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Regulation of Gene Expression in PC12 Cells via an Activator of Dual Second Messengers: Pituitary Adenylate Cyclase Activating Polypeptide

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In this study we demonstrate that the activator protein-1 (AP-1) DNA motif, initially considered to be unresponsive to cyclic AMP (cAMP), does function as a cAMP-response element in PC12 cells. A luciferase reporter gene driven by the collagenase promoter that contains the AP-1 motif is responsive to cAMP as well as phorbol esters when transfected in PC12 cells. We have recently shown that pituitary adenylate cyclase activating peptide (PACAP) has neurotrophic properties and activates both adenylylcyclase and the inositol lipid cascade in PC12 cells. Consistent with these actions, we demonstrate that PACAP is an effective activator of luciferase reporter genes whose promoters bear the AP-1 motif, as well as the related DNA element that binds the protein CREB. Both the cAMP and inositol lipid pathways appear to play a role in the activation of these motifs by PACAP. Mutation of the AP-1 motif and its juxtaposition to a heterologous promoter proves that the AP-1 motif is a locus for response to cAMP and PACAP. The luciferase reporter genes bearing the AP-1 motif are not cAMP responsive in HeLa tk^- cells, indicating that the mode of second-messenger responsiveness is cell-type specific.

INTRODUCTION

Many agents have been identified that cause pluripotential cells of the sympathoadrenal lineage, such as rat PC12 pheochromocytoma cells, to extend long processes termed neurites, characteristic of a neuronal phenotype (Greene and Tischler, 1976). Recently multiple neurotrophic agents have been identified that, like the historic prototype nerve growth factor (NGF),[1] activate tyrosine kinases of the trk family (Barbacid *et al.*, 1991). However, it is important not to overlook observations indicating that other signaling pathways also have activity in stimulating neurite outgrowth in PC12 cells, including the cyclic AMP (cAMP) and inositol lipid cascades (Schubert *et al.*, 1978; Gunning *et al.*, 1981; Richter-Landsberg and Jastorff, 1986). We have recently identified the 38-amino acid neuropeptide pituitary adenylate cyclase–activating polypeptide (PACAP38) as a potent stimulator of neurite outgrowth as well as both the inositol lipid and cAMP signaling pathways in PC12 cells (Deutsch and Sun, 1992). PACAP also exists in alternatively processed active form that is truncated after the N-terminal 27 amino acids. PACAP27 shares 70% amino acid identity (Miyata *et al.*, 1990) with the 28-amino acid peptide VIP (vasoactive intestinal peptide).

Long-term maintenance of the neuronal phenotype likely involves changes in gene expression. Among the many genes induced by neurotrophic agents like NGF are those encoding metalloproteinases, which may function in reorganizing extracellular matrix during neurite outgrowth (Machida *et al.*, 1989). The activator protein-1 (AP-1) DNA motif with consensus sequence 5′-TGA(G or C)TCA-3 is the major target of regulation of the expression of genes encoding the metalloproteinases stromelysin and interstitial collagenase (Angel *et al.*, 1987a) and is presumed to play a role in the activation of metalloproteinase gene expression by NGF (Machida *et al.*, 1989). NGF also activates gene expression in PC12 cells via the closely related motif that binds the cAMP-response element binding protein CREB, 5′-TGACGTCA-3 (Montminy *et al.*, 1986). Although NGF and PACAP stimulate different signaling cascades, their

[1] Abbreviations used: AP-1, activator protein-1; CPT-cAMP, chlorophenylthio cyclic AMP; Kinase A, cyclic AMP-dependent protein kinase; NGF, nerve growth factor; PACAP, pituitary adenylate cyclase activating peptide; TPA, tetradecanoyl phorbol acetate.

ultimately similar effects on neuronal phenotype (Deutsch and Sun, 1992) suggest that these different signaling events might converge at common intracellular events, such as activation of gene expression regulated by these two DNA motifs.

The AP-1 motif was originally characterized as conferring transcriptional stimulation in response to activators of kinase C (Angel *et al.*, 1987b; Lee *et al.*, 1987), and not cAMP (Imagawa *et al.*, 1987), and thus was designated as the tetradecanoyl phorbol acetate (TPA)-response element. In contrast the related CREB-binding motif was initially found to respond only to activators of cAMP-dependent protein kinase (kinase A) and thus termed the cAMP-response element (CRE) (Montminy *et al.*, 1986; Deutsch *et al.*, 1987). Subsequent work suggests that in fact more than one signal transduction system can activate each of these motifs, perhaps in cell-type specific modes of regulation. In PC12 cells, the CREB motif is activated by calmodulin-dependent kinases (Dash *et al.*, 1991; Ginty *et al.*, 1991; Sheng *et al.*, 1991) as well as by kinase A. In some cell types modest activation of the AP-1 motif in response to cAMP has been observed (Deutsch *et al.*, 1988; Hoeffler *et al.*, 1989). Given that the expression of the genes encoding both c-*fos* and c-*jun*, the best characterized components of AP-1, is markedly increased by cAMP in PC12 cells (Wu *et al.*, 1989), we hypothesized that PC12 would be a cell type in which cAMP responsiveness of the AP-1 motif would be substantial. In the current study we demonstrate that the AP-1 motif does in fact act as a cAMP-response element as well as a TPA-response element in PC12 cells. Consistent with the supra-additive effects of cAMP and phorbol esters on AP-1–mediated gene expression in PC12 cells, PACAP38 robustly stimulates the expression of reporter genes bearing the AP-1 motif in PC12 cells. We also demonstrate that the CREB-binding DNA motif is a PACAP38-responsive element in PC12 cells.

MATERIALS AND METHODS

Plasmid Constructions

A luciferase expression vector was engineered with a trimerized polyadenylation signal upstream of the luciferase coding sequence (de Wet *et al.*, 1987) to prevent transcription from occult initiation sequences in the vector. The trimerized SV40 polyadenylation signal was excised as a *Bgl*II-*Hin*dIII fragment from pUC.A.1.5. (Maxwell *et al.*, 1989), generously provided by Dr. I. Maxwell (University of Colorado Health Sciences Center, Denver, CO) and inserted into the *Bam*HI and *Hin*dIII sites of poLUC (Brasier *et al.*, 1989), a promoterless luciferase expression vector in a pGEM3 backbone, generously provided by Dr. A. Brazier (University of Texas, Galveston, TX). The resultant plasmid pZLuc (depicted schematically in Figure 1) contains a poly linker for insertion of promoter fragments between trimerized polyadenylation signal and the luciferase coding sequence and served as the starting material for subsequent constructions.

