

# Identification of a cyclic-AMP-responsive element within the rat somatostatin gene

(gene regulation/protein kinase 2/promoter)

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**ABSTRACT** We have examined the regulation of somatostatin gene expression by cAMP in PC12 rat pheochromocytoma cells transfected with the rat somatostatin gene. Forskolin at 10  $\mu$ M caused a 4-fold increase in somatostatin mRNA levels within 4 hr of treatment in stably transfected cells. Chimeric genes containing the somatostatin gene promoter fused to the bacterial reporter gene encoding chloramphenicol acetyltransferase were also induced by cAMP in PC12 cells. To delineate the sequences required for response to cAMP, we constructed a series of promoter deletion mutants. Our studies defined a region between 60 and 29 base pairs upstream from the transcriptional initiation site that conferred cAMP responsiveness when placed adjacent to the simian virus 40 promoter. Within the cAMP-responsive element of the somatostatin gene, we observed an 8-base palindrome, 5'-TGACGTCA-3', which is highly conserved in many other genes whose expression is regulated by cAMP. cAMP responsiveness was greatly reduced when the somatostatin fusion genes were transfected into the mutant PC12 line A126-1B2, which is deficient in cAMP-dependent protein kinase 2. Our studies indicate that transcriptional regulation of the somatostatin gene by cAMP requires protein kinase 2 activity and may depend upon a highly conserved promoter element.

Somatostatin is a 14-amino acid peptide that modulates the secretion of other regulatory peptides in the pituitary, pancreatic islets, and gastrointestinal tract. Somatostatin secretion is increased by agents that stimulate adenylate cyclase activity, including vasoactive intestinal peptide (VIP) (1), glucagon (2), and epinephrine (3), suggesting that cAMP is a second messenger for somatostatin release. cAMP also regulates somatostatin biosynthesis in primary cultures of fetal rat hypothalamic cells and in clonal isolates of NIH-3T3 fibroblast cells transfected with the rat somatostatin gene (4). To examine the molecular basis of cAMP regulation of the rat somatostatin gene, we have transfected the gene into the neuroendocrine cell line PC12.

PC12 cells, derived from a rat pheochromocytoma (5), synthesize a variety of neuropeptides, including neurotensin and neuropeptide Y (6). These cells provide a particularly useful model in which to evaluate the effects of cAMP on gene expression. Several genes normally expressed in PC12 cells, including the tyrosine hydroxylase gene (7), *c-fos* (8), and possibly the ornithine decarboxylase gene (9), are transcriptionally regulated by cAMP. Additionally, mutant PC12 cell lines that are deficient in cAMP-dependent protein kinases have recently been characterized (10). These kinases have been proposed to mediate many of the transcriptional effects of cAMP (11).

In the present study we show that somatostatin gene expression is regulated by cAMP in stably transfected PC12 cells. To test the hypothesis that regulation of somatostatin expression by cAMP depends on specific promoter sequences, we have transfected PC12 cells with a series of fusion genes containing portions of the somatostatin promoter linked to the bacterial reporter gene encoding chloramphenicol acetyltransferase (CAT). The role of cAMP-dependent protein kinase type 2 in mediating the effects of cAMP on gene expression was determined by comparing the activity of the somatostatin promoter in wild-type and A126-1B2 mutant PC12 cells. The latter cells have normal levels of cAMP-dependent protein kinase type 1 activity but are markedly deficient in type 2 activity (10).

Our experiments define a sequence within the somatostatin gene that is necessary for transcriptional regulation by cAMP. This regulation appears to require cAMP-dependent protein kinase type 2 activity. The sequence within the somatostatin gene is also present in several other cAMP-inducible genes.

## PROCEDURES

**Cell Lines.** PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 5% horse serum, penicillin at 100 units/ml, and streptomycin at 100  $\mu$ g/ml. Cells were fed every 3-4 days and passaged every 7 days. A126-1B2 cells were maintained under the same conditions.

