the presence of 0.5% FCS. Downregulation of PKC was achieved by treatment with TPA (200 nM) for 48 h. When indicated, the cells were incubated with 100 nm TPA for 6 h after 48 h in the presence of TPA or 0.5% FCS. The CAT activity of the extracts was determined and the fold induction over NIH3T3 untreated cells calculated. **B:** NIH3T3 and RN1a cells were serum-depleted for 48 h and treated as follows: TPA: 100 nM TPA for 1 h; forskolin: 10 μM for 1 h; and H-7: 10 μM for the last 2 h. Nuclear extracts were prepared and incubated with a ^{32}P-labelled oligo corresponding to the c-fos AP-1 binding site for the band shift assay.

ations of the cell lines [Perona et al., 1990] and transactivating activity, because a site-directed mutant pma1-213, whose proton pumping activity is decreased to 10%, shows very weak activity transactivating the c-fos promoter. As a consequence of the increased expression of c-fos, the *PMA1* gene induces the formation of transcriptionally active AP-1 complexes that could be involved in the transformation of the transfected cells.

The transactivation of the c-fos promoter induced by the *PMA1* gene seems to be mediated by the SRE. The SRE can be activated by growth factors in a manner dependent and independent of protein kinase C. Our results suggest that the expression of the *PMA1* gene activates a signal transduction pathway that induces transactivation of the c-fos promoter through the SRE independently of protein kinase C activity. The induction of the SRE can be mediated by binding of the phosphorylated p62^{tcf} to a p67^{SRF} bound to the SRE [Ryan et al., 1989]. MAP-2 kinases have been shown to phosphorylate the p62^{tcf} [Gille et al., 1992]. The role of MAP-2

kinases in the *PMA1* signal transduction pathway is currently being investigated.

We think that these results show for the first time that there is a link between the elevation of intracellular pH, a common signal transduction event observed in normal cell proliferation [Burns and Rozengurt, 1993] and transformation [Doppler et al., 1987; Hagag et al., 1987], and the activation of gene transcription. In agreement with our results, Prpic et al. [1989] have demonstrated that physiological doses of IFN-γ induce a rapid Na^+/H^+ exchange in murine macrophages and that these amiloride-sensitive fluxes are important for mediating some subsequent genomic responses to IFN-γ. There are several enzymes that can serve as targets for activation by an increase in pHin because their activity is regulated by pH changes within the physiological range. Examples are Phospholipase C [Murase et al., 1988], Phospholipase A2 [Balsinde et al., 1988], and tyrosine kinases [Wanping et al., 1991]. Evidence has been presented in the literature of activation of tyrosine kinases by elevation of pHin during sea urchin fertilization [Wanping et al., 1991]. Physiologic changes in pH regulate IP3 binding to its receptor with a sharp increase in binding throughout the physiologic range, tripling between pH 7.5 and 8.5 [Worley et al., 1987]. Increases in pHin augment IP3's ability to release Ca^{++} [Joseph et al., 1989] which presumably reflects the influence of pH in IP3 binding to its receptor. In the case of RN1a cells, we still do not know the mechanism that transduces the signal of elevation of pHin to the nucleus, but we are checking the possibilities mentioned above. Another way in which pH changes can be transduced into changes in gene expression is by affecting the chromatin aggregation state. In relation with this, it has been reported that, at least in vitro, changes of pH within the physiological range are important in regulating the chromatin aggregation [Guo et al., 1989]. As the pH is increased less and less, chromatin is aggregated, and also histone dissociation from the chromatin is pH dependent. Interestingly, His-Pro-rich (HX)n repeats have been found to be protein sequences sensitives to pH changes in the physiological range [Janknecht et al., 1991]. These sequences, first observed in PRD domain of the *Drosophila* paired protein, have been found in other proteins such as bicoid [Frigerio et al., 1986], deformed [Sasaki et al., 1990], old skipped [Angiolillo et al., 1985], Chrox 1.4 [Frigerio et al., 1986], and the rabbit T-cell receptor β-chain precursor [Coulter et al., 1990]. It has been suggested that (HX) repeats mediate in pH-dependent fashion protein-protein interactions, which are known to be necessary for the function of transcription factors. Independently of the way in which the transactivation of c-fos by the *PMA1* gene is triggered, the observation that a protein involved in the cellular responses to several proliferative stimuli, such as Fos, is also implicated in cell transformation by *PMA1* could be important and opens a way to the study of these regulatory pathways.