Col-Luc (Figure 1) was constructed by inserting a *Hin*dIII fragment of the human interstitial collagenase gene from −1.2 kB to +63 bp (excised from Col-CAT, generously provided by Drs. P. Angel and P. Herrlich, University of Karlsuhe, Karlsuhe, Germany) in pZLuc. All other reporter plasmids were constructed by the following general strategy employing the polymerase chain reaction (PCR). Desired wild-type promoter fragments were amplified by the use of pairs of primers whose 3′-termini contained ~15 bp that bracketed the promoter sequence of interest. The 5′-terminus of the upstream primer of each pair was designed with a *Bam*HI recognition sequence plus two additional bases to facilitate subsequent digestion; the 5′-terminus of the downstream (antisense) primer of the pair contained a *Kpn* I recognition sequence plus two additional bases. PCR was performed in an MJ Research (Watertown, MA) thermal cycler in a 100 μl volume containing 25 mM tris(hydroxymethyl)aminomethane (Tris), 37.5 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), 90 pmol of each primer, 200 μM of each dNTP, and ~10 pg of template DNA. Cycling parameters were denaturation at 94°C for 3 min, annealing at 50°C for −2 min, and extension at 72°C for 3 min for 30 cycles. Some PCR-amplified fragments were designed with a wild-type or mutated version of a specific motif by incorporating the desired DNA sequence at the 5′-terminus of the upstream primer. In all cases, the desired PCR product was resolved from primers via electrophoresis on 2% Biogel (BIO 101, La Jolla, CA) agarose gels, excised, and purified by adsorption to glass powder (Mermaid; BIO 101) that efficiently purifies fragments of <200 bp. After incubation with the large (Klenow) fragment of DNA polymerase to extend recessed 3′-ends, PCR products were again purified on 2% Biogel agarose electrophoresis, excised, and adsorbed to glass powder (BIO 101) followed by ligation into *Bam*HI-*Kpn* I–digested pZLuc. After screening transformants for insert by restriction digestion, accurate synthesis and incorporation of the desired PCR-amplified promoter fragment was confirmed by DNA sequencing via the dideoxynucleotide chain termination method with the use of the Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH). The sequencing primer employed (5′-GGCTGTCTTCCATTTTACCAACAGT-3′) was complementary to the proximal portion of the luciferase cDNA.

The specific primers and template employed in the PCR reactions that provided the promoter fragments for each plasmid construction are as follows. AP-1-Col-Luc employed Col-Luc as template and as upstream primer 5′-AAGGATCCAGCTTCATGAGTCAGACACC-3′ that includes the collagenase sequence from −74 to −63 in sense orientation. The downstream primer containing a *Kpn* I recognition sequence and the sequence from +50 to +63 in the collagenase gene in antisense orientation was 5′-TTGGTACCAGCTTCGGCC-TTTGTCTTCT-3′. Mut-AP-1-Col-Luc was constructed with AP-1-Col-Luc as template, the identical downstream primer, and a very similar upstream primer with the underlined three-base substitution in the AP-1 motif (5′-AAGGATCCAGCTTCATGCTCCAGACACC-3′). A reporter plasmid containing a heterologous minimal promoter, −53fos-Luc, was constructed with pFC700 (Fisch *et al.*, 1987), a plasmid containing the human c-*fos* promoter as template, an upstream primer containing human c-*fos* gene (Van Straaten *et al.*, 1983) sequences from −53 through −34 in sense orientation (5′-TTGGATCCACT-CATTCATAAAACGCTT-3′), and a downstream primer containing human c-*fos* gene sequences from +26 through +42 in antisense orientation (5′-GTGGTACCGCTCAGTCTTGGCTTCTCAG-3′). AP-1-fos-Luc was constructed with −53fos-Luc as template, the identical fos downstream primer, and an upstream primer that included an AP-1 sequence as well as fos sequences from −53 to −34: 5′-TTGGATCCAGCTTCATGAGTCAGACACCACTCATTCA-TAAAACGCTT-3′. The promoter fragment for Mut-AP-1-fos-Luc was amplified by PCR with AP-1-fos-Luc as template, the common downstream fos primer, and an upstream primer containing the underlined 3-bp substitution in the AP-1 element, as well as fos sequences from −53 to −34 (5′-TTGGATCCAGCTTCATG-CTCCAGACACCACTCATTCATAAAACGCTT-3). All of the above constructions are illustrated schematically in Figure 1. Additionally, CRE-fos-Luc was synthesized with AP-1-fos-Luc as template, the common downstream fos primer, and an upstream primer containing the CREB binding motif TGACAGTCA, as well as fos sequences from −53 to −41 (5′-TTGGATCCAGCTT-CATGACGTCAGACACCACTCATTCATAA-3′). All oligonucle-

otide primers were synthesized by Operon Technologies (Alameda, CA).

Cell Culture, Transfections, Luciferase Assays, and cAMP Radioimmunoassays

PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin, and 0.03% glutamine. HeLa tk^- cells (generously provided by Drs. P. Mitchell and R. Tjian, San Francisco, CA) were grown in the same medium supplemented with 10% fetal bovine serum. PC12 cells were transfected by the calcium phosphate precipitation method (Graham and van der Erb, 1973), transfecting monolayers with plasmid at 20–30 μg/ml, 24 h after seeding at 20 000 cells/cm^2. PC12 cells were subjected to hyperosmotic shock with 15% glycerol for 1 min after 20-h exposure to the calcium phosphate precipitate. The hyperosmotic shock was omitted for HeLa tk^- cells. After treatment with modulators, cells were lysed and luciferase activity assayed as previously described (Brasier *et al.*, 1989). Luciferase activity, measured as relative light units detected after 10 s, was determined in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA). As in previous studies (Deutsch *et al.*, 1988, 1990), we endeavored to maximize the reproducibility of transfection efficiency in a given experiment by plating cells in parallel and preparing calcium phosphate precipitates under comparable conditions to obviate the need for corrections for transfection efficiency. The reproducibility of data obtained from independent transfections, as reflected by small standard errors, validates that within individual experiments high reproducibility of transfection efficiency was achieved.

Intracellular cAMP levels were determined by radioimmunoassay as previously described (Deutsch and Sun, 1992).

Reagents

PACAP27 and PACAP38 were obtained from Peninsula (Belmont, CA). Chlorophenylthio cyclic AMP (CPT-cAMP) and TPA were obtained from Sigma (St. Louis, MO). NGF (2.5 S form) was obtained from Bioproducts for Science (Indianapolis, IN). All other chemicals were of the highest reagent grade available.

RESULTS

The AP-1 Motif-Containing Collagenase Promoter is cAMP Responsive in PC12 Cells

To allow clear identification of the second-messenger–responsive properties of the AP-1 motif, luciferase reporter genes engineered with wild-type and mutant AP-1 motifs were employed, resulting in orders of magnitude higher sensitivity than chloramphenicol acetyltransferase (CAT) reporters. The construction of several of these reporter plasmids is schematically illustrated in Figure 1; methodology is detailed in MATERIALS AND METHODS.

The gene encoding the interstitial fibroblast form of human collagenase contains an AP-1 motif at −72 to −66 in its promoter (Angel *et al.*, 1987a) and has been a paradigm for study of the AP-1 element. A fragment of the collagenase promoter from −1200 to +63 confers substantial phorbol ester responsiveness but not cAMP responsiveness on reporter genes transfected in HeLa tk^{-1} cells (Angel *et al.*, 1987a). We found that a luciferase reporter plasmid containing that same collagenase promoter fragment (Col-Luc) was also TPA responsive after transfection into PC12 cells (Figure 2), stimulated 14-fold by a maximal (100 nM) concentration of TPA. A closely related AP-1 motif–containing promoter fragment, that from the rat gene encoding the metalloproteinase stromelysin (also called transin), is NGF responsive in PC12 cells, presumably via the AP-1 motif (Machida *et al.*, 1989), and we predicted that the collagenase promoter would also confer NGF responsiveness. That prediction was borne out; luciferase activity directed by the Col-Luc plasmid transfected into PC12 was stimulated 34-fold by NGF (Figure 2). In view of the cAMP responsiveness of c-*jun* as well as c-*fos* mRNA in PC12, we hypothesized that the collagenase promoter would also confer cAMP responsiveness in PC12. Consistent with that prediction we found that Col-Luc, after transient transfection in PC12, was stimulated 33-fold by the cAMP analogue CPT-cAMP employed at a maximal dose of 0.5 mM, comparable in magnitude to the stimulation of Col-Luc by NGF (Figure 2). Thus, the transfected collagenase promoter represented one locus for convergence of NGF and cAMP on gene expression in PC12 cells.