**Production of Stable Lines.** PC12 cells were transfected by electroporation using 1  $\mu$ g of pSV2-neo (12) and 10  $\mu$ g of a somatostatin gene subclone pSal that extends from -250 base pairs (bp) to +4 kilobases (kb) downstream of the gene (13). Cells ( $1.5 \times 10^6$  per 100-mm dish) were harvested by centrifugation at  $1000 \times g$  for 5 min. The cells were washed, and resuspended in 0.5 ml of cold (4°C) phosphate-buffered saline (PBS, 145 mM NaCl/5 mM NaP<sub>i</sub>, pH 7.4). DNA was precipitated in ethanol, resuspended in 20  $\mu$ l of sterile PBS, and added to the cell suspension. A 3500-V, 0.9-mA shock was delivered to the suspension, as described by Potter *et al.* (14), and the cells were plated directly in DMEM. Two days later, the neomycin analogue G418 was added to the medium, and resistant colonies were isolated after 2 to 3 weeks of selection. Colonies were individually analyzed for production of immunoreactive somatostatin according to published techniques (15).

**S1-Nuclease and RNA Blot Analysis.** Total cytoplasmic RNA was isolated and analyzed by S1-nuclease treatment and blot hybridization as described previously (4). Samples

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Abbreviations: CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s); VIP, vasoactive intestinal peptide; SV40, simian virus 40; LTR, long terminal repeat.

were normalized by densitometry of ribosomal RNA bands on native agarose gels (4).

**Plasmid Construction.** The somatostatin-CAT fusion genes are a set of promoter deletion mutants designated  $\Delta(-750)$ ,  $\Delta(-250)$ ,  $\Delta(-71)$ , and  $\Delta(-48)$  (Fig. 3). The numbers in parentheses indicate the 5' termini of somatostatin gene flanking sequences relative to the somatostatin mRNA transcriptional start point. Somatostatin promoter fragments were prepared from the plasmid pSRIF, which contains the 5'-flanking region and 5' untranslated sequences extending from -750 to +53 (Fig. 3).  $\Delta(-750)$  was constructed by inserting a *Hind*III fragment from pSRIF into the *Hind*III site of pSV0-CAT (16).  $\Delta(-250)$  was constructed by inserting a 300-bp *Sal*I-*Hind*III fragment from pSRIF into the *Sal*I-*Hind*III sites of pSV0-CAT.  $\Delta(-71)$  was constructed by ligating a *Bgl*II-*Hind*III pSRIF fragment and a *Hind*III-*Bam*HI CAT gene fragment into the *Bam*HI site of pUC12.  $\Delta(-48)$  was prepared by deleting a 560-bp *Aat*II fragment from  $\Delta(-71)$ .  $\Delta(-49/-39)$  was constructed by inserting a synthetic oligonucleotide into the *Bgl*II site of pA10-CAT 2 (17). All constructions were confirmed by restriction endonuclease mapping, nucleotide sequencing, or both (18).

**Transient Assays.** PC12 cells ( $10^6$  cells per 100-mm plate) were transfected with 40  $\mu$ g of DNA (consisting of a CAT plasmid and pBR322 carrier) by calcium phosphate precipitation followed by glycerol shock (16). Cells were treated for 36-48 hr with forskolin or ethanol vehicle and then assayed for CAT activity. Cells were harvested in 10 ml of cold PBS, centrifuged, and resuspended in 100  $\mu$ l of 0.25 M Tris-HCl, pH 7.6. Cell lysates were prepared as described by Gorman *et al.* (16). One-half of the lysate (representing approximately 40  $\mu$ g of total protein) from each transfection was assayed for CAT activity. Lysates were incubated with 1.0  $\mu$ Ci (1 Ci = 37 GBq) of [ $^{14}$ C]chloramphenicol and 0.2 mM acetyl-CoA in 150 mM Tris-HCl, pH 7.6, for 60 min at 37°C. Under these conditions, CAT activity was linear with time. CAT activity was analyzed by thin-layer chromatography as described by Gorman *et al.* (16).

## RESULTS

**Regulation of the Somatostatin Gene in Stable Lines.** Stably transfected PC12 clones were screened for their ability to produce immunoreactive somatostatin. Blot hybridization analysis of total cytoplasmic RNA from stably transfected PC12 cells showed a single 650-bp somatostatin mRNA band, which comigrated with that in Ca-77 cells, a somatostatin-producing medullary thyroid carcinoma cell line (19). S1-nuclease analysis demonstrated that the 5' terminus of the somatostatin mRNA mapped to the same position in PC12 and Ca-77 cells (Fig. 1A). These results indicate that PC12 cells use the normal transcriptional initiation site for expression of the foreign somatostatin gene.