ACKNOWLEDGMENTS

This work was supported by a grant from the Fundación Ramón Areces and the Comunidad de Madrid. J.R.M. is a fellow of the Gobierno Vasco. We gratefully acknowledge Juan Carlos Lacal for support and critical reading of this manuscript. We also thank to James Feramisco, Inder Verma for the c-fos constructs, and Ana Aranda, Leandro Sastre, and Ramón Serrano for helpful discussions.

REFERENCES

Andrews NC, Faller D (1991): A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499.

Angel P, Karin M (1991): The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129–157.

Angiolillo AL, Lamoyi E, Bernstein KE, Mage RG (1985): Identification of genes for the constant region of rabbit T-cell receptor β chains. Proc Natl Acad Sci USA 82:4498–4502.

Balsinde J, Diez E, Schüller A, Mollinedo F (1988): Phospholipase A2 activity in resting and activated human neutrophils. J Biol Chem 263:1929–1936.

Burns CP, Rozengurt E (1983): Serum, platelet-derived growth factor, vassopressin and phorbol esters increase intracellular pH in Swiss 3T3 cells. Biochem Biophys Res Commun 116:931–938.

Chen C, Okayama H (1987): High-efficiency transformation of mammalians cells by plasmid DNA. Mol Cell Biol 7:2745–2752.

Chiu R, Boyle WJ, Meek J, Hunter T, Karin M (1988): The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54:541–542.

Coulter D, Swaykus EA, Beran-Koehn MA, Goldberg D, Wieschaus E, Schedl P (1990): Molecular analysis of odd-skipped, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. EMBO J 8:3795–3804.

Deschamps J, Meijlink F, Verma IM (1985): Identification of transcriptional enhancer element upstream from the proto-oncogene fos. Science 230:1174–1177.

Doppler W, Jaggi R, Groner B (1987): Induction of v-mos and activated Ha-ras oncogene expression in quiescent NIH 3T3 cells causes intracellular alkalinization and cell-cycle progression. Gene 54:147–153.

Fisch TB, Prywes R, Roeder RG (1989): An AP-1 binding site in the c-fos gene can mediate induction by epidermal growth factor and 12-O-tetradecanoyl-phorbol-13-acetate. Mol Cell Biol 9:1327–1331.

Franza BR, Rauscher FJ III Jr, Curran T (1988): The fos complex and fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science 239:1150–1153.

Frigerio G, Burri M, Bopp D, Baumgartner S, Noll M (1986): Structure of the segmentation gene paired and the Drosophila PRD gene set as part of a gene network. Cell 47:735–746.

Gille H, Shamrrocks A, Shaw P (1992): Phosphorylation of p62tcf by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature 358:414–417.

Gillies RJ, Martinez-Zaguilan R, Martinez GM, Serrano R, Perona R (1990): Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions. Proc Natl Acad Sci USA 87:7414–7418.

Gorman CM, Moffat LF, Howard BH (1982): Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2:1044–1051.

Guo X, Cole RD (1989): Chromatin aggregation changes substantially as pH varies within the physiological range. J Biol Chem 264:11653–11657.

Hagag N, Lacal JC, Graber M, Aaronson S, Viola MV (1987): Microinjection of ras p21 induces a rapid rise in intracellular pH. Mol Cell Biol 7:1984–1988.

Hidaka H, Wanatabe M, Kobayashi R (1991): Properties and use of H-series of compounds as protein kinase inhibitors. Methods Enzymol 201:328–339.

Hori Y, Kaibuchi K, Fukumoto Y, Oku N, Takai Y (1990): Activation of the serum response element and TPA-response element by expression of the v-abl protein: Comparison of the mode of action of the v-abl protein with those of protein kinase C, cyclic AMP-dependent protein kinase, and the activated c-raf gene. Oncogene 5:1201–1206.

Janknecht R, Sander C, Pong O (1991): $(HX)_n$ repeats: A pH-controlled protein-protein interaction motif of eukaryotic transcription factors? FEBS Lett 295:1–2.

Joseph SK, Rice HL, Williamson JR (1989): The effect of axternal calcium and pH on inositol triphosphate-mediated calcium release from cerebellum microsomal fractions. Biochem J 258:261–265.