The cAMP responsiveness of the collagenase promoter in PC12 cells suggested that its activity would also be stimulated by neuropeptides that activate adenylylcyclase. Vasoactive intestinal peptide (VIP) has been proposed to be an activator of adenylylcylase in sympathoadrenal cells like PC12, but the high concentrations ($\geq 10^{-6}$ M) required suggest that it is cross-reacting with the receptor for another related neuropeptide that stimulates PC12 cells at more physiological concentrations, which we and others propose is likely to be pituitary adenylate cyclase activating polypeptide (PACAP) (Watanabe *et al.*, 1990; Deutsch and Sun, 1992). Consistent with that view, PACAP38 at 10^{-8} M and VIP at 10^{-6} M but not VIP at 10^{-8} M stimulated luciferase activity in PC12 cells transiently transfected with Col-Luc (Figure 3). The magnitude of stimulation of Col-Luc activity by PACAP38 was comparable to the stimulation by a maximal dose of CPT-cAMP. This suggested that stimulation of Col-Luc activity by PACAP38 may not be mediated by cAMP alone, in view of the attenuation of cAMP levels after persistent stimulation with PACAP38 (Figure 9) and the general requirement for persistent elevation of cAMP levels for maximal stimulation of second-messenger–responsive gene expression (Deutsch *et al.*, 1990). The higher level of luciferase activity after Col-Luc-transfected PC12 are coincubated with 10^{-8} M PACAP38 and 0.5 mM CPT-cAMP versus incubation with 0.5 mM CPT-cAMP alone (Figure 4) is consistent with an additional mechanism of stimulation in response to PACAP38. Our previous studies provide evidence that treatment of PC12 cells with PACAP38 at 10^{-8} M stimulates the inositol lipid cascade in addition to elevating cAMP levels (Deutsch and Sun, 1992), and both pathways are likely contributing to the PACAP responsiveness of Col-Luc in PC12.

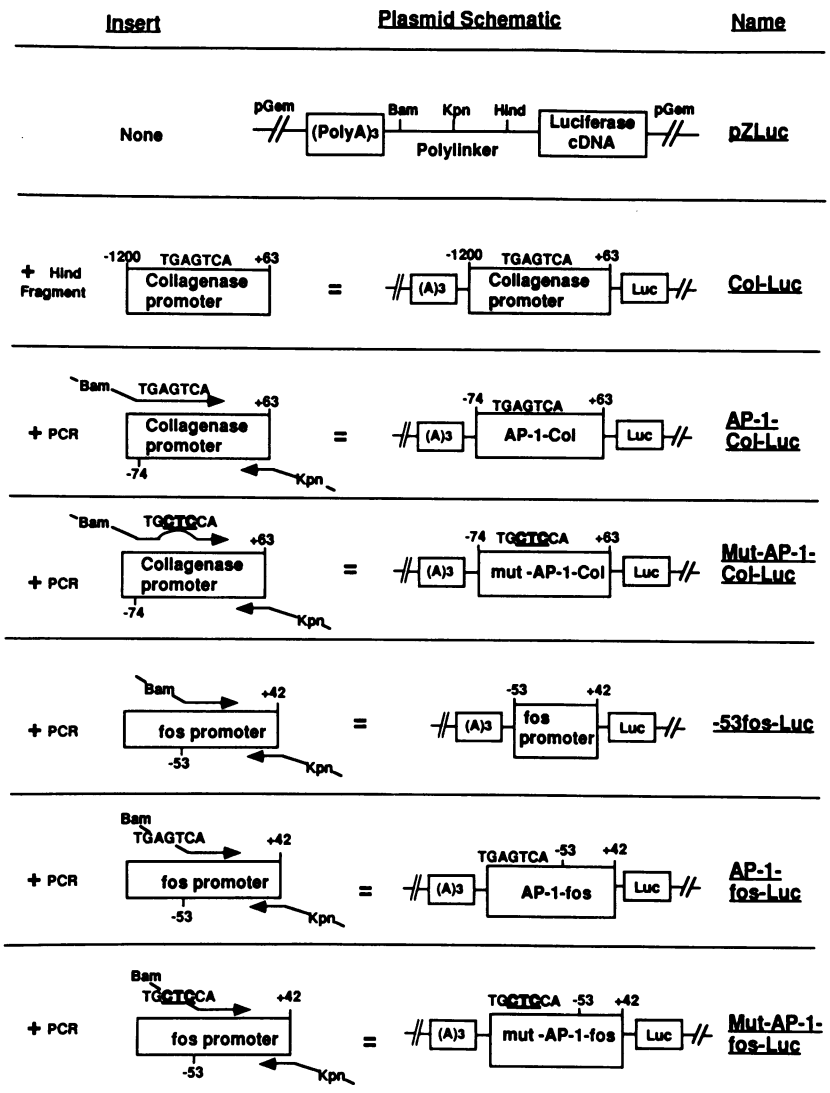

Figure 1. Schematic of luciferase reporter plasmids. Promoter fragments containing wild-type and mutated versions of specific motifs were synthesized by PCR and inserted into the vector designated pZLuc. Details of construction of each plasmid are provided in MATERIALS AND METHODS.

In additional experiments, stimulation of Col-Luc-transfected PC12 cells with maximal concentrations of both cAMP and TPA results in supra-additive effects compared with incubation in either agent alone.

The AP-1 Motif is the Major Locus of cAMP and PACAP Responsivity of the Collagenase Promoter

We presumed that the AP-1 motif was likely to be a major locus of the cAMP and PACAP responsivity of the collagenase promoter in PC12 cells, and we constructed other reporter genes to test this hypothesis explicitly. A collagenase promoter truncated just upstream of the AP-1 motif at −74 was synthesized with PCR, as was a parallel promoter fragment containing a 3-bp substitution in the middle of the AP-1 motif. These two promoter fragments were inserted into the pZLuc vector creating AP-1-Col-Luc and Mut-AP-1-Col-Luc, respectively (Figure 1). After transient transfection in PC12 cells, luciferase activity directed by AP-1-Col-Luc was substantially stimulated by both CPT-cAMP and PACAP38, and the extent of stimulation was markedly attenuated after transfection with the AP-1–mutated reporter gene (Figure 5), consistent with the conclusion that the AP-1 motif per se is responsive to the modulators. Comparable results were seen when transiently transfected PC12 cells were stimulated with modulators for 4.5 or 24 h (Figure 5). After 4 h of stimulation with PACAP38, elevation of cAMP levels is attenuated (Figure 9), leaving open the possibility that stimulation of gene expression by PACAP38 at 24 h could involve other signal transduction pathways than at early time points, but at both 4.5 and 24 h, stimulation of AP-1-Col-Luc by PACAP38 does appear to act predominantly via the AP-1 motif (Figure 5).