Forskolin, a direct activator of adenylate cyclase, was used to examine cAMP responsiveness of the transfected somatostatin gene. Transfected PC12 cells treated with 10  $\mu$ M forskolin over a 4-hr period increased their levels of somatostatin mRNA 4-fold (Fig. 1B). Wild-type PC12 cells treated with forskolin did not produce somatostatin mRNA.

**Identification of a cAMP-Responsive Element.** To characterize cAMP-responsive promoter sequences within the somatostatin gene, we fused 750 bases of the somatostatin 5' flanking region to the bacterial reporter gene for CAT. This plasmid is designated  $\Delta(-750)$ . Promoter activity was determined by transient assay after transfection of the fusion gene into PC12 cells. To control for nonspecific effects of cAMP on gene expression, a pRSV-CAT plasmid (20) was also studied. This plasmid contains the Rous sarcoma virus promoter fused to the CAT structural gene.

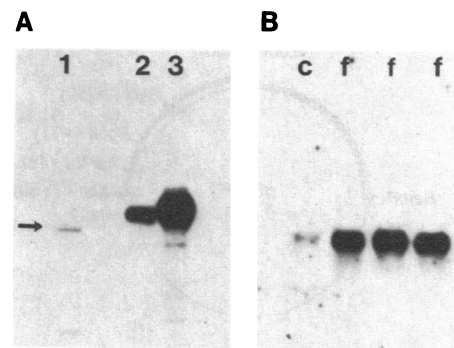


FIG. 1. (A) Localization of the transcriptional initiation site of the rat somatostatin gene in stably transfected PC12 cells and in Ca-77 rat medullary thyroid carcinoma cells. RNA from both cell lines was hybridized to a 5'-end-labeled *Bss*H2 fragment of the cloned somatostatin gene (13). The hybrid was digested with S1 nuclease and the products were analyzed on an 8% polyacrylamide/urea gel. Lane 1 is a *Hae*III-digested pBR322 size marker. Arrow indicates position of a 123/124-bp doublet. Lanes 2 and 3 show the DNA fragments protected by mRNA from transfected PC12 cells and Ca-77 cells, respectively. (B) Effect of forskolin on somatostatin mRNA levels. Cytoplasmic RNA was prepared from stably transfected PC12 cells after treatment with either ethanol vehicle control (c) or 10  $\mu$ M forskolin (f) for 4 hr. Three separate forskolin treatments are shown. One microgram of cytoplasmic RNA from each sample was electrophoresed, transferred to nitrocellulose, and hybridized to  $^{32}$ P-labeled antisense somatostatin RNA.

The constitutive level of expression of plasmid  $\Delta(-750)$  was about 3% of the level of pRSV-CAT. The basal expression of CAT under the control of either the somatostatin or Rous sarcoma virus promoter was proportional to the amount of DNA transfected up to 40  $\mu$ g, the highest amount tested. As shown in Fig. 2, forskolin increased CAT activity 7.5-fold in the transfected PC12 cells at all concentrations of  $\Delta(-750)$  tested. In contrast, forskolin had no effect on pRSV-CAT expression.

To delineate the sequences in the somatostatin promoter responsible for induction by cAMP, we progressively deleted sequences from the 5'-flanking region of plasmid  $\Delta(-750)$ . CAT activity derived from  $\Delta(-250)$  and  $\Delta(-71)$  was induced 8- to 10-fold in response to forskolin (Fig. 3). In contrast, the induction of CAT activity in  $\Delta(-48)$  after forskolin treatment was only 2-fold. There were no differences in the basal levels of CAT activity among  $\Delta(-750)$ ,  $\Delta(-250)$ ,  $\Delta(-71)$ , and  $\Delta(-48)$ . These results suggest that the 5' boundary of the cAMP-responsive element is located between 71 and 48 nucleotides upstream from the somatostatin transcriptional initiation site.

