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Dual enhancer activities of the cyclic-AMP responsive element with cell type and promoter specificity

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ABSTRACT

The role of the cyclic-AMP (cAMP) responsive element (CRE) in eukaryotic gene transcription was investigated in several cell lines transfected by constructs containing the chloramphenicol acetyltransferase (CAT) gene linked to the three different promoters, simian virus(SV) 40, human c-Ha-ras-1, or chicken β-actin promoter, with or without CRE. CRE had inducible enhancer activity only when it was linked to the SV40 promoter and in a few cell lines such as PC12. CRE functioned as a constitutive enhancer with the human c-Ha-ras-1 promoter in all cell lines examined. CRE also had constitutive enhancer activity when it was linked to the chickenβ-actin promoter, but this activity was observed only in KB, HeLa, and A431 cells. The different types of enhancer activities of CRE depending on the cell and promoter may be caused by interaction with different trans-acting factors that were demonstrated by gel retardation analyses.

INTRODUCTION

Enhancers, which have been identified in numerous viral and cellular genes, are important elements for gene expression in eukaryotic cells (for reviews, see refs. 1-3). These enhancers are often, though not always, located 5' from the promoter sites for RNA polymerase II initiation and have a long-range ability to activate transcription of cis-linked genes. The enhancers have been classified into two types, constitutive and inducible. Some constitutive enhancers are specific to tissues or cell type. The inducible enhancers mediate selective expression of genes in response to appropriate signals.

cAMP mediates the hormonal induction of numerous eukaryotic genes. CRE is an inducible enhancer of the genes responsible for the induction of transcription in response to increases in the intracellular cAMP concentration (4, 5). CRE of the rat somato-

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statin gene is in the region between 60 and 29 bp upstream from the transcriptional initiation site, and it contains an 8-base palindrome, 5'-TGACGTCA-3', which is highly conserved in many other genes the expression of which is regulated by cAMP (5). A sequence similar to CRE was also found in the transcriptional regulatory region of various genes such as the adenoviral early genes (6-9). To understand the role of CRE in eukaryotic gene transcription at the molecular level more precisely, we examined the level of transcription from three different promoters with or without CRE in seven cell lines. Our studies showed that CRE had both inducible and constitutive enhancer activities that depended on the promoter and cell line. The interaction between CRE and multiple *trans*-acting factors, which were demonstrated by gel retardation analyses, could cause these dual enhancer activities.

MATERIALS AND METHODS

Cell cultures. The human epidermoid carcinoma cell lines A431 and KB, the human epitheloid carcinoma cell line HeLa, the African green monkey kidney cell line CV1, and the mouse connective tissue cell line L were maintained in ES medium (Nissui Seiyaku Co.) supplemented with 10% fetal bovine serum. NIH3T3 mouse fibroblasts were grown in ES medium supplemented with 10% calf serum. The rat pheochromocytoma cell line PC12 was maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum and 5% horse serum.

Plasmid construction. The structures of the plasmids used are shown in Fig. 1. To construct pA10CATCRE, 30 bp of the synthetic duplex oligonucleotide containing CRE shown in Fig. 1, which contains the sequence between positions -32 and -57 of the rat somatostatin gene (5), were inserted into the *Bgl*II site of pA10CAT2 (10), upstream of the SV40 promoter. The plasmid prasCAT1 contains the 550-bp fragment of the 5' regulatory region of the human c-*Ha*-*ras*-1 proto-oncogene linked to the CAT gene (11). In the plasmid pactCAT, 1350 bp of a fragment containing the 5' regulatory region of the chicken β-cytoplasmic actin gene has been fused to a segment containing the CAT gene (12, 13). To generate the pactCATCRE and prasCATCRE plasmids, 30 bp of synthetic duplex oligonucleotide containing CRE was inserted into

the BamHI site of pactCAT and prasCAT1, about 100 bp downstream of the poly(A) addition signal for CAT mRNA.

DNA transfection and CAT assay. Cells (except for PC12 cells) were plated at a density of 4×10^5 per 10 cm Nunc tissue culture plate. Mixtures of 13 μg of a plasmid DNA and 2 μg of pRSV-β-gal plasmid DNA were transfected by the Ca-PO_4 precipitation method (14) 16-20 h after plating of the cells. The plasmid pRSV-β-gal, which carries the β-galactosidase gene linked to the Rous sarcoma virus LTR promoter, was used to provide an internal control for differences in transfection efficiency between different precipitates by measurement of β-galactosidase activity (15). PC12 cells were plated at a density of 1×10^6 per 6 cm collagen-coated tissue culture plates as described by Schweitzer and Kelly (16) and then transfectd with mixtures of 6 μg of a plasmid DNA and 2 μg of pRSV-β-gal plasmid DNA 16-20 h later. The 8-Br-cAMP treatment was for 16-20 h starting 20 h after transfection at a final concentration of 1 mM.

