Corticotropin-Releasing Hormone Stimulates Proopiomelanocortin Transcription by cFos-Dependent and -Independent Pathways: Characterization of an AP1 Site in Exon 1

A. L. Boutillier*, D. Monnier*, D. Lorang, J. R. Lundblad, J. L. Roberts, and J. P. Loeffler

Institut de Physiologie et de Chimie Biologique
URA 1446 du Centre Nationale de Recherche Scientifique
(A.L.B., D.M., J.P.L.)
67 084 Strasbourg Cédex, France

Fishberg Research Center for Neurobiology
Mount Sinai Medical Center (D.L., J.R.L., J.L.R.)
New York, New York 10029

The POMC gene, encoding a hormonal precursor protein, is primarily expressed in the pituitary in a tissue-specific manner. The POMC gene is transcriptionally regulated by a variety of hormones and neuropeptides and the second messengers cAMP and Ca^{++}. Using the corticotrope-derived AtT20 cell line, we have previously shown that overexpression of cFos stimulates POMC transcription. The aim of this work was to analyze whether cFos directly interacts with the POMC gene in basal and corticotropin-releasing hormone (CRH) stimulated cells. Using progressively deleted POMC promoter sequences or heterologous promoter constructs coupled to the chloramphenicol acetyl transferase reporter gene, we demonstrate the existence of a major cFos- responsive sequence within the first exon of the POMC gene. This sequence, TGACTAA, appears functionally indistinguishable from the canonical AP1 binding site. When fused to a minimal promoter, this sequence confers inducibility by cFos and CRH. Gel shift analyses with CRH-stimulated AtT20 nuclear extracts or *in vitro* synthesized proteins revealed that this sequence efficiently binds Fos and Jun. Expression of c-*fos* anti-sense mRNA reduced CRH-stimulated POMC transcription, thus indicating that, at least in part, cFos mediates the effect of CRH on POMC transcription. However, deletion of this major exonic AP1 site from the POMC constructs greatly reduced the effect of c-*fos* overexpression but did not suppress POMC stimulation by CRH, indicating that CRH stimulates POMC transcription by one or more cFos-independent mechanism(s). (Molecular Endocrinology 9: 745–755, 1995)

0888-8809/95/$3.00/0
Molecular Endocrinology

INTRODUCTION

The POMC gene, primarily expressed in the pituitary, encodes a variety of peptides including ACTH, the melanotropins (α-, β-, and γ-MSH), and opioid peptides of the β-endorphin family. The diversity of biological activities of these peptides suggests a key role for POMC in complex physiological processes, mainly in response to the "stress." Such a role in turn implicates a fine tuning of hormonal biosynthesis at the posttranslational but also at the transcriptional level. To adapt to such a complex environmental situation, POMC-producing cells in the pituitary are under multihormonal controls. In corticotrope cells of the anterior pituitary, the main physiological regulators are corticotropin-releasing hormone (CRH) and glucocorticoids. These two hormones, respectively, exert positive and negative controls on POMC transcription (reviewed in Ref. 1). In corticotrope cells, CRH increases cAMP levels and Ca^{++} entry (2). These two second messengers, cAMP and Ca^{++}, are well documented stimulators of POMC mRNA levels (3–5), and numerous publications of many laboratories now give us a clear picture of the intracellular pathways (second messengers and kinases) by which CRH stimulates POMC biosynthesis. In contrast, the nuclear activation mechanisms at the level of POMC gene remain largely obscure. The coupling between input signals into

endocrine cells and the subsequent genomic events that transduce short to long-term responses often occur through the activation of early response genes (6, 7). However, the transacting factor(s) by which CRH and its second messengers cAMP and Ca^{++} activate POMC transcription is not known. It is now accepted that cAMP and depolarization (Ca^{++}) signals could be transduced and result in transcriptional stimulation by specific short sequences located in the promoter region of many inducible genes. These sequences, cAMP or Ca^{++}-responsive elements [CRE or CaRE, respectively, 8)], contain the basic consensus sequence TGACGTCA. Using heterologous POMC promoter-reporter fusion gene constructs, we have previously identified two CRH and cAMP-responsive regions at positions −478 to −320 and −234 to −133 base pairs (bp)(9, 10) relative to the major POMC transcription start site. However, the classical cAMP CRE/CaRE of the TGACGTCA type is not present in these regions, suggesting that the transcriptional stimulation by cAMP is not mediated by the classical CRE binding protein (CREB). Indeed, we recently characterized and cloned one transcription factor, distinct from CREB, that mediates cAMP induction and binds to the −234/−133 region of the POMC promoter (10).

Furthermore, we have previously reported the induction of cFos by CRH via cAMP and Ca^{++}-dependent mechanisms in corticotrope cells (11), an observation that allows a possible role of this nuclear effector in mediating POMC transcription. Such an hypothesis would be in line with the observation that overexpression of cFos in corticotrophs does increase POMC transcription (11).

In the present study, we extend this observation by showing that CRH inductions of the POMC gene operates via cFos-dependent and cFos-independent mechanisms. We report the existence of a cFos-responsive element within the first exon of the gene. This sequence (TGACTAA) is a functional phorbol ester-responsive element (TRE) that can stimulate POMC gene transcription upon cFos and cJun binding.

RESULTS

cFos Stimulates Basal and CRH-Induced POMC Transcription

To test whether cFos is responsible for POMC transactivation, the 5′-flanking region of the rat POMC gene (−706/+63) coupled to the chloramphenicol acetyl transferase (CAT) reporter gene was transfected into AtT20 cells with a lipopolyamine-based gene transfer method developed in our laboratory (12, 13). Work from different groups has previously shown that the POMC promoter region used here is sufficient to confer pituitary tissue- specific expression (14), as well as modulation by the main physiological regulators: CRH (9) and glucocorticoids (15). We have previously reported that cFos stimulates POMC transcription (11). The data shown in Fig. 1 confirm and extend this observation. Overexpression of cFos elevates basal and further potentiates CRH-stimulated POMC transcription, although the fold change with CRH remains the same when compared with the elevated basal level.

Additional experiments show that CRH exerts its effects on POMC transcription, at least in part, via cFos. We performed cotransfections of the POMC-CAT construct with a c-*fos* antisense expression vector (16) that we have already shown to block cFos protein production efficiently (17). Figure 2 shows that the antisense significantly ($P \leq 0.05$) reduced the CRH-stimulated POMC transcription. This inhibitory effect by c-*fos* antisense was saturated with higher amounts of the antisense vector.

cFos-Responsive Elements in the POMC Promoter

To delineate cFos-responsive elements within the POMC gene, a progressively deleted rat POMC promoter (from −706 to −31 bases) was fused with a CAT reporter gene and transfected into AtT20 cells. Cotransfection of these constructs with a frame-shifted cFos mutant expression vector (pΔBK28) did not alter basal or CRH-induced POMC promoter activity when compared with transfection studies with reporter genes alone (data not shown). Interestingly, the intact cFos expression vector (pBK28) was able to stimulate basal POMC promoter activity in all of the 5′-deletion constructs, and CRH was capable of further stimulating promoter activity in an approximately additive way (Fig. 3).