To provide more rigorous proof that the AP-1 motif is a locus for stimulation by cAMP analogues and

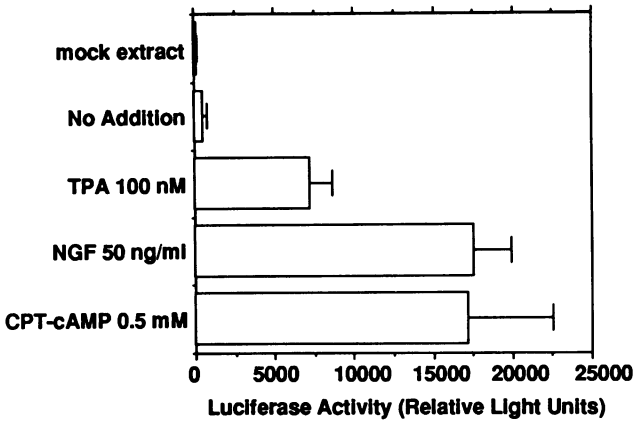

Figure 2. Responsiveness of the collagenase promoter to TPA, NGF, and CPT-cAMP in PC12 cells. Cells were transfected with Col-Luc or no plasmid for the mock extract control, and after stimulation with the indicated modulator for 24 h, harvested and assayed for luciferase activity as described under MATERIALS AND METHODS. For each condition, triplicate plates from individual transfection mixtures were transfected, and mean luciferase activity is depicted. Bars = ±SD.

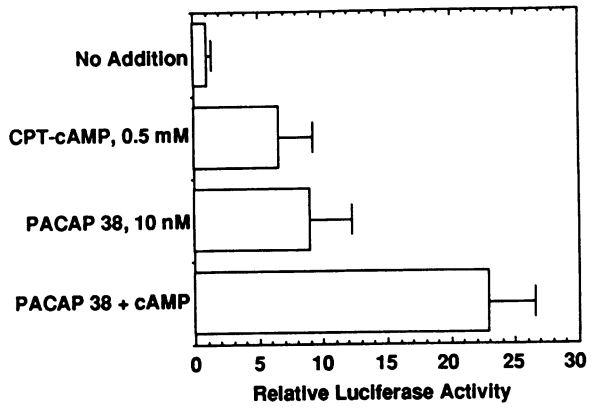

Figure 4. Synergism of PACAP38 and cAMP in activation of collagenase promoter in PC12 cells. Cells were transfected with Col-Luc as in MATERIALS AND METHODS, after incubation with the indicated additive for 24 h, relative luciferase activity determined as in the legend to Figure 3. PACAP38 + cAMP designates plates coincubated in 0.5 mM CPT-cAMP and 10 nM PACAP38.

PACAP38, we juxtaposed the AP-1 motif to another basal promoter. We employed a fragment of the human c-*fos* promoter from −53 to +42. This fragment has a TATA box but no other characterized regulatory motif (Fisch *et al.*, 1989), and indeed transfection of PC12 cells with −53fosLuc resulted in essentially no detectable basal or cAMP-stimulated luciferase activity compared with background (Figure 6A). In contrast, insertion of an AP-1 element upstream of the minimal fos promoter conferred significant cAMP-responsive luciferase activity after transfection into PC12 (Figure 6A), which was essentially absent after transfection with a parallel construction with a 3-bp substitution in the AP-1-motif, Mut-AP-1-fos-Luc (Figure 6A). Luciferase activity directed by AP-1-fos-Luc transfected into PC12 was also stimulated by PACAP38 (Figure 6B).

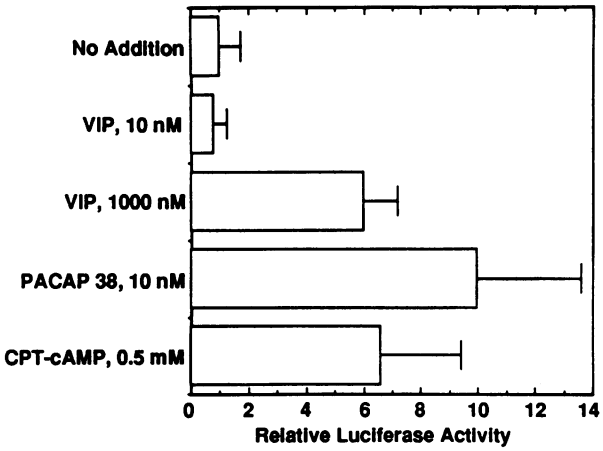

Figure 3. Responsiveness of the collagenase promoter to VIP and PACAP38 in PC12 cells. PC12 cells were transfected as described under MATERIALS AND METHODS. The indicated modulator was added for 4.5 h before harvest and determination of luciferase activity. Relative luciferase activity, normalized to the condition without addition of modulator, was calculated after subtraction of background activity in extracts from mock transfected cells. For each condition, triplicate plates from individual transfection mixtures were transfected, and mean luciferase activity is depicted. Bars = ±SD.

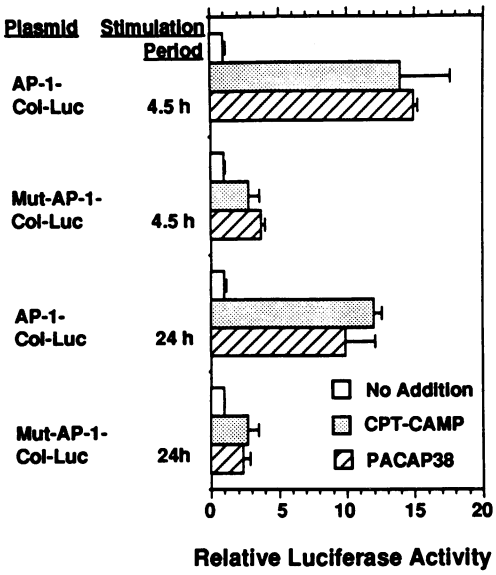

Figure 5. Mutation of the AP-1 motif abrogates cAMP and PACAP38 responsiveness. PC12 cells were transfected with either AP-1-Col-Luc or Mut-AP-1-Col-Luc, as described under MATERIALS AND METHODS. Parallel plates were harvested after incubation in the indicated modulator was added for 4.5 h or 24 h before harvest and determination of relative luciferase activity is the same as in the legend to Figure 3.

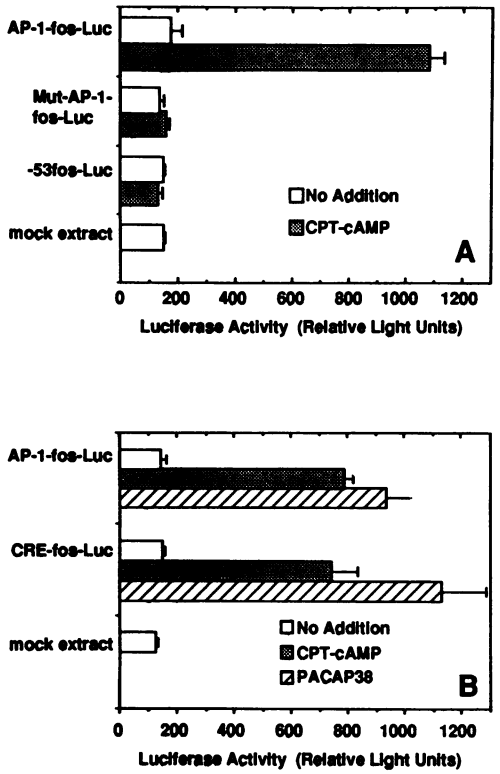

Figure 6. The AP-1 motif is cAMP and PACAP38 responsive when juxtaposed to a heterologous promoter. PC12 cells were transfected with the indicated plasmid and stimulated with modulator for either 4.5 h (A) or 24 h (B) before harvest and determination of luciferase activity, all as described in MATERIALS AND METHODS. To facilitate accurate comparison of the low levels of basal activity after transfection with the various plasmids, background activity (mock extract) was not subtracted but is depicted for reference. Luciferase activity is depicted the same as in the legend to Figure 2.