Forty hours after transfection, cells were collected by scraping with a rubber policeman into 0.25 M Tris-HCl (pH 7.8). Cells were lysed by freeze-thawing and sonication to prepare extracts, and β-galactosidase activities were measured. CAT enzyme reactions were done as described by Gorman et al. (17) with 0.6 μCi of [^{14}C]-chloramphenicol. The amounts of cell extract used for CAT assays were normalized with respect to the β-galactosidase activity. Acetylated reaction products were resolved by TLC analysis on Baker-Flex silica gel IB-FTLC plates. Following autoradiography, the acetylated forms were excised and counted in a scintillation counter.

Southern blot and RNA analyses. To analyze the copy number of the transfected plasmid DNA, nuclei were isolated to prepare DNAs 40h after transfection, and at the same time β-galactosidase activity was measured to confirm that transfection efficiencies were similar. Twenty micrograms of nuclear DNA was digested with EcoRI and analyzed by the method of Southern (18). The hybridization probe was a nick-translated 1.6 kb BamHI-HindIII fragment containing the CAT gene. Forty hours after transfection, RNA was isolated by the guanidium thiocyanate method (19), and at the same time β-galactosidase activities were measured in a sample

of cells to confirm that transfection efficiencies were similar. Primer extension analysis was done with 40 μg of total RNAs as described previously (20). A synthetic 24 base oligonucleotide complementary to nucleotides 26-49 of the coding sequence of the CAT gene was used as a primer. S1 analysis was done as described elsewhere (21) with 40 μg of total RNA. The 528-base single-stranded probe containing the sequences of prasCAT1 from 472 bp upstream of the junction (the HindIII site) between the c-Ha-ras-1 segment and the CAT gene to 56 bp downstream of this junction site was used for S1 analysis.

cAMP analysis. Concentrations of the intracellular cAMP were measured with use of a cAMP assay kit (Amersham Corp.) that contained [^{3}H]cAMP and bovine muscle-binding protein, according to the protocol of the supplier.

Analysis of CRE-binding proteins. Nuclear extracts were made from HeLa and NIH3T3 cells as described by Dignam et al.(22). The gel retardation assay was done as reported by Singh et al. (23). First, 10 μg of HeLa or 20 μg of a nuclear extract of NIH3T3 cells was mixed with 1 μg of poly(dI-dC) and incubated in a 15-μl

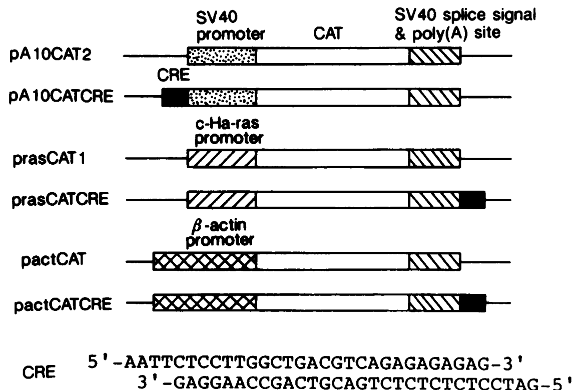

Figure 1. Structure of the CAT constructs used. In the plasmids pA10CAT2, prasCAT1, and pactCAT, the CAT genes were fused to the promoters of the SV40 early gene, the human c-Ha-ras-1 gene, and the chicken β-actin gene, respectively. The 30-bp of the duplex oligonucleotide containing the CRE of the rat somatostatin gene shown at the bottom was chemically synthesized and inserted into the BglII site of pA10CAT2 and the BamHI site of both prasCAT1 and pactCAT, as shown by the filled box. The same oligonucleotide was also inserted into the BamHI site of pA10CAT2, the XhoI site of prasCAT1, or the XhoI site of pactCAT (not shown here).

reaction mixture containing 10 mM Tris-HCl (pH 7.8), 1 mM DTT, 1 mM $MgCl_2$, 50 mM KCl and 10% glycerol for 15 min at 0^oC. The 20 fmol (3×10^4 cpm) of the ^{32}P-labeled 30-bp oligonucleotide shown in Fig. 1 was then added, and incubation continued for 15 min at 25^oC. DNA-protein complexes were separated on a 10% nondenaturing polyacrylamide gel (cross-linking ration, 30:1) containing 6.6 mM Tris-HCl (pH 7.5), 3.3 mM $NaCH_3CO_2$, and 1 mM EDTA. In competition experiments, a 50-fold excess of 30-bp oligonucleotide competitor and [^{32}P]-labeled oligonucleotide probe were mixed together and added to the preincubation mix.