Since the −31/+63 construct gave full cFos and CRH induction, we focused on this region. Within the first exon of POMC, nucleotides +41/+47 (TGACTAA) share substantial homology with the classical AP1 (TGACorGTCA) binding site, and, therefore, this sequence was further characterized. To address whether this putative AP1 site is responsible for maintaining cFos stimulation in the −31/+63 POMC promoter/reporter plasmid, two additional internal deletion constructs were made. These constructs removed the −18/+63 region in the context of the −706 (pJL157) and −31 (pJL160) 5′-deletion POMC promoter/reporter plasmids. When the pJL160 construct is analyzed (Fig. 3), both basal cFos-stimulated and CRH-induced promoter activity was eliminated. This minimal POMC promoter was not, however, without activity, showing a basal level of activity 25% that of the −706/+63 promoter construct (JL145). When the upstream portion of the −706 promoter was replaced (pJL157) in this construct, again, cFos had no effect on basal POMC promoter activity and CRH treatment had no effect on POMC promoter activity beyond what it had with the cFos mutant expression vector. CRH continued to stimulate POMC promoter activity independent of cFos expression vector activity.

Finally, to test whether the observed CAT activity correctly reflects initiated transcription from the various reporter genes, we characterized the transcription start sites of pJL 145, 142, 157, and 160. Figure 4

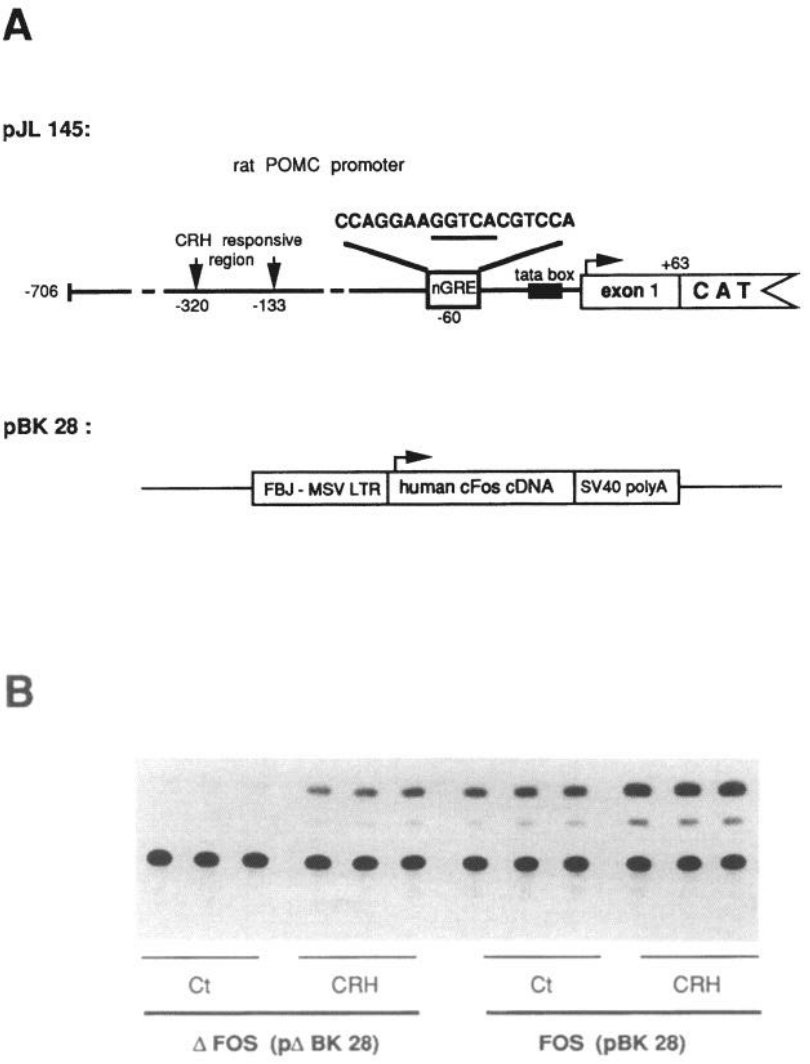

Fig. 1. cFos Stimulates Basal and CRH-Induced POMC Transcription

A, Diagrams of the different constructs. pJL145 contains the 5′-flanking region of the rat POMC promoter (−706 bp) and a part of the noncoding exon 1 (to +63) cloned in front of a CAT reporter gene (Roberts, 1987). The main regulatory regions of the POMC promoter responsive to CRH and to glucocorticoids (negative glucocorticoid responsive element, nGRE) are depicted. pBK28 expresses the human cFos protein (Fos), pΔBK28 is a frame-shift mutant of this construct (ΔFos) and serves as control. B, AtT20 cells were grown to 50% confluency in DMEM supplemented with 10% fetal calf serum and serum starved for 24 h before transfection experiments. Cells were cotransfected for 5 h with pJL145 (2 μg) and ΔFos (2 μg) (*left*) or Fos (2 μg) (*right*), switched to fresh DMEM for 12 h and then treated (CRH, 10^{-8} M) or not (Ct) for 6 h. Autoradiograms show the increased CAT activity of the POMC-CAT construct by Fos, on the basal (Ct) and CRH-induced AtT20 cells.

shows the correct initiation of these reporter genes after deletion of the promoter sequences located 5′ and/or 3′ from the TATA box. The basal CAT activity of these constructs in transient transfection assays is compared in Fig. 3.

The First Exon of the POMC Gene Contains a Functional TRE

To test whether the putative AP1-binding site in the first POMC exon possesses enhancer-like activity, a 23 base pair synthetic oligonucleotide centered on the TGACTAA (+41/+47) sequence was inserted into the minimal POMC promoter (pJL160) in front of the TATA box. When transfected into AtT20 cells, this construct is responsive to both cFos and CRH, and together their effects are again additive (Fig. 5). Mutations within the core TRE sequence (TGACTAA to AGGCTAA) completely abolished cFos- and CRH-mediated inductions (Fig. 5), similar to what was observed for the parent pJL160 construct alone (Fig. 3). Based on its homology with the consensus TRE and its functional regulation by cFos, this newly characterized sequence was subsequently called PTRE (POMC TRE).

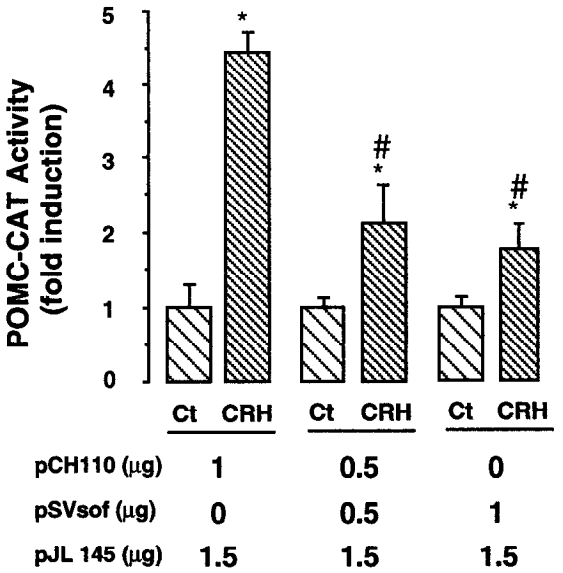

Fig. 2. Blockade of cFos Reduces CRH-Induced POMC-CAT Expression

AtT20 cells were cotransfected as described in Fig. 1 with 1.5 μg of the full length POMC-CAT reporter gene (pJL145) and different amounts of pSVsof (expressing antisense of c-*fos* mRNA, Mercola *et al.*, 1987). pCH110 (expressing β-galactosidase) was used to keep amounts of DNA constant. Cells were treated 8 h with CRH (10^{-8} M). *, When compared with respective untreated cultures and # when compared with CRH stimulation in the absence of fos antisense expression vector ($P < 0.05$, Student's t test). Histograms are means ± SD, n = 3.