Second-Messenger Responsiveness of the AP-1 Motif is Cell-Type Dependent

These results indicating that the AP-1 motif is cAMP responsive in PC12 cells are at variance with the marked TPA responsivity and undetectable cAMP responsivity of the AP-1 motif–containing reporter plasmids transfected into other cells such as HeLa tk^- (Angel *et al.*, 1987a,b; Imagawa *et al.*, 1987). To prove that these differences are inherent to the cell type, rather than reflecting subtle differences in the reporter genes used in these studies, we transfected HeLa tk^- cells with Col-Luc and AP-1-fos-Luc. Luciferase activity directed by both plasmids in HeLa tk^- cells was markedly TPA responsive and insignificantly cAMP responsive (Figure 7), indicating that the mode of second-messenger responsiveness of the AP-1 motif is cell-type dependent. This difference in behavior of HeLa tk^- cells appears not to reflect a general deficiency in the cAMP cascade, because 0.5 mM CPT-cAMP did stimulate c-*fos* mRNA expression in HeLa tk^-.

The CRE Motif is PACAP Responsive in PC12 Cells

In addition to proving that the AP-1 motif, TGAGTCA, was PACAP responsive in PC12 cells, we wished to investigate the PACAP responsiveness of the closely related CREB-binding motif, TGACGTCA, in PC12 cells. A promoter fragment containing this consensus CRE sequence upstream of the minimal fos promoter was synthesized by PCR (MATERIALS AND METHODS) and inserted into the pZLuc vector. Luciferase activity directed by this CRE-fos-Luc plasmid transfected in PC12 cells was, as expected, cAMP responsive (Figures 6B and 8). The extent of stimulation by PACAP38 at 10^{-8} M exceeded the stimulation by a maximal concentrations of CPT-cAMP (Figures 6B and 8), suggestive of stimulation by PACAP38 via another second-messenger pathway in addition to cAMP. Others (Sheng *et al.*, 1990; Dash *et al.*, 1991) have shown that the CRE mediates transcriptional activation in response to membrane depolarization and calcium as well as cAMP in PC12 cells. Membrane depolarization, as induced for example by extracellular KCl, evokes calcium influx via voltage-gated calcium channels. Phosphorylation of CREB by a calcium- (and perhaps calmodulin-)dependent kinase appears to be the locus at which these signaling pathways converge in the activation of CRE-mediated transcription (Sheng *et al.*, 1990; Dash *et al.*, 1991). In our experimental paradigm, a depolarizing concentration of KCl does not result in detectable activation of luciferase activity directed by CRE-fos-Luc transfected into PC12 (Figure 8). However, coincubation of CRE-fos-Luc-transfected PC12 cells with KCl and a maximum dose of CPT-cAMP does result in 4-fold more

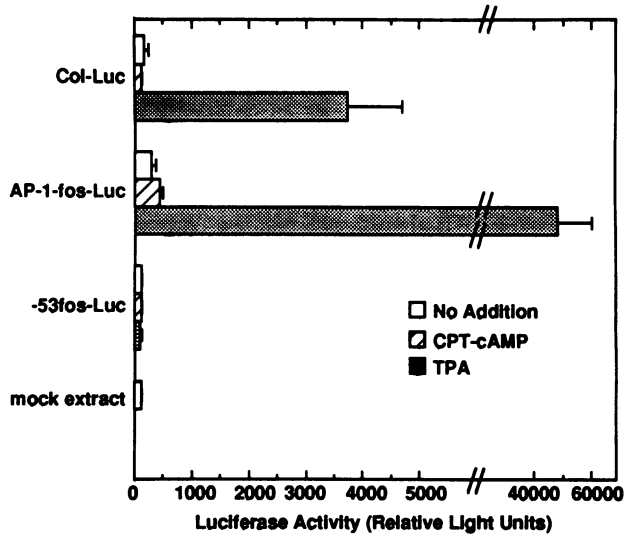

Figure 7. Second-messenger responsiveness of luciferase reporter plasmids transfected in HeLa tk^- cells. HeLa tk^- cells were transfected with the indicated plasmid as described in MATERIALS AND METHODS and stimulated with modulator for 16 h before harvest. Luciferase activity is depicted the same as in the legend to Figure 2.

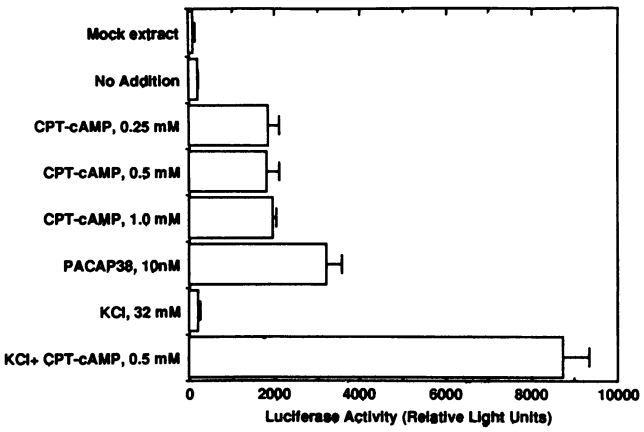

Figure 8. Responsiveness of the CRE motif in PC12 cells. Cells were transfected with CRE-fos-Luc as described under MATERIALS AND METHODS before stimulation with the indicated modulator for 24 h before harvest. KCl was added as a depolarizing agent at 32 mM in addition to the 5 mM in the formulation of Dulbecco's modified Eagle's medium. Luciferase activity is depicted the same as in the legend to Figure 2.

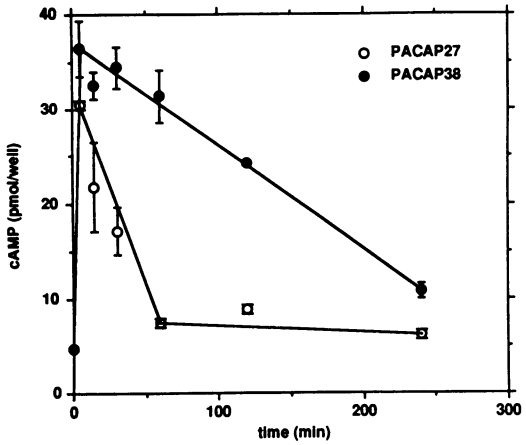

Figure 9. Attenuation of cAMP response during persistent stimulation with PACAP27 vs. PACAP38. PC12 cells plated at 0.5×10^6 cells per 9.6 cm^2 well 4 d previously were stimulated with PACAP27 at 10^{-8} M or PACAP38 at 10^{-8} M without phosphodiesterase inhibitor for the indicated time before lysis and determination of intracellular cAMP by radioimmunoassay as described (Deutsch and Sun, 1992). Data are expressed as picomole cAMP per well, depicted as the mean ± SD of samples from triplicate wells. At the day of stimulation, each well contained 1.9×10^6 cells.

luciferase activity than incubation with CPT-cAMP alone (Figure 8), providing evidence that concomitant activation of two signaling cascades that are activated by PACAP38 (Deutsch and Sun, 1992) can synergistically stimulate CRE-mediated reporter gene expression in PC12 cells.