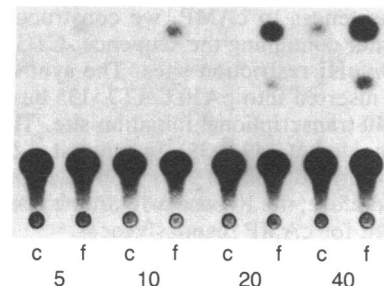


FIG. 2. CAT enzyme activity in PC12 cells transfected with the somatostatin-CAT fusion gene  $\Delta(-750)$ . Plates containing  $10^6$  cells were transfected with 5, 10, 20, or 40  $\mu$ g of  $\Delta(-750)$ . Transfected cells were treated for 48 hr with 10  $\mu$ M forskolin (f) or ethanol vehicle control (c). Cell extracts were assayed for CAT activity by thin-layer chromatography to separate acetylated forms (upper spots) from unreacted chloramphenicol.

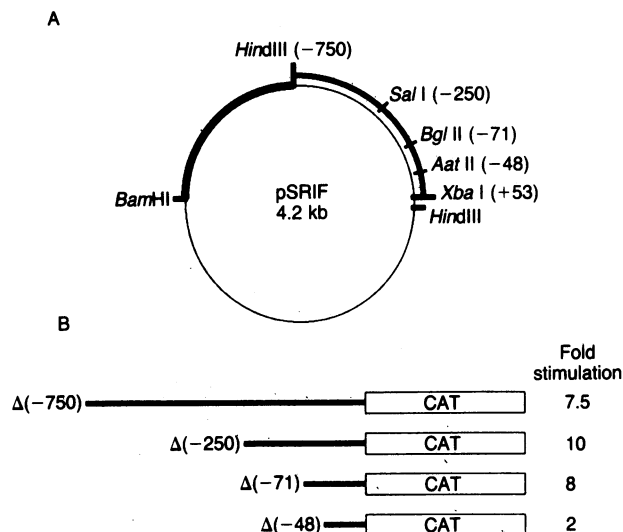


FIG. 3. Effect of forskolin on activity of somatostatin-CAT fusion genes. (A) Fragments of the somatostatin 5'-flanking region were prepared from pSRIF and inserted upstream of the bacterial CAT gene. (B) Four somatostatin-CAT constructions were prepared and analyzed for cAMP responsiveness after transfection into PC12 cells. Fold stimulation for each construction was calculated by dividing the percent conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms in forskolin-treated samples by the percent conversion in control (ethanol-treated) samples.

**The cAMP-Responsive Element of the Somatostatin Gene Regulates a Heterologous Promoter.** To determine whether sequences in the 5'-flanking region of the somatostatin gene could confer cAMP responsiveness to a promoter not normally regulated by this agent, we constructed the fusion gene  $\Delta(-60/-29)$  (Fig. 4A). This construction, which is based on the plasmid pA10-CAT2 (17), contains a region of the somatostatin gene between -60 and -29 inserted into a site 135 bases upstream from the SV40 transcriptional initiation site. Basal expression of  $\Delta(-60/-29)$  transfected into PC12 cells was comparable to that of pA10-CAT2. After treatment with forskolin, wild-type cells transfected with  $\Delta(-60/-29)$  increased their level of CAT activity by 15-fold at all levels of plasmid DNA tested (Fig. 4B). In contrast, cells transfected with the parental vector pA10-CAT2 increased their level of CAT expression by only 1.5-fold after forskolin treatment (data not shown).

Within the somatostatin gene portion of  $\Delta(-60/-29)$ , we observed a palindrome (5'-CTGACGTCAG-3'), which is highly conserved in a number of other cAMP-responsive genes (see Discussion and Table 1). To determine whether the conserved palindrome contained sufficient information to allow responsiveness to cAMP, we constructed a double-stranded 14-mer containing the sequence -CTGACGTCAG-flanked by BamHI restriction sites. The synthetic oligonucleotide was inserted into pA10-CAT2, 135 bases upstream from the SV40 transcriptional initiation site. This construction was designated  $\Delta(-49/-39)$ . In contrast to  $\Delta(-60/-29)$ , CAT activity derived from  $\Delta(-49/-39)$  was not induced by forskolin. Therefore, the 10-base palindrome does not appear to be sufficient for cAMP responsiveness.