Direct Involvement of cFos and cJun in the Nucleoprotein Complex That Binds the PTRE

We next asked whether proteins present in AtT20 cells could form complexes with PTRE and/or Met-TRE, a well characterized TRE from the metallothionein gene (18). Figure 6A shows that both oligonucleotides form one major complex (migrating at the same apparent mol wt), barely detectable in the control culture, but strongly enhanced when nuclear extracts from CRH-stimulated cells are used. Again, oligonucleotides mutated in the met-TRE or PTRE core sequence did not form complexes corresponding to the wild type gel-shift. Figure 6B shows the cross-competition between Met-TRE and PTRE, suggesting the presence of the same proteins binding to both oligonucleotides.

To analyze the nature of the proteins binding the PTRE, AtT20 cell nuclear extracts were preincubated with antisera directed against cFos or cJun proteins. Figure 7A shows that these antisera directed against cFos (Fos Ab, lane 3) and cJun (Jun Ab, lane 4) were able to abolish the formation of the retarded band, suggesting that with AtT20 nuclear extracts, PTRE binds a specific AP1 complex containing cFos and cJun immunoreactive protein. Identical results were obtained with the Met-TRE (data not shown). Suppression of nucleoprotein formation with the cFos or cJun antisera was specific since an unrelated antisera (directed against glucagon) was without effect (Ct Ab, lane 5).

To further address the question of whether the cFos- and CRH-inducible PTRE described above behaved as a classical TRE, binding the nucleoproteins cFos and cJun, we analyzed by gel retardation the interactions of the PTRE with the *in vitro* translated cFos and cJun proteins generated with a rabbit reticulocyte lysate. The characterized metallothionein TRE served as a control. As shown in Fig. 7B, neither cFos nor cJun alone is able to bind either of the two probes under our experimental conditions. However, both Met-TRE and PTRE could form a DNA-protein complex when incubated with the *in vitro* proteins cFos and cJun when translated together. Each retardation was specifically competed by an excess of respective unlabeled oligonucleotide (lane 4) whereas it was not by the unlabeled mutated oligonucleotide (lane 5). Further, cFos and cJun appear to bind to the same core sequence in the Met-TRE and the PTRE since cross-competition experiments with unlabeled oligonucleotides (lane 6) also efficiently compete for the retarded band. In parallel experiments, labeled mutated Met-TRE or PTRE oligonucleotides did not form detectable nucleoprotein complexes (data not shown).

DISCUSSION

CRH is the main hypophysiotropic factor for corticotroph cells. The primary effects of CRH on the corticotrophs are an increase in cAMP levels and the stimulation of POMC mRNA accumulation subsequent to transcriptional activation, most probably via a protein kinase A (PKA) mechanism (19). In our laboratories, we have shown that POMC transcription, as measured with POMC/CAT chimera genes, is abolished by genetic inactivation of PKA (A. L. Boutillier, D. Lorange, J. L. Roberts, and J. P. Loeffler, manuscript in revision) with dominant inhibitory mutants of this enzyme (20). In many genes, cAMP-stimulated gene expression is mediated by the transacting factor CREB, on the cAMP responsive element (CRE) TGACGTCA (21). However, sequence analysis of the rat POMC promoter does not reveal any such consensus CREs. Indeed, the only matches are poor ones (22, 23) and occur in regions of the promoter that are only weakly inducible by cAMP (9). This strongly suggests that CRH stimulates POMC via cAMP-dependent transacting factors different from CREB. Indeed, we have previously shown that such transcription factors are present and functional in AtT20 cells (24). Indirect evidence further shows that the regulatory pathway by which CRH activates POMC includes constitutive but also *de novo* synthesized transcription factors. cFos clearly belongs to this latter class of POMC regulatory proteins, since we show here that expression of antisense c-*fos* mRNA (and subsequent suppression of c-*fos* mRNA translation) reduces CRH effects. However, this blockade with c-*fos* antisense was only

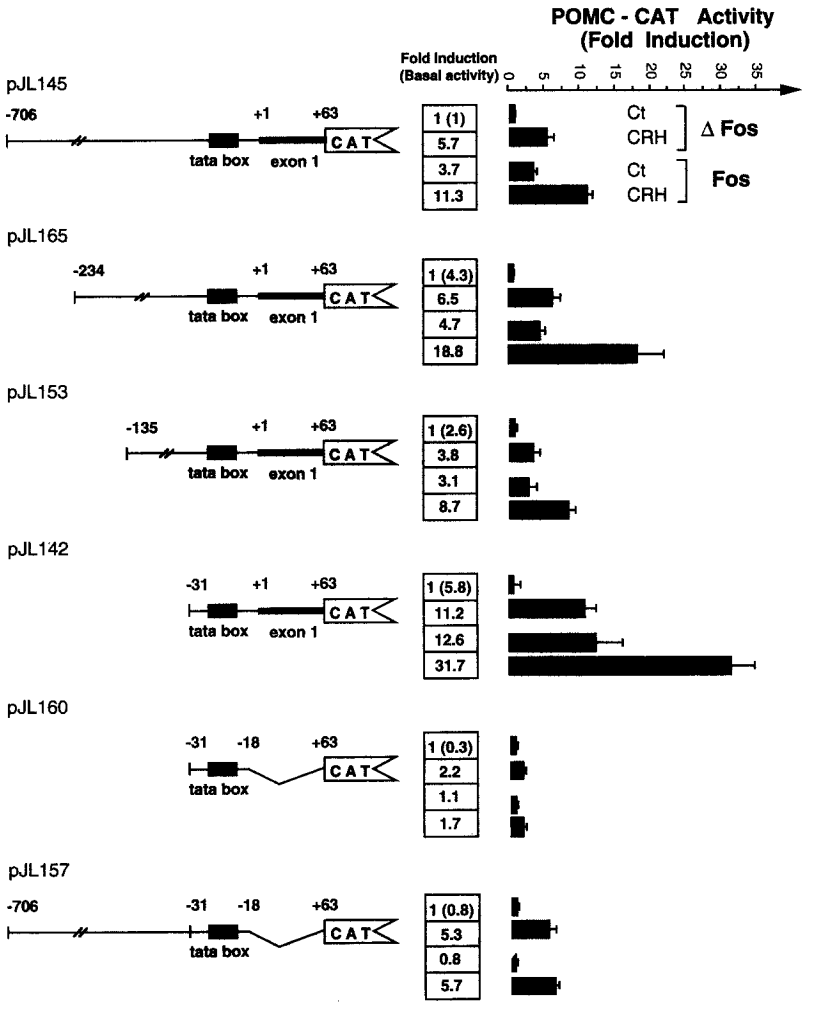

Fig. 3. Delineation of cFos and CRH-Responsive Elements by Mutagenesis of the POMC Promoter POMC-CAT Constructs, Progressively Deleted from the 5′-End of the Promoter Were Cotransfected into AtT20 Cells with Either ΔFos or Fos (Same Protocol as in Fig. 1)