RESULTS AND DISCUSSION

Inducible enhancer activity of CRE linked to the SV40 early gene promoter. First, we used the pA10CAT2 and pA10CAT2CRE constructs in which the SV40 promoter was linked to the CAT gene. The SV40 promoter in these constructs contains a TATA box 30 bp upstream of the RNA start site and six tandemly arranged GC boxes in the region between 50 and 110 bp upstream of the RNA start site, and have no CAAT box (24). Therefore, in the absence of the enhancer, the level of transcription from the SV40 promoter is regulated by the transcriptional factor Sp1 that binds to the GC boxes (25). When pA10CATCRE containing CRE was transfected into PC12 cells, the addition of 8-Br-cAMP stimulated the level of CAT activity 6-fold (Fig. 2, A and B). In the control experiment with pA10CAT2, 8-Br-cAMP did not affect the level of CAT activity (data not shown). These results confirmed that CRE has inducible enhancer activity in PC12 cells when linked to the SV40 promoter (5). To examine the cell-type specificity of inducible enhancer activity of CRE, we then used CV1, NIH3T3, L, HeLa, KB, and A431 cells for transfection. The level of CAT activity expressed from pA10CATCRE was stimulated slightly by 8-Br-cAMP in CV1 and NIH3T3 cells, but was unaffected in L, HeLa, KB, and A431 cells (Fig. 2, A and B). We also obtained the similar results with the construct that was made by insertion of CRE into the BamHI site of the pA10 CAT2 plasmid, about 100 bp downstream of the poly (A) addition signal for CAT mRNA (data not shown). To check whether the CAT enzyme activities were correlated with the level of correctly initiated RNA, primer extension analysis was done with a primer

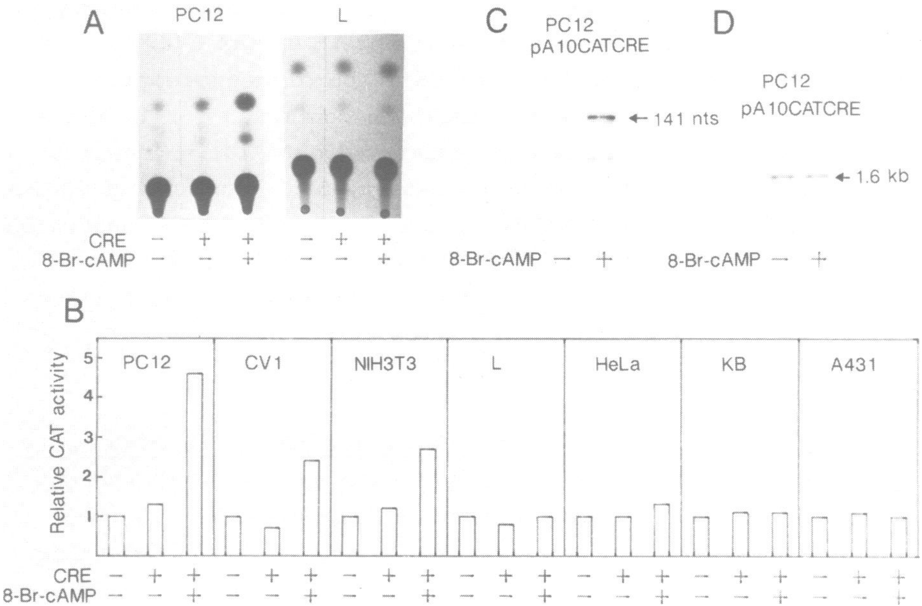

Figure 2. Inducible enhancer activity of CRE when linked to the SV40 promoter. A, B: Effects of CRE and 8-Br-cAMP on expression of the CAT gene from the SV40 promoter. The plasmid pA10CAT2 (CRE(-)) or pA10CATCRE (CRE(+)) was transfected into the cells shown above and treated with (+) 8-Br-cAMP or not (-). CAT reactions were done for 2 h (PC12), 1.5 h (CV1), 0.5 h (NIH3T3 and L), 4 h (HeLa), 12 h (KB) and 18 h (A431), with use of a normalized amount of extract by the β-galactosidase activity. Typical autoradiograms are shown in A. In B, activities are expressed relative to the activity of pA10CAT2 when not treated with 8-Br-cAMP. C: Primer extension analysis of CAT RNA. RNA was prepared from the PC12 cells transfected by pA10CATCRE and treated with (+) 8-Br-cAMP or not (-). The band 141-nucleotide long (nts) corresponding to the predicted length of SV40-CAT mRNA initiated from the correct RNA start site is shown by the arrow. The results were consistent with those from the CAT assays. D: Analysis of the copy number of plasmid DNA after transfection into PC12 cells. DNAs prepared from the PC12 cells transfected by pA10CAT CRE and treated with (+) 8-Br-cAMP or not (-), were used for Southern blot analysis. The 1.6-kb band corresponding to the CAT DNA fragment is shown by the arrow.

complementary to a segment of CAT mRNA. If CAT RNA starts at the correct site in the SV40 early promoter, the extended primer should be 141 nucleotides long (56 nucleotides of SV40 mRNA plus 85 nucleotides of CAT RNA) (10). In PC12 cells transfected with pA10CATCRE, the intensity of the 141-nucleotide band was in-

Table 1. Increase of the intracellular cAMP concentration by 8-Br-cAMP treatment.

