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A Calcium Ionophore-Inducible Cellular Promoter Is Highly Active and Has Enhancerlike Properties

AUGUSTINE Y. LIN, SHIN C. CHANG, AND AMY S. LEE*

Department of Biochemistry, University of Southern California School of Medicine, Los Angeles, California 90033

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We examined the regulatory/promoter sequence of a calcium ionophore-inducible gene isolated from the rat genome. Whereas the promoter of this ubiquitously expressed gene is active under noninduced conditions, after induction by calcium ionophore A23187 this promoter is 10- to 25-fold more active than the simian virus 40 early promoter, as measured by chloramphenicol acetyltransferase activities. Within this regulatory/promoter region, we have identified a DNA fragment with enhancerlike properties immediately 5′ to the TATA sequence. This 291-nucleotide fragment acts in *cis* to enhance expression of the neomycin phosphotransferase (*neo*) gene driven by the herpes simplex virus thymidine kinase promoter in an orientation-independent manner. In addition, this fragment can confer A23187 inducibility to the *neo* gene and effectively compete for positive regulatory factors involved in A23187 induction. Sequence analysis of this promoter reveals homology with viral core enhancer sequences, and the apparent organization of direct repeat domains is similar to those observed in viral enhancers.

Transcriptional regulatory elements referred to as "enhancers" are *cis*-acting elements which have profound effects on the transcriptional activity of nearby genes. These elements have been found within a number of DNA viruses and retroviruses (for review, see reference 15), more recently, cellular enhancers, such as those associated with the immunoglobulin, interferon, and metallothionein genes, have been identified (8, 10, 27; A. Haslinger and M. Karin, Proc. Natl. Acad. Sci. USA, in press). Although it has been established that enhancers act by increasing transcriptional activities, little is yet known about the molecular mechanisms involved. However, one expectation might be that strong cellular enhancers are likely to be found in genes that are expressed at high constitutive levels or in genes that can be readily induced. Unlike the enhancers found in tissue-specific genes, these enhancers may be equally effective in many cell types.

Our laboratory has been interested in the transcriptional regulation of two cellular genes that are highly inducible in a variety of mammalian cell types by the calcium ionophore A23187 (28). These genes are constitutively expressed, but within 3 h of treatment with A23187, a 30-fold increase in the transcriptional rate of one of the genes, p3C5, was observed. At the steady state after induction, the p3C5 transcripts represented about 3.5% of the total polyadenylated RNA of the cell, an increase from 0.1% in the basal state. The p3C5 gene can also be induced by glucose starvation in a variety of cultured cells; however, there is a lag period of 16 h, and a 5- to 10-fold induction is generally observed (20).

A hamster mutant cell line, K12, has the unique property that when incubated at the nonpermissive temperature (40.5°C), the transcription of p3C5 is activated 10-fold within 30 min (19). The availability of this temperature-sensitive (ts) mutant allows us to study the interaction of a *trans*-acting regulatory element with its target sequence, the p3C5 gene. Thus, we have recently reported that the 5′ sequence of the p3C5 gene contains sufficient information for the induction of the p3C5 gene by glucose starvation and by the K12 ts mutation (1) and that a 1.25-kilobase (kb) 5′ flanking sequence can confer A23187 inducibility to heterologous fusion genes (28).

In this report, we present the DNA sequence and structural organization of the regulatory/promoter region of the rat p3C5 structural gene. One striking feature of the sequence is the existence of two imperfect direct repeats, each spanning about 170 nucleotides (nt). A 291-nt fragment immediately 5′ to the TATA sequence has enhancerlike properties. It can confer A23187 inducibility to a heterologous gene and can effectively compete for positive regulatory factors involved in induction by A23187 and the K12 ts mutation. The promoter of this ubiquitously expressed gene is active in different cell lines and, after induction by A23187, is 10- to 25-fold more active than the simian virus 40 (SV40) early promoter.

MATERIALS AND METHODS

Cell culture and conditions. K12, a ts mutant derived from Chinese hamster fibroblast cell line Wg1A (18), is routinely maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% cadet calf serum. The normal rat kidney (NRK) cell line was obtained from P. Vogt and was maintained in DMEM supplemented with 10% cadet calf serum. The mouse fibroblast cell line (mouse LA9) was obtained from R. E. K. Fournier and was a hypoxanthine phosphoribosyltransferase-negative derivative of mouse L cells. The cells were maintained in DMEM supplemented with 10% fetal calf serum. Human hepatoma cell line HepG2 was also obtained from R. E. K. Fournier and was maintained in DMEM supplemented with 10% fetal calf serum. COS 7, an SV40-transformed derivative of established African green monkey kidney cell line CV-1p (9), was obtained from A. Berk. The cell line was maintained in DMEM supplemented with 10% fetal calf serum. NIH 3T3 cells were obtained from S. Rasheed and maintained in DMEM supplemented with 10% cadet calf serum and 2 mM glutamine.