All these constructs are inducible by CRH and Fos. These inductions are lost when the proximal promoter and the first exon were deleted (pJL160, −31/−18). A longer construct containing most of the POMC promoter (−706/−18) is still inducible by CRH whereas the response to Fos is lost. Basal transcription varied between the different POMC-CAT constructs and to compare their responses, the results are represented as fold induction. A typical experiment is represented, in which all the constructs have been tested together, in triplicate. Histograms represent means ± SD (n = 3). Basal activity of each construct is noted in *parentheses* as fold induction, taking pJL145 as reference (1).

partial, even at higher amounts of antisense vector, suggesting that other transcription factors are involved in the CRH stimulus. Indeed, protein synthesis inhibition experiments with cycloheximide, performed in our laboratories, show that the initial phase (15–60 min) of POMC transcription (evaluated by heterologous POMC RNA measurement; see Ref. 1) induced by CRH is not dependent on new protein synthesis. Later time points (>60 min), which would allow comparison with data obtained with CAT reporter genes (>8 h), could not be explored due to toxic side effects of protein synthesis inhibitors. Thus, taken together, these results suggest that CRH-mediated POMC transcription is a complex mechanism initiated by post-translational modification of preexisting transcription factors and then amplified or prolonged by newly synthesized proteins including cFos.

Delineation of a Functional cFos-Responsive Element

We have previously shown that cFos elevation via an expression vector stimulates POMC transcription (11). Moreover, cFos is induced by cAMP and adenylate cyclase-coupled receptors in corticotrope cells (11, 24). In the present work, aimed at deciphering the molecular mechanism by which CRH stimulates POMC transcription, we confirm and extend these observations with cFos. CRH and cFos overexpression were shown to stimulate POMC promoter activity,

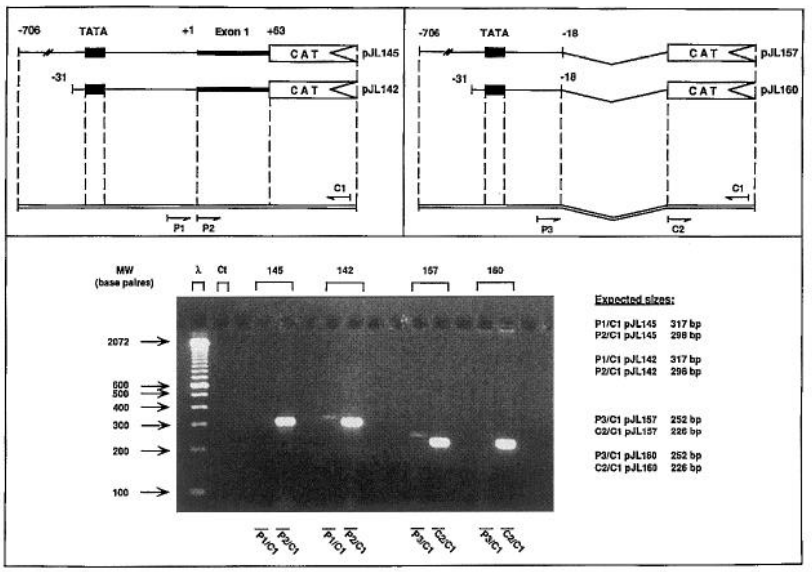

Fig. 4. Confirmation of the Good Initiation of the Transfected POMC Reporter Genes in AtT20 Cells by Combined RT-PCR

Upper panel, The oligonucleotides (P1, P2, P3, C1, and C2, see sequences in *Materials and Methods*) used as primers for RT-PCR are localized on the schematic representation of the four vectors tested (pJL145, pJL142, pJL 157, and pJL 160). P1 is located in front of the POMC gene start site for plasmids pJL145 and 142. P3 is located in front of the CAT gene start site for pJL157 and 160. After transfection of these vectors, AtT20 cells were treated for 1 h with Forskolin (5×10^{-6} M). RNA were then extracted according to the TRIzol reagent from GIBCO. *Lower panel,* Combined RT-PCR was performed on each RNA extract as described in *Materials and Methods* with two specific combinations of primers. One tenth of the RT-PCR products was loaded on a 3% agarose/Tris-acetate-EDTA gel and electrophoresed under 90 V for 2 h. A typical gel is represented. λ, 100-bp ladder (GIBCO-BRL); Ct, nontransfected AtT20 cells used as control; The following lanes represent the PCR products obtained from the different constructs (noted above) with the different combinations of primers as noted below. Combinations of P1/C1 and P3/C1 allow determinations of the incorrect initiation levels (read through) of pJL 145, pJL 142, pJL 157, and pJL 157, respectively. Proportion of incorrectly initiated transcripts is less than 50-fold compared with the correctly initiated ones. Expected sizes of RT-PCR products deduced from the different vectors' sequences and primers are noted on the *right*.

and their effects together were additive (Figs. 1 and 3). Dissection of the promoter with 5′-deletion constructs showed that this effect was maintained even in the minimal −31/+63 promoter/reporter construct, suggesting the presence of a CRH/cFos-responsive element within. Deletion of the −18/+63 portion of this minimal CRH/cFos-responsive element abolished the response, confirming the presence of the CRH/cFos element within.

Two elements of the −18/+63 rat POMC promoter region have previously been shown to bind nuclear proteins and affect POMC basal activity. The POB element at −15/−8 appears to have major effects on basal activity (25), but there have been no reports as to its CRH inducibility. Possibly its absence in the pJL160 plasmid can account for the low basal activity observed for this promoter/reporter construct. Therrien and Drouin (26) reported the presence of a AP1-like element in the rat POMC promoter (+41/+47) that footprinted with AtT20 nuclear extracts, but again, no CRH or cFos inducibility was presented. Our studies show that the POMC AP1-like element (+41/+47) is responsive to both CRH and cFos (Fig. 4) in a fashion identical to that of the −31/+63 construct, suggesting that this element is capable of mediating the CRH/cFos response alone. In addition, a double mutation in the element (Fig. 5) abolished both CRH and cFos responses, indicating a common mechanism of action on this element. These data suggest that the +41/+47 element in the −18/+63 region is mediating the CRH/cFos responses observed.