Cells	8-Br-cAMP treatment	Concentration of cAMP pmol/ mg protein	pmol/ 10^6 cells	relative value[a]
PC12	-	23	2	1
	+	4,414	388	199
NIH3T3	-	60	34	1
	+	5,793	3,307	97
HeLa	-	6	2	1
	+	3,027	817	480

[a] Relative value was calculated by division of cAMP concentration by that in control samples without 8-Br-cAMP treatment.

creased about 5-fold by 8-Br-cAMP (Fig.2C), indicating that 8-Br-cAMP stimulated specific and correct initiation at the SV40 early gene promoter. The intensity of the 141-nucleotide band was not affected by 8-Br-cAMP in HeLa cells (data not shown).

We also tested the possibility that 8-Br-cAMP was acting by increasing the copy number of the transfected plasmid. After PC12 cells were transfected with pA10CATCRE and treated with 8-Br-cAMP or not, the DNA was extracted from nuclei and used for Southern blotting analysis. In both cases, the intensities of 1.6-kb bands detected by a CAT probe were about the same (Fig. 2D), showing that the addition of 8-Br-cAMP did not affect the copy number of pA10CATCRE plasmid DNA after transfection. Taken together, these results suggest that the inducible enhancer activity of CRE linked to the SV40 early gene promoter is specific to cell type.

To find whether this cell type specificity resulted from differences in the extent of increase in the intracellular cAMP concentration between different cell lines, cAMP in the various cells was measured by radioimmunoassay (Table 1). In PC12 and NIH3T3 cells in which CRE functioned as an inducible enhancer, the level of cAMP was increased about 200- and 100-fold, respectively, by 8-Br-cAMP treatment. The addition of 8-Br-cAMP increased the level of cAMP about 480-fold in HeLa cells in which CRE had no inducible enhancer activity. These results indicated that the cell-type specificity of the inducible enhancer activity

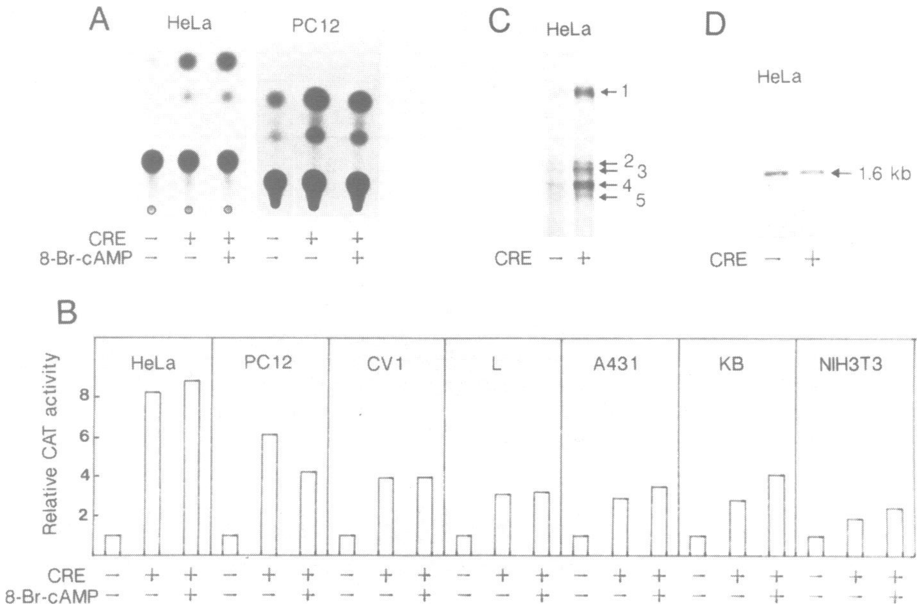

Figure 3. Constitutive enhancer activity of CRE when linked to the human c-Ha-ras-1 promoter. A,B: Effects of CRE and 8-Br-cAMP on expression of the CAT gene from the human c-Ha-ras-1 promoter. The plasmid prasCAT1 (CRE(-)) or prasCATCRE (CRE(+)) was transfected into the cells shown above and treated with (+) 8-Br-cAMP or not (-). CAT reactions were done for 1 h (HeLa, PC12, and CV1), 0.7 h (L), 18 h (A431), 2 h (KB), and 0.5 h (NIH3T3), with use of a normalized amount of extract by the β-galactosidase activity. Typical autoradiograms are shown in A. In B, activities are expressed relative to the activity of prasCAT1 when not treated with 8-Br-cAMP. C: S1 analysis of CAT RNA. RNA was prepared from HeLa cells transfected with prasCAT1 (CRE(-)) or prasCATCRE (CRE(+)). The five major bands are shown by arrows 1-5. The results were consistent with those from CAT assays. D: Analysis of the copy number of plasmid DNA after transfection into HeLa cells. DNAs prepared from the HeLa cells transfected by prasCAT1 (CRE(-)) or prasCATCRE (CRE(+)) were used for Southern blot analysis. The 1.6-kb band corresponding to CAT DNA fragment is shown by the arrow.

of CRE was not related to the extent of increase in the intracellular cAMP level.