PACAP38 and PACAP27 Differ in the Duration of Their Actions on PC12 Cells

Because the shorter 27-amino acid form of PACAP appears to be an authentic (Miyata *et al.*, 1990) biologic form, we have been interested in comparing its actions on PC12 cells with those of PACAP38. As measured by accumulation of inositol phosphate, PACAP38 is more potent than PACAP27 in stimulating inositol lipid turnover (Deutsch and Sun, 1992), but when added with a maximal dose of cAMP analogue, PACAP27, like PACAP38 (Figure 4), results in an augmentation of the stimulation of luciferase activity directed by the AP-1 motif, indicating that stimulation of dual cascades also plays a role in mediating effects on gene expression by PACAP27. Another difference between the action of PACAP27 versus PACAP38 on PC12 cells is the persistence of elevation of cAMP levels. The decline of cAMP levels after stimulation of PC12 cells with PACAP27 is much more rapid than after stimulation with PACAP38 (Figure 9).

PACAP27 is also much less potent than PACAP38 in inducing neuronal morphology in PC12 cells (Deutsch and Sun, 1992), an induction that proceeds after several days of stimulation with the neuropeptides. We investigated whether these differences in kinetic profiles of cAMP elevation after stimulation with the two peptides would be reflected in the kinetic profiles of activation of gene expression. In fact, after 4.5 h of stimulation of transfected PC12 cells, luciferase activity directed by the collagenase promoter was stimulated by both PACAP38 and PACAP27 (Figure 10), with only a modest difference, of questionable significance, between the two peptides. On the other hand, after 24 h of treatment, luciferase activity was four-fold higher in PACAP38- versus PACAP27-treated cells (Figure 10). This more sustained stimulation of gene expression by PACAP38 might have consequences for differences in

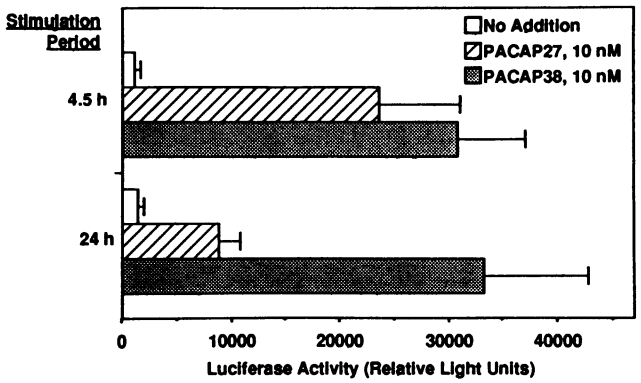

Figure 10. Persistence of stimulation after treatment of Col-Luc-transfected PC12 cells with PACAP27 vs. PACAP38. PC12 cells were transfected with Col-Luc, as described under MATERIALS AND METHODS. Parallel plates were harvested after incubation in the indicated modulator for 4.5 or 24 h before harvest and determination of luciferase activity is the same as in the legend to Figure 2.

the phenotype of cells treated with prolonged periods of time with PACAP38 as compared with treatment with PACAP27.

DISCUSSION

In this study we have demonstrated that in PC12 cells the AP-1 DNA regulatory motif is a locus for stimulation by cAMP as well as TPA. The AP-1 motif–containing collagenase promoter was cAMP responsive, and a 3-bp substitution in that motif greatly abrograted that responsiveness. In addition the wild-type, but not mutated, AP-1 motif conferred cAMP responsivity on a heterologous minimal promoter fragment in PC12. The AP-1 motif was initially characterized in HeLa tk^- cells as responsive to TPA but not cAMP (Imagawa *et al.*, 1987), and we confirmed these observations, indicating that the modes of second-messenger responsiveness of this motif are cell-type specific. Among the multiple mechanisms by which cAMP might conceivably activate transcription via the AP-1 motif, one of the simplest to envision is the activation by cAMP of the expression of the c-*fos* and c-*jun* proto-oncogene products. The corresponding heterodimeric Fos-Jun protein is the best characterized activator of transcription via the AP-1 motif (Bohmann *et al.*, 1987; Angel *et al.*, 1988a; Curran and Franza Jr., 1988; Kouzarides and Ziff, 1989). Expression of c-*fos* at the mRNA level is transiently activated by cAMP in a wide variety of cell types (Muller *et al.*, 1984; Greenberg *et al.*, 1985) but in many of those cells, does not suffice for activation of AP-1–bearing promoters. Activation of c-*jun* gene expression by cAMP has been documented in a much more restricted set of cell types. PC12 is one such cell line in which cAMP activates both c-*fos* (Muller *et al.*, 1984; Greenberg *et al.*, 1985) and c-*jun* mRNA expression (Wu *et al.*, 1989), whereas cAMP does not induce c-*jun* mRNA expression in HeLa tk^- cells (Angel *et al.*, 1988b). These observations support the model that the cell-specific mode of second-messenger responsiveness of c-*jun* expression may be a critical determinant of the mode of responsiveness of AP-1 activity.

In addition to PC12 cells, the AP-1 motif appears to be cAMP responsive in other cell types. In JEG-3 choriocarcinoma cells (Deutsch *et al.*, 1988; Hoeffler *et al.*, 1989), AP-1 motif–containing CAT reporter genes are responsive to activators of kinase A as well as phorbol esters, which activate kinase C, although the magnitude of cAMP responsiveness is less than that observed in the current study. Additionally, the AP-1 motif–containing promoter of the 422 (aP2) adipocyte specific gene is cAMP responsive in preadipocyte cell lines (Yang *et al.*, 1989), but the mechanism of responsiveness in that case appears to involve derepression of a 40-bp negative regulatory element that flanks the AP-1 motif, and the AP-1 motif itself is not sufficient for such cAMP responsiveness. The network of transcription factors and other regulatory molecules that participate in the transduction of second-messenger signals into changes in the activity of promoters containing AP-1 motifs is quite complex. Cell-specific differences in any of these factors could in theory underlie the cell specificity of cAMP responsivity of the AP-1 motif. For example, multiple other fos- and jun-related gene products may contribute to AP-1 activity, and several of these have been shown to be cAMP regulated in at least some cell types (Datta *et al.*, 1991; Kobierski *et al.*, 1991). Additionally, AP-1 activity has been shown to be regulated by an inhibitory protein designated IP-1, which itself is regulated by phosphorylation (Auwerx and Sassone-Corsi, 1991). Moreover, the ability of the specific CRE-binding protein, CREB, to bind to and activate the AP-1 motif may be modulated by other accessory factors (Muchardt *et al.*, 1990; Hoeffler *et al.*, 1991) whose expression may be cell specific. Certainly, multiple other mechanisms for conditional cAMP responsivity of the AP-1 motif might be operative, and the detailed elucidation of the mechanism of different regulatory modes in PC12 versus HeLa tk^- cells will require considerable analysis. Others have demonstrated that in PC12 cells, the CRE DNA motif, like AP-1, is activated by both the cAMP and inositol lipid cascades. Thus, ability of PACAP38 to stimulate both cascades in PC12 cells (Deutsch and Sun, 1992)likely contributes to its vigorous stimulation of both AP-1– and CRE—ediated gene expression in PC12.