This POMC TRE is not the only CRH-responsive element in the −706/+63 region of the POMC promoter, although the 5′-deletion studies would suggest that the −31/+63 region is sufficient for full regulation. Deletion of the −18/+63 portion (pJL157) of a −706/+63 POMC promoter/CAT reporter plasmid caused a loss of the cFos overexpression- stimulated basal and additive effect of CRH on POMC promoter activity (Fig. 3). While this confirmed that the −18/+63 region was necessary for the cFos response, it also showed that the −706/−18 region contained at least one additional CRH-responsive element. We can not say whether this element is completely cFos independent, since CRH treatment alone induces cFos in AtT20 cells (11). However, cFos alone is not capable of the induction, so either it is an effect specific to CRH or to one mediated by cFos that has been modified (*e.g.* PKA phosphorylation) by CRH treatment. Indeed, phosphorylation of cFos by PKA and subsequent modification of its biological activity has been reported (27). This also implies that the TRE-like element present at −321/−315 (TGCCTCA) in the rat POMC

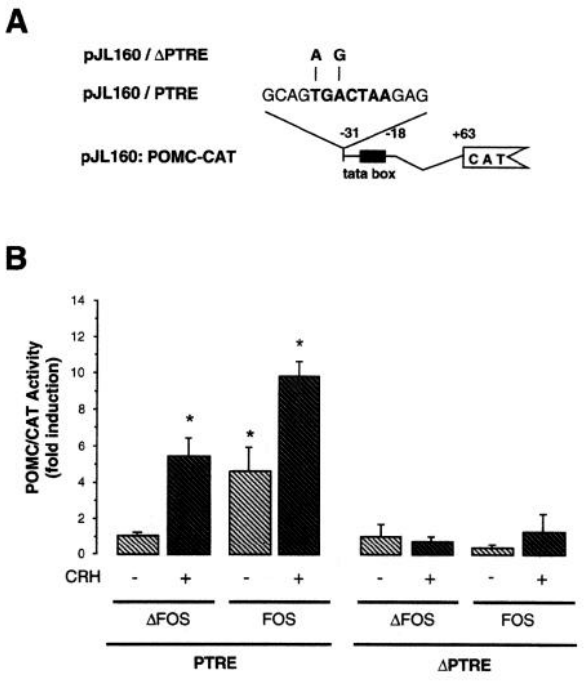

Fig. 5. The POMC TRE Is Functional

A, Diagram of the ΔPTRE and the PTRE constructs. The POMC TRE: PTRE (TGACTAA) present within the first exon or a mutated form: ΔPTRE (AGGCTAA) were cloned in front of a minimal POMC promoter CAT reporter gene (pJL160). B, POMC/CAT activity represented as fold induction from CAT assays carried out in AtT20 cells cotransfected with the pJL160/PTRE or pJL160/ΔPTRE fusion genes and either pBK28 (Fos) or pΔBK28 (ΔFos) expression vectors. Cotransfection experiments were performed as described (see legend of Fig. 1). Cells were left untreated (−) or treated with 10 nM CRH (+).

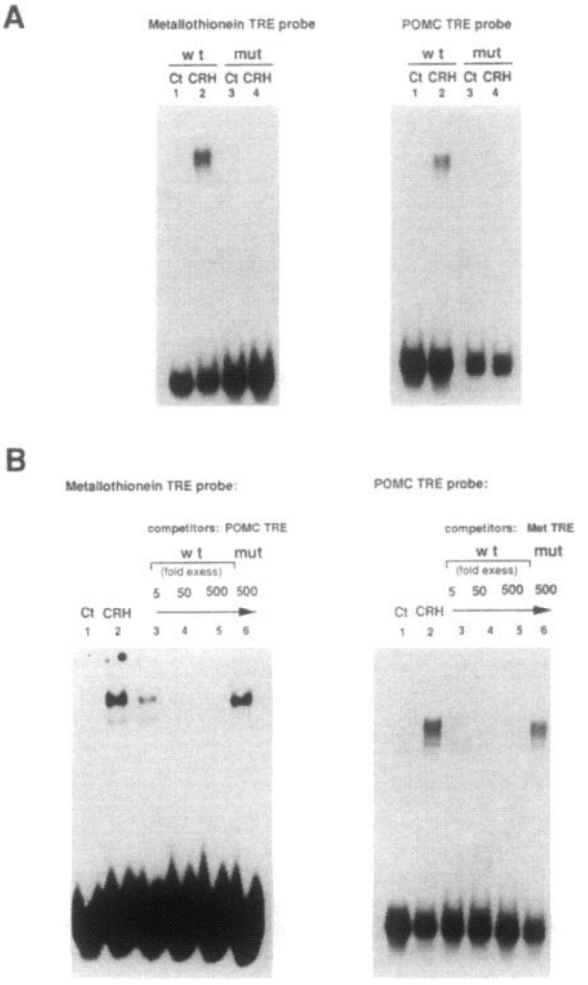

Fig. 6. The POMC TRE Functions as a Classical TRE

A, Gel-shift analyses with nuclear extracts (15 μg protein) from untreated (Ct) or 10 nM CRH-treated (CRH) AtT20 cells. *Left*, A retarded band is obtained with the wild type (wt) metallothionein TRE probe whereas the mutated form of the TRE oligonucleotide (mut) is not retained. *Right*, Same experiment with a labeled oligonucleotide containing the POMC TRE sequence (wt) or a mutated form (mut). B, Binding activity and competitions assays with wild type or mutated probes. *Left*, Binding of the metallothionein TRE probe using 15 μg of nuclear protein extracts from AtT20 cells, untreated (lane 1, Ct) or CRH-treated (lanes 2–6). Lanes 3–5, Competition with different concentrations of cold double-stranded POMC TRE oligonucleotide (5-, 50-, 500-fold molar excesses). Lane 6, Competition with a 500-fold molar excess of cold mutant POMC TRE oligonucleotide. *Right*, Binding of the POMC TRE probe using 15 μg nuclear protein extracts from AtT20 cells. The same conditions as for the Met TRE probe except that the cold double-stranded metallothionein TRE oligonucleotide served as competitor.

promoter does not behave as a functional AP1 site in this context. Therefore, we believe that the −706/+63 POMC promoter region is stimulated by CRH both through a TRE-like element in the first exon and through a different mechanism(s) in the −706/−18 region.

That the +41/+47 TRE-like element is actually a classical TRE element was further investigated. Therrien and Drouin (26) have previously shown that this element binds protein(s) in untreated AtT20 extracts. In our experiments, nuclear extracts from both control or CRH-stimulated AtT20 cells form one major nucleoprotein complex that is efficiently competed with the consensus metallothionein TRE and shows a major CRH induction of the shift. The nature of this nucleoprotein was immunologically characterized with cFos and cJun-directed antisera, both of which have been previously shown to suppress AP1 binding activity in gel shift assay (E. Gizang-Ginsberg, personal communication). Pretreatment of CRH-stimulated AtT20 nuclear extracts with these antisera suppressed the retarded complex, implying that it contained cFos and cJun protein. Finally, the POMC TRE can bind efficiently *in vitro* synthesized cFos and cJun proteins. In summary, this series of experiments shows that cFos and cJun can physically interact with the POMC gene on the TRE-like sequence (TGACTAA) located in the first exon at +41/+47. This sequence is functional in AtT20 cells and CRH stimulates POMC transcription, at least in part, through this element.