Constitutive enhancer activity of CRE linked to the human c-Ha-ras-1 promoter. To examine the promoter specificity, we analyzed the effect of CRE on expression from another promoter, the human c-Ha-ras-1 promoter (Fig. 3). The human c-Ha-ras-1 promoter

in the prasCAT1 has six Sp1-binding sites (146, 157, 266, 326, 377, and 404 bp upstream of the junction between the promoter fragment and the CAT gene, respectively), one CAAT box (304 bp upstream of the junction between the promoter fragment and the CAT gene), and no TATA box (26). The transcription factor Sp1 and the CTF (CAAT transcriptional factor) bound to the CAAT box are positive regulators of this promoter (26,27, Nagase and Ishii; unpublised results). The plasmids prasCAT1 and prasCATCRE containing CRE were used for transfection. When HeLa cells were transfected with prasCAT1 or prasCATCRE, the CAT activity obtained with prasCATCRE was 8-fold higher than that with prasCAT1. The 8-Br-cAMP treatment did not affect the level of CAT activity obtained by prasCAT1 or prasCATCRE (Fig. 3, A and B). These results suggested that CRE had not inducible enhancer activity but constitutive enhancer activity when linked to the human c-*Ha*-*ras*-1 promoter. To evaluate the cell-type specificity of this activity of CRE, we used other cell lines for transfection. The addition of CRE stimulated the level of CAT activity 6-fold in PC12 cells, 4-fold in CV1 cells, 3-fold in L, A431, and KB cells, and 2-fold in NIH3T3 cells. The similar results were obtained with the construct that was made by insertion of CRE into the *Xho*I site of the prasCAT1 plasmid, about 70 bp upstream of the most upstream Sp1 binding site (data not shown).

To confirm that CAT activities were correlated with the level of correctly initiated RNA, S1 analysis was done. RNA from HeLa cells transfected with prasCAT1 or prasCATCRE produced the same five major protected DNAs (Fig. 3C, bands 1-5). The most abundant protected DNAs were 240, 220, 145, 135, and 127 bases long (Fig. 3C, bands 1-5, respectively). We reported the start site of the human c-*Ha*-*ras*-1 gene is heterogeneous, occurring 40, 28, 23, and 16 bp upstream from the boundary between the first exon and the first intron (11). The 127-base protected DNA (Fig. 3C, band 5) corresponded to the predicted length of c-*Ha*-*ras*-CAT mRNA initiated from the most upstream RNA start site reported previously. The four protected DNAs (Fig. 3C, bands 1-4) corresponded to the length of transcripts initiated from sites 10, 20, 95, and 115 bp upstream from the most upstream RNA start site identified previously. This difference between the observed length and the