The molecular mechanisms by which neurotrophic agents elicit neurite outgrowth in PC12 and other pluripotential neuronal cells is a subject of intensive investigation and may lead to the development of novel therapies for neurodegenerative disorders. Much recent work has focused on an expanding list of neurotrophic agents that act via the trk family of tyrosine kinases (Barbacid *et al.*, 1991), but agents like PACAP38 that act via G protein coupled receptors clearly have neurotrophic properties as well (Deutsch and Sun, 1992). These diverse signaling cascades may converge at several loci. The activation of the inositol lipid cascade via selective phosphorylation of phospholipase C-γ by the NGF receptor tyrosine kinase (Kim *et al.*, 1991) is one example of convergent signaling. The observation of PACAP activation of AP-1 activity in this report indicate that this more distal signaling event is another locus of convergence. Others have shown that NGF activates the AP-1 motif–containing transin promoter in PC12 cells (Machida *et al.*, 1989), and we found that NGF activates the collagenase promoter as well. The stimulation of expression of metalloproteinases like collagenase and stromelysin via the AP-1 motif by neurotrophic factors in developing neurons might play a role in the early phase of neurite outgrowth by clearing a path through extracellular matrix. The list of genes whose regulation may involve AP-1 is likely to be a large one, and the investigations of the current study merely em-

ploy collagenase as a model AP-1–containing promoter. Certainly the activity of PACAP38 in stimulating both AP-1– and CRE-mediated gene expression could result in the induction of a wide variety of distinct genes in PC12 cells. Other sequences with similarities to the consensus AP-1 motif and CRE appear to confer responsiveness to cAMP and phorbol esters in PC12 cells (Velcich and Ziff, 1990; Fink *et al.*, 1991), further expanding the number of potential PACAP-regulated genes in PC12 cells.

The existence of a naturally occurring (Miyata *et al.*, 1990), albeit less abundant (Arimura *et al.*, 1991), alternatively processed form of PACAP, the 27-amino acid form, provides a variant form to investigate which biochemical features of PACAP action are necessary for its effects on phenotype. PACAP38 is more potent than PACAP27 in stimulating the inositol lipid cascade in PC12 (Deutsch and Sun, 1992), and at 10^{-8} M, PACAP38 also leads to more sustained elevations of cAMP than PACAP27 (Figure 9). Both of those attributes may contribute to the greater activity of PACAP38 versus PACAP27 both on AP-1–mediated gene expression after 24 h of stimulation (Figure 10), as well as neurite outgrowth (Deutsch and Sun, 1992). Sustained activation of gene expression, typically requiring protracted stimulation of second-messenger levels (Deutsch *et al.*, 1990), is likely important in the long-term effects of neuropeptides such as the induction of a differentiated phenotype. In an analogous system, treatment with single doses of diacylglycerol (an activator of protein kinase C) or calcium ionophore leads to transient elevation of c-*fos* and c-*jun* mRNAs without activation of AP-1 enhancer activity in U937 monoblasts, whereas multiple doses of those agents activate AP-1 enhancer activity and induce differentiation into the macrophage phenotype (William *et al.*, 1990).

It is noteworthy that at physiological concentrations, 24 h treatment of PC12 cells with PACAP38 has effects on AP-1– (Figures 4–6) and CRE– (Figure 8) mediated gene expression that are quantitatively comparable to the effects of maximum doses of the pharmacological cAMP analogue. Presumably the stimulation of dual signaling cascades by PACAP38 counterbalances the attenuation in the cAMP signal in the resultant quantitative effect on reporter gene activity. Multiple other peptide hormone receptors appear to activate adenylyl cyclase as well as the inositol lipid cascade (Van Sande *et al.*, 1990; Abou-Samra *et al.*, 1992; Chabre *et al.*, 1992). Interestingly, this has been shown quite clearly for the parathyroid hormone (Abou-Samra *et al.*, 1992) and calcitonin receptors (Chabre *et al.*, 1992) expressed via cDNA transfection, and those two receptors (Juppner *et al.*, 1991; Lin *et al.*, 1991) share considerable sequence similarity with the secretin receptor, a receptor for another neuropeptide of the VIP-PACAP-glucagon family whose cDNA sequence has elucidated (Ishihara *et al.*, 1991) and which may well be structurally similar to PACAP receptors. In our ever increasing appreciation of the complexity of cell regulatory mechanisms, it will be interesting to explore more fully the consequences of activation of dual signaling pathways on gene expression and cell phenotype.

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REFERENCES

Abou-Samra, A.-B., Juppner, H., Force, T., Freeman, M.W., Xong, X.-F., Schipani, E., Urena, P., Richards, J., Bonventre, J.V., Potts, J.T., Jr, Kronenberg, H.M., and Segre, G.V. (1992). Expression cloning of a PTH/PTHrP receptor from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc. Natl. Acad. Sci. *89*, 2732–2736.

Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T., and Karin, M. (1988a). Oncogene jun encodes a sequence specific trans-activator similar to AP-1. Nature *332*, 166–171.

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H.J., and Herrlich, P. (1987a). 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol. Cell. Biol. *7*, 2256–2266.

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988b). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 875–885.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987b). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell *49*, 729–739.

Arimura, A., Somogyvari-Vigh, A., Miyata, A., Mizuno, K., Coy, D.H., and Kitada, C. (1991). Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. Endocrinology *129*, 2787–2789.

Auwerx, J., and Sassone-Corsi, P. (1991). IP-1: a dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation. Cell *64*, 983–993.

Barbacid, M., Lamballe, F., Pulido, D., and Klein, R. (1991). The trk family of tyrosine kinase receptors. Biochim. Biophys. Acta *1072*, 115–127.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science *238*, 1386–1392.

Brasier, A.R., Tate, J.E., and Habener, J.F. (1989). Optimized use of firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques *7*, 1116–1122.

Chabre, O., Conklin, B.R., Lin, H.Y., Lodish, H.F., Wilson, E., Ives, H.E., Catanzariti, L., Hemmings, B.A., and Bourne, H.R. (1992). A recombinant calcitonin receptor independently stimulates 3′-5′-cyclic adenosine monophosphate and Ca2+/inositol phosphate signaling pathways. Mol. Endocrinol. *6*, 551–556.

Curran, T., and Franza, B.R., Jr. (1988). Fos and Jun: the AP-1 connection. Cell *53*, 395–397.

Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R., and Kandel, E.R. (1991). cAMP response element-binding protein is activated by calcium/calmodulin as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *88*, 5061–5065.

Datta, R., Nakamura, T., Sherman, M.L., and Kufe, D.L. (1991). Regulation of jun-B expression by a cAMP-dependent mechanism in human myeloid cells. Blood *78*, 83–88.

Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., and Habener, J.F. (1988). Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements which bind distinct proteins. Proc. Natl. Acad. Sci. USA *85*, 7922–7926.