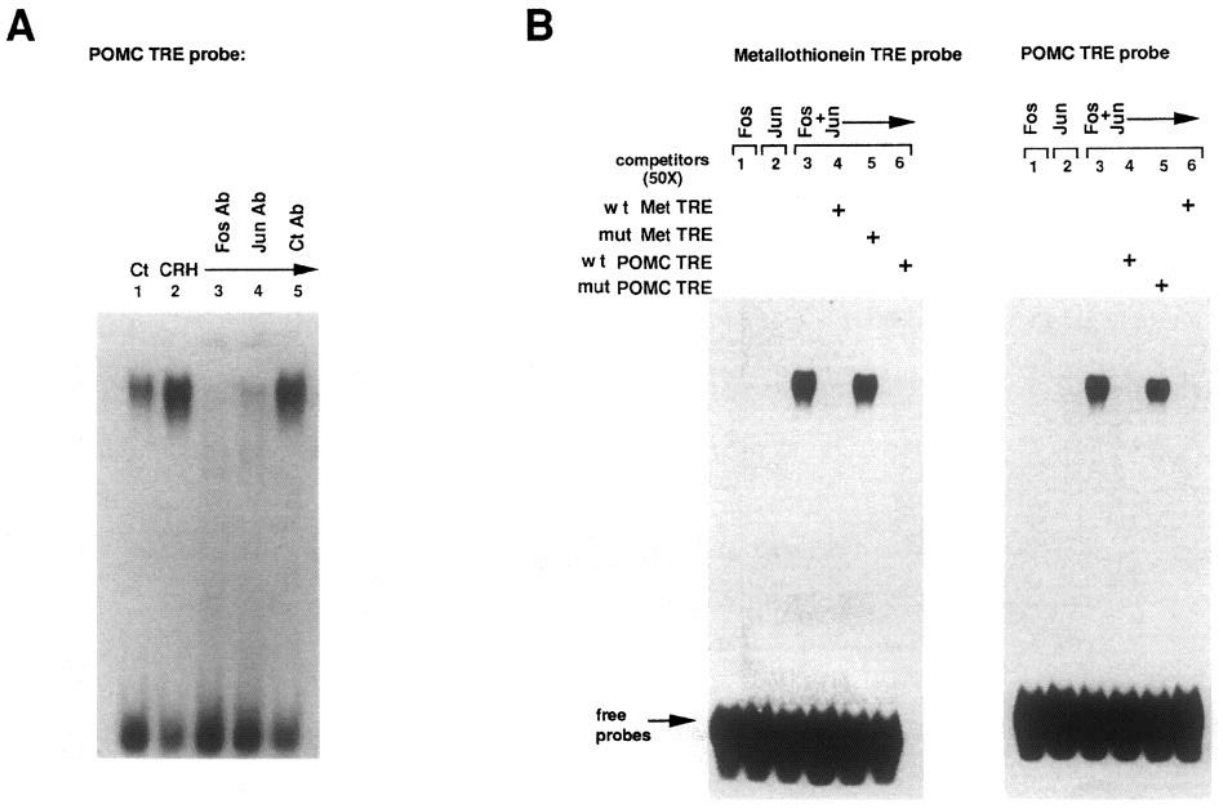

Fig. 7. Gel-Shift Analysis of the Exon I TRE

A, Nuclear proteins cFos and cJun bind to the POMC TRE gel-shift analyses were performed with 15 μg nuclear protein extracts from unstimulated (Ct, lane 1) or 10 nM CRH-stimulated AtT20 cells (CRH, lanes 2–5) preincubated with either no (lane 2), cFos (Fos Ab, lane 3), cJun (Jun Ab, lane 4), or glucagon (Ct Ab, lane 5) antisera. The binding is disrupted with cFos or cJun antisera. An unrelated antibody, directed against glucagon, does not affect the binding. Supershifts were clearly visible upon longer exposure of the autoradiogram (not shown). B, The POMC TRE binds *in vitro*-translated cFos and cJun proteins. *Left*, Binding activity of the metallothionein TRE probe using 3 μg of each translation mixture containing cFos alone (lane 1), cJun alone (lane 2), or cFos and cJun together (lanes 3–6) (see *Materials and Methods*). A specific band appears when cFos and cJun are cotranslated (lane 3). This band is efficiently competed by the cold metallothionein TRE (wt Met TRE, lane 4) and the wild type POMC TRE (wt POMC TRE, lane 6) whereas it is not by the mutated metallothionein TRE (mut Met TRE, lane 5). *Right*, Same type of experiment, but using the POMC TRE probe. Competitions of the gel-shifted band were performed with the cold probe (wt POMC TRE, lane 4), the mutated cold probe (mut POMC TRE, lane 5), or the wild type metallothionein TRE (wt Met TRE, lane 6).

Physiological Role of cFos in POMC Regulation by CRH

Our deletion studies show that CRH stimulates POMC transcription by a cFos-dependent mechanism at the +41/+47 site and also through another, possibly cFos-independent, mechanism upstream. However, these two mechanisms do not appear to operate independently since blockade of cFos expression by c-*fos* antisense vectors significantly reduces CRH-induced POMC transcription from the complete (−706 to +63) POMC promoter. One possible interpretation is that the exonic TRE has to function to allow maximal transcriptional stimulation by CRH. However, we can not exclude by our experimental approach a more subtle role of cFos in the 5′-region where cFos would not play a stimulatory effect on its own but rather exert a permissive effect on other transacting factors. This hypothesis is currently being investigated by a detailed mapping of the many 5′-binding sites by footprint and gel-shift analyses.

MATERIALS AND METHODS

Cell Cultures

AtT20/D16–16 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, at 37 C under 95% O_2-5% CO_2. Cells were serum deprived for 24 h before experiments.

Plasmids and Synthetic DNA Oligonucleotides

POMC promoter/CAT reporter fusion vectors: A 10-kilobase *Eco*RI fragment of DNA containing the rat genomic POMC gene was isolated from a Sprague-Dawley rat genomic library (28). The 5′-flanking region of the POMC gene from this 10-kilobase sequence was isolated and ligated to pCAT to generate pJL145 (Fig. 1) fusion construct. The pJL145 vector

contains the 5′-flanking POMC sequences (−706/+63), including the TATA box, the transcription initiation site, and a portion of the exon 1, fused to the bacterial CAT reporter gene in a pUC19-derived plasmid. This POMC construct also contains SV40 splicing and polyadenylation signals derived from pSV2CAT. The series of 5′-deletions of the POMC promoter sequences were generated using existing restriction sites in the parent pJL145 construct or Bal 31 digestion followed by blunt end repair with the Klenow fragment of DNA polymerase I. The pJL157 and pJL160 plasmids were constructed from the pJL145 and pJL142, respectively, by removing the −31/+63 region (*Pst*I and *Xho*I) and replacing it with a synthetic DNA fragment that recreated the POMC promoter region from −31 to −18. pBK28, expressing the human cFos protein and pΔBK28, a frame-shift mutant of pBK28, were provided by I. Verma (San Diego, CA).

pT7cfos (29) contains the full-length rat c-*fos* cDNA, subcloned into pGEM3. pJH19 (30) contains a partial human c-*jun* cDNA (cJun core, containing the leucine zipper and DNA-binding domains) subcloned in Bluescript SK$^{(+)}$. These plasmids were generously provided by E. Ziff (New York University, New York, NY).