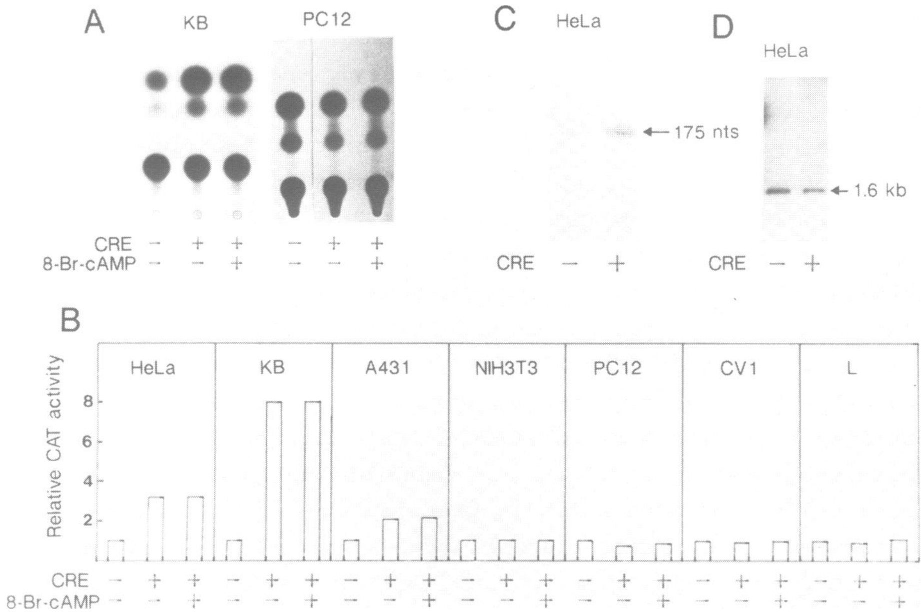

Figure 4. Constitutive enhancer activity of CRE when linked to the chicken β-actin promoter. A, B: Effects of CRE and 8-Br-cAMP on expression of the CAT gene from the chicken β-actin promoter. The plasmid pactCAT (CRE(-)) or pactCATCRE (CRE(+)) was transfected and treated with (+) 8-Br-cAMP or not (-). CAT reactions were done for 1 h (HeLa and KB), 3 h (A431), 0.2 h (NIH3T3), 0.6 h (PC12), 0.5 h (CV1), and 0.25 h (L), with use of a normalized amount of extract by the β-galactosidase activity. Typical autoradiograms are shown in A. In B, activities are expressed relative to the activity of pactCAT when not treated with 8-Br-cAMP. C: Primer extension analysis of CAT RNA. RNA was prepared from HeLa cells transfected by pactCAT (CRE(-) or pactCATCRE (CRE(+)). The band 175-nucleotide long (nts) corresponding to the predicted length of β-actin-CAT mRNA initiated from the correct cap site is shown by the arrow. The results were consistent with those from the CAT assays. D: Analysis of the copy number of plasmid DNA after transfection into HeLa cells. DNAs prepared from HeLa cells transfected by pactCAT (CRE(-)) or pactCATCRE (CRE(+)) were used for the Southern blot analysis. The 1.6-kb band corresponding to the CAT DNA fragment is shown by an arrow.

predicted length of protected DNAs could be caused by differences in the assay: in this study, RNA was expressed transiently from transfected extrachromosomal DNA, but earlier experiments used RNA transcribed permanently from the gene in a chromosome. To measure the densities of the protected bands, autoradiograms were

traced and the peak areas for each band were measured. CRE enhanced the level of the protected products about 8-fold in HeLa cells. Using NIH3T3 cells, we observed the same protected bands, and CRE stimulated the level of the protected products about 3-fold (data not shown). These results indicated that the levels of CAT RNA were correlated with those of CAT enzyme activities.

To check the possibility that CRE was acting by increasing the copy number of the transfected plasmid, Southern blotting analysis was done again. Insertion of CRE into the BamHI site of prasCAT1 did not affect the copy number of transfected DNA (Fig. 3D). The results indicated that CRE functioned as a constitutive enhancer when linked to the human c-Ha-ras-1 promoter; this activity was observed in all of the seven cell lines examined.

Cell-type specificity of constitutive enhancer activity of CRE linked to the chicken β-actin promoter. To examine the promoter specificity of the enhancer activity of CRE in more detail, we used the chicken β-actin promoter (Fig.4). The chicken β-actin promoter in the pactCAT has a TATA box, a CAAT box 30 and 90 bp upstream of the RNA start site, respectively, and four GC boxes in the region between 115 and 145 bp upstream of the RNA start site (12). Insertion of CRE into the BamHI site of pactCAT stimulated the level of CAT activity 8-fold in KB cells, 3-fold in HeLa cells, and 2-fold in A431 cells. The 8-Br-cAMP treatment did not affect the level of CAT expression from pactCATCRE (Fig. 4, A and B). On the other hand, the level of CAT activity obtained by pactCATCRE was almost the same as that with pactCAT in NIH3T3, PC 12, CV1, and L cells (Fig. 4, A and B). In these cells, 8-Br-cAMP did not stimulate the CAT expression from pactCATCRE. We also obtained the similar results with the construct that was made by insertion of CRE into the XhoI site of the pactCAT plasmid, about 280 bp upstream of the RNA start site (data not shown).

To confirm that CAT activities were correlated with the level of correctly initiated RNA, primer extension analysis was done again with a primer complementary to a segment of CAT mRNA. If CAT RNA starts at the correct site in the β-actin promoter, the extended primer should be 175 nucleotides long (90 nucleotides of β-actin mRNA plus 85 nucleotids of CAT RNA) (12, 13). RNA from HeLa cells transfected with pactCAT or pactCATCRE produced ex-

Table 2. Dual enahncer activities of CRE.

Promoter \ Cells	PC12	NIH3T3 CV1	HeLa KB	A431	L
SV40	I[a]	I	-	-	-
c-Ha-ras 1	C[b]	C	C	C	C
β-actin	-[c]	-	C	C	-

[a]Inducible enhancer activity
[b]Constitutive enhancer activity
[c]No enhancer activity

tended primers 175 nucleotides long, and the intensity of the 175-nucleotide band was increased about 4-fold by insertion of CRE into the BamHI site of pactCAT (Fig. 4C). Using NIH3T3 cells, we observed the same 175-nucleotide extended primer, and CRE did not stimulate the level of the extended product (data not shown), indicating that the levels of CAT RNA were correlated with those of CAT enzyme activities. Southern blotting analysis was also done and showed again that CRE did not affect the copy number of transfected DNA (Fig. 4D). These results indicated that CRE functioned as a constitutive enhancer that was cell-type specific when linked to the β-actin promoter.