Deutsch, P.J., Jameson, J.L., and Habener, J.F. (1987). Cyclic AMP-responsiveness of human gonadotropin-alpha gene transcription is directed by an 18 base pair enhancer: alpha promoter receptivity to the enhancer confers cell-preferential expression. J. Biol. Chem. *262*, 12169–12174.

Deutsch, P.J., and Sun, Y. (1992). The 38-amino acid form of pituitary adenylate cyclase activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. J. Biol. Chem. *267*, 5108–5113.

Deutsch, P.J., Sun, Y., and Kroog, G.S. (1990). Vasoactive intestinal peptide increases intracellular cAMP and gonadotropin-alpha gene activity in JEG-3 syncytial trophoblasts: constraints posed by desensitization. J. Biol. Chem. *264*, 10274–10281.

de Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. *7*, 725–737.

Fink, J.S., Verhave, M., Walton, K., Mandel, G., and Goodman, R.H. (1991). cAMP- and phorbol ester-induced transcriptional activation are mediated by the same enhancer element in the human VIP gene. J. Biol. Chem. *266*, 3882–3887.

Fisch, T.M., Prywes, R., and Roeder, R.G. (1987). c-fos sequences necessary for basal expression and induction by epidermal growth factor, 12-O-tetradecanoyl phorobol-13-acetate, and the calcium ionophore. Mol. Cell. Biol. *7*, 3490–3502.

Fisch, T.M., Prywes, R., Simon, M.C., and Roeder, R.G. (1989). Multiple sequences in the c-fos promoter mediate induction by cAMP. Genes Dev. *3*, 198–211.

Ginty, D.D., Glowacka, D., Bader, D.S., Hidaka, H., and Wagner, J.A. (1991). Induction of immediate early genes by Ca++ influx requires cAMP-dependent protein kinase. J. Biol. Chem. *266*, 17454–17458.

Graham, F., and van der Erb, A. (1973). Infectivity of adenovirus DNA. Virology *52*, 456–467.

Greenberg, M.E., Greene, L.A., and Ziff, E.B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. *260*, 14101–14110.

Greene, L.A., and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73*, 2424–2428.

Gunning, P.W., Landreth, G.E., Bothwell, M.A., and Shooter, E.M. (1981). Differential and synergistic actions of nerve growth factor and Cyclic AMP in PC12 cells. J. Cell. Biol. *89*, 240–245.

Hoeffler, J.P., Deutsch, P.J., Lin, J.C., and Habener, J.F. (1989). Distinct adenosine 3',5'-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. Mol. Endocrinol. *3*, 868–880.

Hoeffler, J.P., Lustbader, J.W., and Chen, C.-Y. (1991). Identification of multiple nuclear factors that interact with cAMP Response element-binding protein and ATF-2 by protein-protein interactions. Mol. Endocrinol. *5*, 256–266.

Imagawa, M., Chiu, R., and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell *51*, 251–260.

Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, K., and Nagata, S. (1991). Molecular cloning and expression of a cDNA encoding the secretin receptor. EMBO J. *10*, 1635–1641.

Juppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X.F., Schipani, E., Richards, J., Kolakowski, L.F., Hock, J., Potts, J.T., Kronenberg, H.M., and Segre, G.V. (1991). A G-protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science *254*, 1024–1026.

Kim, U.H., Fink, D., Kim, H.S., Park, D.J., Contreras, M.L., Guroff, G., and Rhee, S.G. (1991). Nerve growth factor stimulates phosphorylation of phospholipase C-gamma in PC12 cells. J. Biol. Chem. *266*, 1359–1362.

Kobierski, L.A., Chu, H.M., Tan, Y., and Comb, M.J. (1991). cAMP-dependent regulation of proenkephalin by JunD and JunB: positive and negative effects of AP-1 proteins. Proc. Natl. Acad. Sci. USA *88*, 10222–10226.

Kouzarides, T., and Ziff, E. (1989). Leucine zippers of fos, jun, and GCN4 dictate dimerization specificity and thereby control DNA binding. Nature *340*, 568–571.

Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell *49*, 741–752.

Lin, H.Y., Harris, T.L., Flannery, M.S., Aruffo, A., Kaji, E.H., Gorn, A., Kolakowski, L.F., Lodish, H.F., and Goldring, S.R. (1991). Expression cloning of a calcitonin receptor, a novel adenylyl cyclase-coupled receptor. Science *254*, 1022–1024.

Machida, C.M., Rodland, K.D., Matrisian, L., Magun, B.E., and Ciment, G. (1989). NGF induction of the gene encoding the protease transin accompanies neuronal differentiation in PC12 cells. Neuron *2*, 1587–1596.

Maxwell, I.H., Harrison, G.S., Wood, W.M., and Maxwell, F. (1989). A DNA cassette containing a trimerized SV40 polyadenylation signal which efficiently blocks spurious plasmid-initiated transcription. Biotechniques *7*, 276–280.

Miyata, A.T., Jiang, L., Dahl, R.R., Kikada, C., Kubo, K., Fujino, M., Minamino, N., and Arimura, A. (1990). Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochem. Biophys. Res. Commun. *170*, 643–648.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H. (1986). Identification of a cAMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA *83*, 6682–6686.

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

Richter-Landsberg, C., and Jastorff, B. (1986). The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. J. Cell. Biol. *102*, 821–829.

Schubert, D., LaCorbiere, M., Whitlock, C., and Stallcup, W. (1978). Alterations in the surface properties of cells responsive to nerve growth factor. Nature *273*, 718–723.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). c-fos transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571–582.

Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a calcium-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427–1430.

Van Sande, J., Raspe, E., Perret, J., Lejeune, C., Maenhaut, C., Vassart, G., and Dumont, J.E. (1990). Thyrotropin activates both the cyclic AMP and the PIP2 cascades in CHO cells expressing the human cDNA of the TSH receptor. Mol. Cell. Endocrinol. *74*, R1–R6.

Van Straaten, F., Muller, R., Curran, T., Van Beveren, C., and Verma, I.M. (1983). Complete nucleotide sequence of a human c-onc gene: deduced amino acid sequence of the human c-fos protein. Proc. Natl. Acad. Sci. USA *80*, 3183–3187.

Watanabe, T., Ohtaki, T., Itada, C., Tsuda, M., and Fujino, M. (1990). Adrenal pheochromocytoma PC12h cells respond to pituitary adenylate cyclase activating polypeptide. Biochem. Biophys. Res. Commun. *173*, 252–258.

William, F., Wagner, F., Karin, M., and Kraft, A.S. (1990). Multiple doses of diacylglycerol and calcium ionophore are necessary to activate AP-1 enhancer activity and induce markers of macrophage differentiation. J. Biol. Chem. *265*, 18166–18171.

Wu, B.-Y., Fodor, E.J.B., Edwards, R.H., and Rutter, W.J. (1989). Nerve growth factor induces the proto-oncogene c-jun in PC12 cells. J. Biol. Chem. *264*, 9000–9003.

Yang, V.W., Christy, T.J., Cook, J.S., Kelly, T.J., and Lane, M.D. (1989). Mechanism of regulation of the 422 (aP2) gene by cAMP during prejadipocyte differentiation. Proc. Natl. Acad. Sci. USA *86*, 3629–3633.