The oligonucleotides containing the exonic rat POMC TRE (+41/+47), PTRE, and the mutated form, ΔPTRE, were synthesized as 23-mers and then cloned in front of the POMC TATA box (−31/+1) fused to the CAT reporter gene (see POMC/CAT fusion vectors) to generate PTRE/CAT and ΔPTRE/CAT. Recombinants were verified by DNA sequencing. Their sequences are as follow: PTRE, 5′-AGCTTAGCAGTGACTAAGAGAGT-3′ and ΔPTRE, 5′-AGCTTAGCAGAGGCTAAGAGAGT-3′. These oligonucleotides were also used as double-stranded DNA probes for gel retardation assays.

The sequences of the oligonucleotides for combined reverse transcriptase-polymerase chain reaction (RT-PCR) reaction are as follow: C1, 5′-GCATTCATCAGGCGGGC-3′; C2, 5′-CAGGAGCTAAGGAAGC-3′; P1, 5′-GAGAAGAGTGACAGGAAC-3′;P2,AAACGGGAGGCGACGGAG-3′;P3,AGCCTCGAGGAGCTTGC-3′.

Transfections and Reporter Assays

Transfections of AtT20 cells were carried out with a lipopolyamine-based method as previously described (11, 12). Briefly, cells cultured in six-well plates (Falcon) were grown to 50% confluency and serum deprived for 24 h before each experiment. Two to 4 μg of DNA per well were transfected for about 5 h. Cells were then grown in fresh DMEM for 12 h. The treatment with appropriate drugs lasted for 6–7 h. CAT activity was determined by the method of Gorman *et al.* (31) and quantitated as previously described (11). CAT activity was calculated as the percentage of chloramphenicol converted to acetylated forms and represented as fold induction relative to controls. All experiments were repeated at least three times.

Reverse Transcriptase and PCR

Cells were transfected as described above in 10-cm diameter dishes (Falcon) with 2 μg/ml of plasmid DNA. Cells were then stimulated 1 h with 5×10^{-6} M forskolin. Total RNA was extracted according to the TRIzol TM Reagent method from GIBCO (Grand Island, NY) and subsequently DNAse treated (20 U, 15 min at 37 C), phenol-chloroformed, and ethanol-precipitated. The combined RT-PCR was set up on ice in 50 mM KCl, 1.5 mM $MgCl_2$, 25 mM Tris-HCl, pH 8.3, 100 μg/ml BSA, 50 μM of each deoxynucleoside trisphosphate with 15 pmol/primer plus 500 ng of the RNA template in a final volume of 50 μl. Denaturing and annealing were performed in a monocycler apparatus (Hybaid, Logan, UT) for 10 min at 94 C, followed by a programmed step to 55 C (10 min). One microliter of a mix of SuperScript RNaseH$^-$ RTase (100 U, GIBCO) and *Taq* polymerase (1.25 units, GIBCO) was added and the reaction lasted 1 h at 55 C. The reverse transcription step was stopped by a 5-min incubation at 94 C. The subsequent amplification program was as follows: 30 sec at 94 C, 30 sec at 55 C, 45 sec at 72 C, for 30 cycles. RT-PCR products were electrophoresed through a 3% agarose/Tris-acetate-EDTA gel and bands were visualized with ethidium bromide under UV.

In Vitro Transcription and Translation

In vitro transcription of pT7cfos (linearized with *Hin*dIII) and pJH19 (linearized with *Xho*I) was carried out using T7 and T3 RNA polymerases, respectively (Boehringer Mannheim, Mannheim, Germany). After denaturation, 10 min at 67 C, 2 μg of RNA substrates were mixed with 1/3 final volume of rabbit reticulocyte lysate (Promega, Madison, WI), 1 mM amino acid mixture - methionine, 1 mM methionine, and 2 mM dithiothreitol (DTT), in a final volume of 50 μl. The reaction was incubated at 30 C for 1 h. cFos and cJun were synthesized either alone or together. A parallel translation with [^{35}S]methionine was performed, and labeled protein products were confirmed by sodium dodecyl sulfate gel electrophoresis (data not shown).

Nuclear Extracts

AtT20 cells were grown in 10-cm diameter wells (Falcon) until 70–80% confluency and serum deprived 24 h before treatment with CRH (10^{-8} M) for 1 h. Cells were then harvested in cold PBS. Nuclear extracts were prepared as described (32). Final concentrations were typically 2–3 μg/μl as determined by the Bio-Rad protein assay (Munich, Germany).

Gel Retardation Assays

Five picomoles of each double-stranded oligonucleotide (PTRE and ΔPTRE as shown above, metallothionein TRE and ΔTRE as reported in Ref. 18) were end-labeled with [gamma-^{32}P]dCTP (3000 Ci/mmol), resolved on a 10% acrylamide gel, and eluted from the gel at 4 C in TE buffer (10 mM Tris-HCl pH8, 1 mM EDTA). Approximately 0.2 ng of ^{32}P-labeled DNA (15,000 to 20,000 cpm) was added to the preincubated *in vitro* translated products or nuclear extracts.

For the *in vitro* translated proteins, 3 μl of each assay (cFos alone, cJun alone, or cFos and cJun together) were preincubated 10 min on ice, in 15 μl of binding buffer (10 mM HEPES pH 7.9, 4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM spermidine, 2 mM DTT, and 15% glycerol) with 1 μg poly(dI-dC) and 800 ng unlabeled, single-stranded PTRE; 200 fg of ^{32}P-labeled probe were added, and the binding reaction was incubated for an additional 15 min on ice. In competition experiments, a 50-fold molar excess of unlabeled competitor oligonucleotides was added with the poly(dI-dC). Protein-DNA complexes were resolved on a 4% polyacrylamide gel electrophoresis in 0.5× Tris-borate-EDTA.

For the cell nuclear extracts, 15 μg proteins were preincubated in 12 μl binding buffer (6 mM KCl, 20 mM DTT, 5 mM spermidine, 8% glycerol, and 2% Ficoll) with 1 μg poly(dI-dC) and 0.2 ng ^{32}P-labeled probe. In competition experiments, 5, 50, or 500-fold molar excess of unlabeled competitor oligonucleotides were added with the poly(dI-dC). The binding reaction was allowed to remain at room temperature for 20 min, and the protein-DNA complexes were resolved on a 4% polyacrylamide gel electrophoresis in 0.5× TBE. The gels were dried and autoradiographed with intensifying screens at −70 C with Kodak X-OMAT AR films.

Fos and Jun polyclonal antibodies used in gel-shift analyses (Fig. 7) were a generous gift from Dr. E. Ziff (New York University, New York, NY). These antibodies were generated with Trp fusion protein produced from pFOSTRPA (33) and pTE-vJun (34) expression vectors. Both antibodies have been

shown to suppress AP1 binding activity in gel-shift assays with a labeled TRE (E. Gizang-Ginsberg, personal communication).

Acknowledgments

We are indebted to Dr. I. Verma (San Diego, CA) for the kind gift of cFos expression vectors and to Drs E. Ziff and E. Ginzang-Ginsberg (New York, NY) for the generous gift of cFos and cJun antibodies and the vectors for *in vitro* cFos and cJun protein synthesis. We thank Dr. W. D. Jin for the cloning of the pJL160/PTRE and pJL160/ΔPTRE constructs.