**Expression of Somatostatin-CAT Fusion Genes in cAMP-Dependent Protein Kinase 2-Deficient PC12 Cells.** To determine whether induction of the somatostatin promoter by cAMP requires cAMP-dependent protein kinase activity, we examined the expression of the somatostatin-CAT fusion genes in the PC12 mutant cell line A126-1B2. Expression of both the pRSV-CAT and somatostatin-CAT fusion genes was 5 times higher in A126-1B2 cells than in wild-type PC12 cells. Baseline CAT activity of the fusion genes remained

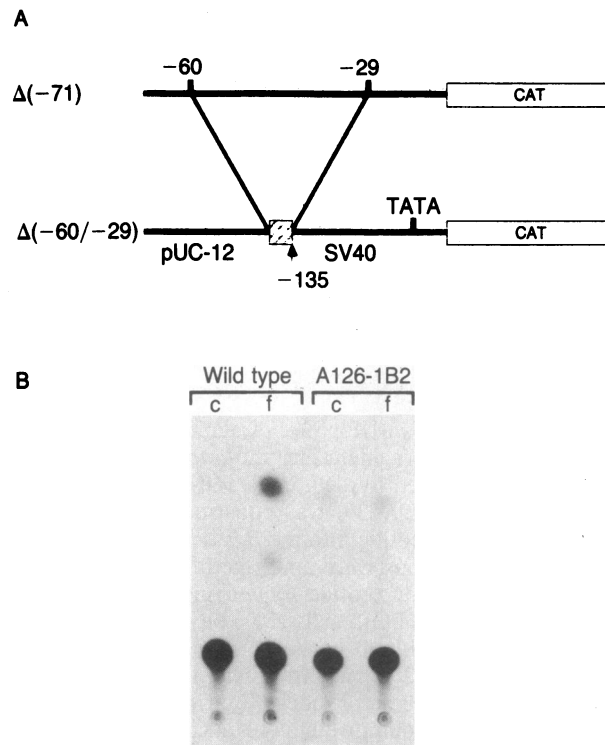


FIG. 4. Effects of forskolin on CAT activity of plasmid  $\Delta(-60/-29)$  in wild-type and A126-1B2 PC12 cells. (A) A 31-bp *Nru*I-*Dra*I fragment from  $\Delta(-71)$  was inserted into the *Bgl*II site of pA10-CAT2, 135 bases upstream of the simian virus 40 (SV40) transcriptional initiation site. (B) Conversion of [ $^{14}$ C]chloramphenicol to acetylated forms in wild-type and A126-1B2 cells after transfection with  $\Delta(-60/-29)$ . An f indicates cells treated for 48 hr with 10  $\mu$ M forskolin; c indicates control cells treated with ethanol vehicle. This experiment represents CAT activity derived from 20  $\mu$ g of  $\Delta(-60/-29)$ . Identical results were obtained with 5, 10, and 40  $\mu$ g of plasmid.

proportional to the amount of DNA transfected, however. Fig. 4B shows the effect of forskolin on activity of plasmid  $\Delta(-60/-29)$  in wild-type and A126-1B2 cells. In contrast to the 15-fold stimulation of somatostatin-CAT activity in wild-type PC12 cells, CAT activity in A126-1B2 cells increased by only 1.5-fold.  $\Delta(-750)$ ,  $\Delta(-250)$ , and  $\Delta(-71)$  were also only minimally induced in A126-1B2 cells (data not shown).

## DISCUSSION

Our studies define an element within the 5'-flanking region of the rat somatostatin gene that is required for regulation by cAMP. This 31-bp element confers cAMP inducibility when placed upstream from the SV40 promoter, which is not normally regulated by cAMP. Comparison of the cAMP-responsive sequence within the somatostatin gene to sequences near the promoters of several other genes known to be regulated by cAMP reveals a highly conserved palindrome, 5'-TGACGTCAG-3' (Table 1). A similar sequence is found in some genes that are known to be regulated by cAMP in PC12 cells, including the tyrosine hydroxylase gene (ref. 7; C. Harrington, E. Lewis, and D. Chikaraishi, personal communication) and *c-fos* (8, 28). Other genes that are regulated by cAMP when transfected into PC12 cells, such as the VIP gene, also contain the palindromic sequence (unpublished observations).