Multiple trans-acting factors bound to CRE. The enhancer activities of CRE are summarized in Table 2. The enhancer activities observed are position-independent that is consistent with other reports published so far (4,28). The inducible enhancer activity in response to cAMP was observed only in PC12, NIH3T3, and CV1 cells when they were linked to the SV40 promoter. These results raised the possibility that the trans-activator bound to CRE, which is responsible for inducible-enhancer activity, exists only in cells such as PC12 cells. The cAMP regulatory pathway in mammalian cells has been studied in considerable detail. CAMP-dependent protein kinases are activated in response to increased c-AMP concentrations. These activated kinases then phosphorylate proteins that presumably modulate transcription. The pathway to generate the trans-activator responsible for the inducible enhancer activity of CRE may work in a limited number of cell lines such as PC12 cells. CRE did not function as an inducible enhancer

when linked to the c-Ha-ras-1 or the β-actin promoter even in PC 12 cells. These results may suggest that interaction between the trans-acting factor bound to CRE and the specific sequence(s) of promoter or factor(s) bound to the promoter is necessary for the inducible enhancer activity of CRE.

CRE also functioned as a constitutive enhancer in a cell-type specific way when linked to the β-actin promoter, but constitutive enhancer activity of CRE linked to the c-Ha-ras-1 promoter was observed in all of the cell lines examined. Two mechanisms are possible for this cell type specificity of the constitutive enhancer activity of CRE. First, there are at least two different trans-activators for the constitutie enhancer activity that exist with different cell type specificity, and the selection of trans-activator bound to CRE depends on the promoter because the interaction between a trans-activator bound to CRE and the promoter sequence or the promoter factor is necessary. Another possibility is that there is only one trans-activator bound to CRE, but that interaction between the trans-activator bound to CRE and promoter factors that exist with various cell type specificities is necessary for constitutive enhancer activity. The second possibility seems unlikely, because the β-actin gene is expressed in almost all cell lines, suggesting that the promoter factors for the β-actin gene are not specific to cell type.

According to these speculations, HeLa cells and NIH3T3 cells may have both the common and distinct CRE-binding proteins. To examine the possibility of multiple trans-acting factors for CRE, the gel retardation assays were used. The double-stranded oligonucleotide containing CRE of the somatostatin gene shown in Fig.1 was [^{32}P]-labeled at the 5'-end and incubated with a nuclear extract of NIH3T3 or HeLa cells; the complexes formed were analyzed by polyacrylamide gel electrophoresis at low ionic strength. Multiple bands were observed for each extract. A self-competition experiment with unlabeled homologous competitor DNA showed that two bands with the NIH3T3 extract (Fig.5, bands a and b) and two bands with the HeLa extract (Fig. 5, bands c and d) represent specific complexes, although band d was less competed with 50-fold more amount of the competitor. The electrophoretic mobilities of bands a and c were similar, suggesting that the

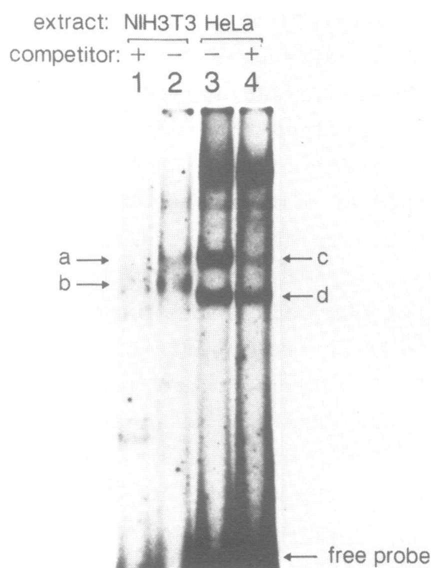

Figure 5. Binding of multiple nuclear factors to CRE. The 30-bp end-labeled oligonucleotide containing CRE shown in Fig. 1 was used for a gel retardation assay. For the binding reaction, 10 μg of a nuclear extract of HeLa (lanes 1 and 2) or 20μg of a nuclear extract of NIH3T3 cells (lanes 3 and 4) was included. For competition experiments, a 50-fold excess of the 30-bp oligonucleotide containing CRE was added (lanes 1 and 4). The retarded bands, which were competed out in a self-competition experiment, are shown by arrows a-d.

same protein existed in both HeLa and NIH3T3 cells and was bound to CRE. The bands b and d, which have different electrophoretic mobilities, indicated the presence of different proteins bound to CRE.