Received April 2, 1993. Re-revision received March 15, 1995. Accepted March 21, 1995.

Address requests for reprints to: J. P. Loeffler, Institut de Physiologie et de Chimie Biologique, URA 1446 du Centre Nationale Recherche Scientifique, 67 084 Strasbourg Cedex, France.

This work was supported by the Institut National de la Santé et de la Recherche Médicale, NIH Grant DK-27484 and Association pour la Recherche contre le Cancer Grant ARC 6089.

*Supported by the Ministère de la Recherche et de la Technologie.

REFERENCES

1. Autelitano DJ, Lundblad JR, Blum M, Roberts JL 1989 Hormonal regulation of POMC gene expression. Annu Rev Physiol 51:715–726
2. Luini A, Lewis D, Guild S, Corda D, Axelrod J 1985 Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin secreting cells. Proc Natl Acad Sci USA 82:8034–8038
3. Loeffler JP, Kley N, Pittius CW, Höllt V 1985 Corticotropin-releasing factor and forskolin increase proopiomelanocortin messenger RNA levels in rat anterior and intermediate cells *in vitro*. Neurosci Lett 62:383–387
4. Guild S and Reisine T 1987 Molecular mechanisms of corticotropin-releasing factor stimulation of calcium mobilisation and adenocorticotropin release from anterior pituitary tumor cells. J Pharmacol Exp Ther 241:125–129
5. Von Dreden G, Loeffler JP, Grimm C, Höllt V 1988 Influence of calcium ions on proopiomelanocortin mRNA levels in clonal anterior pituitary cells. Neuroendocrinology 47:32–37
6. Karin M and Smeal T 1992 Control of transcription factors by signal transduction pathways: the beginning of the end. Trends Biochem Sci 17:418–422
7. Ginty DD, Bading H, Greenberg ME 1992 Trans-synaptic regulation of gene expression. Curr Opin Neurobiol 2:312–316
8. Sheng M, Mc Fadden, Greenberg ME 1990 Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. Neuron 4:571–582
9. Roberts JL, Lundblad JR, Eberwine JH, Fremeau RT, Salton SRJ, Blum M 1987 Hormonal regulation of proopiomelanocortin gene expression in the pituitary. Ann NY Acad Sci 512:275–285
10. Jin WD, Boutillier AL, Glucksman MJ, Salton SRJ, Loeffler JP, Roberts JL 1994 Characterization of a corticotropin-releasing hormone-responsive element in the rat proopiomelanocortin gene promoter and molecular cloning of its binding protein. Mol Endocrinol 8:1377–1388
11. Boutillier AL, Sassone-Corsi P, Loeffler JP 1991 The protooncogene c-*fos* is induced by corticotropin-releasing factor and stimulates proopiomelanocortin gene transcription in pituitary cells. Mol Endocrinol 5:1301–1310
12. Behr JP, Demeneix BA, Loeffler JP, Perez-Mutul J 1989 Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proc Natl Acad Sci USA 86:6982–6986
13. Loeffler JP and Behr JP 1993 Gene transfer into primary and established mammalian cell lines with lipopolyamine-coated DNA. Methods Enzymol 217:599–618
14. Jeannotte L, Trifino MA, Plante RK, Chamberland M, Drouin J 1987 Tissue-specific activity of the proopiomelanocortin gene promoter. Mol Cell Biol 7:4058–4064
15. Charron J and Drouin J 1986 Glucocorticoids inhibition of transcription from episomal proopiomelanocortin gene promoter. Proc Natl Acad Sci USA 83:8903–8912
16. Mercola D, Rundell A, Westwich J, Edwards SA 1987 Antisense RNA to the c-*fos* gene: restoration of density dependent growth arrest in a transformed cell line. Biochem Biophys Res Commun 147:288–294
17. Demeneix BA, Kley N, Loeffler JP 1990 Differentiation to a neuronal phenotype in bovine chromaffin cells is repressed by protein kinase C and is not dependent on cFOS oncoproteins. DNA Cell Biol 9:335–345
18. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Ramhsdorf HJ, Jonat C, Herrlich P, Karin M 1987 Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49:729–739
19. Reisine T, Rougon G, Barbet J, Affolter HV 1985 Corticotropin-releasing factor-induced adenocorticotropin hormone release and synthesis is blocked by incorporation of the inhibitor of cyclic AMP-dependent protein kinase into anterior pituitary tumor cells by liposomes. Proc Natl Acad Sci USA 82:8261–8266
20. Mc Knight GS, Cadd GG, Clegg CH, Otten AD, Correll LA 1988 Expression of wild-type and mutant subunits of the cAMP-dependent protein kinase. Cold Spring Harbor Symp Quant Biol 53:111–119
21. Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH 1986 Identification of cyclic-AMP responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 83:6682–6686
22. Young SL, Nierlsen CP, Lundblad JR, Roberts JL, Melner MH 1985 Gonadotropin regulation of the rat proopiomelanocortin promoter: characterization by transfection of primary ovarian granulosa cells. Mol Endocrinol 3:15–21
23. Drouin J, Chamberland M, Charron J, Jeanotte L, Nemer M 1985 Structure of the rat pro-opiomelanocortin (POMC) gene. FEBS Lett 183:54–58
24. Boutillier AL, Barthel F, Roberts JL, Loeffler JP 1992 β-Adrenergic stimulation of cFos via protein kinase A is mediated by cAMP regulatory element binding protein (CREB)-dependent and tissue-specific CREB-independent mechanisms in corticotrope cells. J Biol Chem 267: 23520–23526
25. Riegel AT, Remenick J, Wolford RG, Berard DS, Hager GL 1990 A novel transcriptional activator (PO-B) binds between the TATA box and CAP site of the pro-opiomelanocortin gene. Nucleic Acids Res 18:4513–4521
26. Therrien M and Drouin J 1991 Pituitary pro-opiomelanocortin gene expression requires synergistic interactions of several regulatory elements. Mol Cell Biol 11:3492–3503
27. Tratner I, Ofir R, Verma I 1992 Alteration of a cyclic AMP-dependent protein kinase phosphorylation site in the c-*fos* protein augments its transforming potential. Mol Cell Biol 12:998–1006
28. Eberwine JH and Roberts JL 1984 Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. J Biol Chem 259:2166–2170
29. Kouzarides T and Ziff E 1988 The role of the leucine

zipper in the fos-jun interaction. Nature 336:646–651
30. Bohman D, Bos TJ, Admon A, Nishimura T, Vogt PK, 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
31. Gorman CM, Moffat LM, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2:1044–1051
32. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
33. Vosatka RJ, Hermanowski-Vosatka A, Metz R, Ziff EB 1989 Dynamic interactions of c-fos protein in serum-stimulated 3T3 cells. J Cell Physiol 138:493–502
34. Angel P, Allegretto EA, Okino ST, Hattori K, Boyle WJ, Hunter T, Karin M 1988 Oncogene jun encodes a sequence-specific trans-activator similar to AP1. Nature 332:166–171