Surprisingly, several viral LTRs and enhancer elements contain the same palindromic sequence that we have identi-

Table 1. Homology in 5'-flanking regions of genes containing the somatostatin palindrome

Gene	Sequence	Ref(s).
Somatostatin*	CTGGGGGCGCCTCCTTGGCT <b>GACGTCAGAGAGAGAG</b> (-32)	13, 21, 22
PEPCK*	TGATCCAAAGGCCGGCCCT <b>TACGTCAGAGGCGAGC</b> (-74)	23
VIP*	TCCCATGGCCGTCATACTGT <b>GACGTC</b> TTTCAGAGCA (-60)	24
Parathyroid hormone	GGGAGT <b>GACGTC</b> ATCT (-65)	25
Proenkephalin*	GGGCCT <b>GCGTCAGC</b> (-87)	26
$\alpha$ -Chorionic gonadotropin	AAAATT <b>GACGTC</b> ATGG (-113)	27
<i>c-fos</i> *	CCGCCAGT <b>GACGTAGGA</b> (-57)	28
Cytomegalovirus enhancer	CCACCC <b>ATTGACGTC</b> AAATGGGAGTT (-124)	29
BLV LTR	ACCAGACAGAG <b>ACGTCAGCTGCCAGA</b> (-144)	30
HTLV-II LTR	CCACGGCC <b>TGACGTC</b> CCCTCCCCC (-162)	31
Intracisternal A particle	CCTCTCCCG <b>TGACGTC</b> ATCTGGGG (-86)	32

Asterisks indicate genes that are known to be transcriptionally regulated by cAMP. Boldface sequences indicate homology with the palindrome. The position of the 3'-most nucleotide is listed in parentheses at the end of each sequence. PEPCK, phosphoenolpyruvate carboxykinase; BLV, bovine leukemia virus; LTR, long terminal repeat; HTLV-II, human T-cell leukemia virus type II.

fied in the somatostatin gene. For example, the 3' and 5' LTRs of the bovine leukemia virus and human T-cell leukemia virus type II contain the the palindrome at positions -144 and -162, respectively (Table 1). The palindrome is also found within the 19-bp repeat of the mammalian cytomegalovirus enhancer. It is unknown whether these sequences actually mediate cAMP responsiveness in viruses, however.

Although the palindrome is a component of the regulatory region of many cAMP-responsive genes, it is not in itself sufficient for cAMP inducibility. The somatostatin-CAT fusion gene  $\Delta(-48)$  contains the palindrome but is considerably less responsive than  $\Delta(-71)$ ,  $\Delta(-250)$ , or  $\Delta(-750)$ . Furthermore, a 10-bp DNA fragment representing only the palindrome does not confer cAMP inducibility when placed upstream of the SV40 promoter. The palindrome is frequently flanked at its 5' end by a G+C-rich domain and at its 3' end by sequences containing the dinucleotide AG (Table 1). These flanking sequences may be necessary for cAMP responsiveness.

It is possible that the element we have identified binds a specific transcription factor whose level or binding affinity is regulated by cAMP. Alternatively, cAMP could act through a different element in the somatostatin gene (the polymerase 2 binding site, for example) that is dependent on the palindrome or adjacent sequences for maximal activity.

Studies using A126-1B2 cells indicate that cAMP-dependent protein kinase activity may be necessary for regulation of somatostatin gene expression by cAMP. There are two mechanisms by which cAMP-dependent protein kinase might activate gene expression. First, the regulatory subunit of cAMP-dependent protein kinase may influence gene expression directly. In response to cAMP, the regulatory subunits dissociate from the catalytic subunits and appear to be transported into the nucleus (33). Constantinou *et al.* (34) have reported that the type 2 regulatory subunits possess topoisomerase activity and may regulate gene expression by altering chromatin structure near or within responsive genes. Alternatively, cAMP may stimulate gene transcription by phosphorylation of a protein intermediate. Free catalytic subunit microinjected into hepatoma cells, for example, causes an increase in tyrosine aminotransferase biosynthesis (35). Our results with the mutant PC12 line A126-1B2 support the hypothesis that cAMP-dependent protein kinase type 2 is required for cAMP-induced effects on eukaryotic gene expression. The present study does not distinguish between effects mediated by the catalytic or regulatory subunits, however.

In bacteria, cAMP binds to catabolite repressor protein, and this complex regulates gene expression directly by interacting with specific DNA sequences. Our observations suggest that transcriptional regulation of eukaryotic gene

expression by cAMP may also involve conserved promoter sequences. However, the palindrome is not found near the promoters of several eukaryotic genes known to be responsive to cAMP (e.g., growth hormone, prolactin). There may therefore be multiple mechanisms by which cAMP regulates gene expression in eukaryotes.

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