Kaibuchi K, Fukumoto Y, Oku N, Hori Y, Yamamoto T, Toyoshima K, Takai Y (1989): Activation of the serum response element and 12-O-tetradecanoylphorbol-13-acetate response element by the activated c-raf-1 protein in a manner independent of protein kinase C. J Biol Chem 264:20855–20858.

Kaibuchi K, Tsuda T, Kikuchi A, Tanimoto T, Yamashita T, Takai Y (1990): Possible involvement of protein kinase C and calcium ion in growth factor–induced expression of c-myc oncogene in Swiss 3T3 fibroblasts. J Biol Chem 261:1187–1192.

L'Allemain G, Franchi A, Cragoe E, Pouysségur J (1984): Blockade of the Na+/H+ antiport abolishes growth factor induced DNA suynthesis in fibroblasts. J Biol Chem 259: 4313–4319.

Müller R, Bravo R, Burckhardt J, Curran T (1984): Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature 312:716–720.

Murase S, Yamada K, Naito Y, Hirose J, Okuyama H (1988): Calcium requirement and its pH dependence of lysophospholipase C in porcine platelets membranes. J Biochem 104:270–275.

Nishikura K, Murray JM (1987): Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol Cell Biol 7:639–649.

Perona R, Serrano R (1988): Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. Nature 334:438–440.

Perona R, Portillo F, Giraldez F, Serrano R (1990): Transformation and pH homeostasis of fibroblasts expressing yeast H^+-ATPase containing site-directed mutations. Mol Cell Biol 10:4110–4115.

Pouysségur J (1985): The growth factor activable Na+/H+exchange system: A genetic approach. Trends Biochem Sci 10:453–455.

Pouysségur J, Sardet C, Franchi A, L'Allemain G, Paris S (1984): A specific mutation abolishing Na+/H+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. Proc Natl Acad Sci USA 81:4834–4837.

Prpic V, SheAn-Fung Y, Figuiredo F, Hollenbach PW, Gawdi G, Herman B, Uhing RJ, Adams DO (1989): Role of Na+/H+ exchange by interferon-γ in enhanced expression of JE and I-Ab genes. Science 244:469–471.

Rao GN, Lassegué B, Griendling KK, Alexander RW, Berk BC (1993): Hydrogen peroxide–induced c-fos expression is mediated by arachidonic acid release: Role of protein kinase C. Nucleic Acids Res 21:1259–1263.

Riabowol KT, Vosatka RJ, Ziff EB, Lamb NJ, Feramisco JR (1988): Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblasts cells. Mol Cell Biol 8:1670–1676.

Rozengurt E, Mendoza SA (1985): Synergistic signals in mitogenesis: Role of ion fluxes, cyclic nucleotides and protein kinase C in Swiss 3T3 cells. J Cell Sci Suppl 3:229–242.

Sasaki H, Yokoyama E, Kuroiwa A (1990): Specific DNA binding of the two chicken deformed family homeodomain proteins, Chox-1.4 and Chox-a. Nucleic Acids Res 18:1739–1744.

Sassone-Corsi P, Lamph WW, Verma IM (1988): Regulation of proto-oncogene fos: A paradigm for early response genes. Cold Spring Harbor Symp Quant Biol 53:749–760.

Schönthal A, Herrlich P, Rhambsdorf HJ, Ponta H (1988): Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 54:325–334.

Schönthal A, Sugarman J, Brown JH, Hanley MR, Feramisco JR (1991): Regulation of c-fos and c-jun protooncogene expression by the Ca^{2+}-ATPase inhibitor thapsigargin. Proc Natl Acad Sci USA 88:7096–7100.

Sheng M, Dougan ST, McFadden G, Greenberg ME (1980): Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4:451–587.

Voyno-Yasenetskaya T, Conkin BR, Gilbert RL, Hooley R, Bourne HR, Barber DL (1994): Gα13 stimulates Na-H exchange. J Biol Chem 269:4721–4724.

Wanping J, Veno PA, Wood PW, Peaucellier G, Kinsey WH (1991): pH regulation of an egg cortex tyrosine kinase. Dev Biol 146:81–88.

Worley PF, Baraban JM, Supattapone S, Wilson VS, Snider SH (1987): Characterization of inositol triphosphate receptor binding in brain. Regulation by pH and calcium. J Biol Chem 262:12132–12136.