During preparation of this manuscript, Hardy and Shenk also reported finding multiple DNA-protein complex bands in gel retardation assays using the CRE of adenovirus early genes and HeLa cell nuclear extracts (29). Recently, Yamamoto et al. have purified nuclear phosphoprotein (CREB) from rat brain with a molecular weight of 43,000 which binds to the CRE of the rat somatostatin gene, and demonstrated that the dimerization and transcriptional efficacy of CREB in vitro are regulated by phosphorylation (30). Jalinot et al. (31) also purified a 38-kDa protein that binds to the CRE of the adenovirus E2a promoter, which is

therefore smaller than the CREB purified by Yamamoto et al.. The results suggested that there are multiple forms of the CRE-binding protein (CREB). In fact, two genes encoding different CREBs were recently isolated from a human placenta (32 and Ishii et al., unpublished results) and a human brain cDNA library (Maekawa et al., manuscript submitted). These two CREBs have no sequence homology except for the leucine residues in the leucine zipper motif and a cluster of basic amino acids adjacent to the leucine zipper motif, and appears to have the similar DNA binding properties. Heterogeneity of CREB may explain dual enhancer activities of CRE and may be important to our understanding of the regulation of eukaryotic gene expression by CRE.

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REFERENCES

1. Khoury, G. and Gruss, P. (1983) Cell 33, 313-314.
2. Serfling, E., Jasin, M., and Schaffner, W. (1985) Trends Genet. 1, 224-230.
3. Voss, S. D., Schlokat, V., and Gruss, P. (1986) Trends Biochem. Sci. 11, 287-289.
4. Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E., and Goodman, H. M. (1986) Nature 323, 353-356.
5. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. USA 83, 6682-6686.
6. Imperial, M. J., Hart, R. P., and Nevins, J. R. (1985) Proc. Natl. Acad. Sci. USA 82, 381-385.
7. Murthy, S. C. S., Bhat, G. P., and Thimmappaya, B. (1985) Proc. Natl. Acad. Sci. USA 82, 2230-2234.
8. Zajchowski, D. A., Boeuf, H., and Kedinger, C. (1985) EMBO J. 4, 1293-1300.
9. Lee, K. A. W. and Green, M. R. (1987) EMBO J. 6, 1345-1353.
10. Laimins, L. A., Gruss, P., Pozzati, R., and Khoury, G. (1984) J. Virol. 49, 183-189.
11. Ishii, S., Merlino, G. T., and Pastan, I. (1985) Science 230, 1378-1381.
12. Seiler-Tuyns, A., Eldridge, J. D., and Paterson, B. M. (1984) Proc. Natl. Acad. Sci. USA 81, 2980-2984.
13. Schmidt, A., Setoyama, C., and de Crombrugghe, B. (1985) Nature 314, 286-289.
14. Gorman, C. M., Padmanabhan, R., and Howard, B. H. (1983) Science 221, 551-553.
15. Edlund, T., Walker, M. D., Barr, P. J., and Rutter, W. J. (1983) Science 230, 912-916.

16. Schweitzer, E. S. and Kelly, R. B. (1985) J. Cell Biol. 101, 667-676.
17. Gorman, C. M., Moffat, L. M., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044-1051.
18. Southern, E. (1975) J. Mol. Biol. 98, 503-517.
19. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299.
20. Ishii, S., Xu, Y.-H., Stratton, R. H., Roe, B. A., Merlino, G. T., and Pastan, I. (1985) Proc. Natl. Acad. Sci. USA 82, 4920-4924.
21. Ishii, S., Imamoto, F., Yamanashi, Y., Toyoshima, K., and Yamamoto, T. (1987) Proc. Natl. Acad. Sci. USA 85, 4171-4175.
22. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1489.
23. Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986) Nature 319, 154-158.
24. Tooze, J. (1981) Molecular Biology of Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., ed.2.
25. Gidoni, D., Dynan, W. S., and Tjian, R. (1984) Nature 312, 409-413.
26. Ishii, S., Kadonaga, J. T., Tjian, R., Brady, J. N., Merlino, G. T., and Pastan, I. (1986) Science 232, 1410-1413.
27. Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., and Tjian, R. (1987) Cell 48, 79-89.
28. Silver, B. J., Bokar, J. A., Virgin, J. B., Vallen, E. A., Milsted, A., and Nilson, J. H. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2198-2202.
29. Hardy, S. and Shenk, T. (1988) Proc. Natl. Acad. Sci. USA 85, 4171-4175.
30. Yamamoto, K. K., Gonzalez, G. A. S., Biggs, W. H.III, and Montominy, M. R. (1988) Nature 334, 494-498.
31. Jalinot, P., Wintzerith, M., Gaire, M., Hauss, C., Egly, J. M., and Kedinger, C. (1988) Proc. Natl. Acad. Sci. USA 85, 2484-2488.
32. Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L., Habener, J. F. (1988) Science 242, 1430-1433.