Plasmids and their constructions. (i) pI10. Plasmid pI10 was constructed by fusing a 1.25-kb fragment of the 5′ flanking sequence of the rat p3C5 structural gene to the bacterial chloramphenicol acetyltransferase (CAT) gene as described (28).

* Corresponding author.

(ii) pSV2CAT. Plasmid pSV2CAT contained 520 nt of the SV40 early promoter and origin of replication fused to the CAT gene (11) and was obtained from B. Howard (National Institutes of Health).

(iii) phMTCAT. Plasmid phMTCAT contained 770 nt of the human metallothionein II_A gene promoter fused to the Cat gene (14) and was generously provided by M. Karin (University of Southern California).

(iv) pNEO3. The hybrid gene in plasmid pNEO3 contained 680 nt of the herpes simplex virus thymidine kinase (HSV-tk) promoter fused to the neomycin resistance gene (1) and was generously provided by B. Wold (California Institute of Technology).

(v) pNESS41. Plasmid pNESS41 contained the 291-nt *Sma*I/*Stu* fragment inserted 680 nt 5′ to the HSV-tk promoter in pNEO3. The direction of transcription of the *Sma*I/*Stu* fragment is the same as that of the *neo* transcriptional unit (see Fig. 3). Essentially, the 291-nt fragment was isolated from pUC6.2 (1) after digestions with *Sma*I and *Stu*I. The DNA fragment, recovered from low-melting agarose gels and purified over Elutip-d columns (Schleicher and Schuell), was treated with the Klenow fragment of DNA polymerase I before ligation with the *Bam*HI-treated, dephosphorylated pNEO3. The resulting recombinants were transfected into *Escherichia coli* HB101 cells by the Kushner procedure (16). Ampicillin-resistant colonies were screened with a 1.1-kb *Pvu*II subfragment of pUC6.2 which contained the *Sma*I/*Stu* fragment (1).

(vi) pNESS17. Plasmid pNESS17 was constructed identically to pNESS41 except that the *Sma*I/*Stu* fragment was ligated with *Bam*HI synthetic linker before insertion into the *Bam*HI site in pNEO3. The orientation of the *Sma*I/*Stu* fragment in pNESS17 is opposite that of the *neo* transcriptional unit (see Fig. 3).

(vii) pNELK1. Plasmid pNELK1 contained the 291-nt *Sma*I/*Stu* fragment inserted at the *Nde*I site at the 3′ end of the *neo* transcriptional unit on pNEO3 (see Fig. 3). The construction scheme was similar to that described for pNESS41.

(viii) pUC291W. Plasmid pUC291W contained the 291-nt *Sma*I/*Stu* fragment inserted at the *Sma*I site of the polylinker on pUC8 (33).

Transformation conditions. The conditions for DNA transfection have been described (1, 35). Briefly, for stable transformants the transfecting DNA was mixed with 10 μg of high-molecular-weight HeLa cell DNA as carrier in the transformation buffer and added to K12 cells grown as monolayers in 75-cm^2 flasks. Incubation was at 35°C for 20 min. Fresh DMEM was then added to the cells, and incubation was continued for 16 h before glycerol shock. G418 was added to 200 μg/ml 2 days after transfection for the selection of stable transformants. Individual G418-resistant colonies were picked after 2 to 3 weeks and expanded into mass culture. For transient transformants, the transfecting plasmid DNA was mixed with 7 μg of HeLa cell DNA and added to cells grown in 10-cm-diameter cell culture dishes. After incubation at 35°C for 20 min, 5 ml of fresh DMEM was added to the cells, and incubation was continued for 4 h at 35°C before glycerol shock. The cells were harvested 48 to 50 h after transfection without any selection.

Induction conditions. The cells were maintained as monolayers in normal culture medium until they reached about 80% confluency. To test for the effect of calcium ionophore, 7 μM A23187 (Sigma) was added to the culture medium for 16 h. To test for the K12 ts mutation effect, the cells were shifted to 39.5°C for 16 h. To test for the glucose-starvation effect, the cells were incubated in glucose-free medium (20) for 16 h at 35°C.

Isolation of cytoplasmic RNA and hybridization. At the end of the induction period, total cytoplasmic RNA was isolated from the cells as previously described (19). A 10-μg portion of each RNA sample was electrophoresed on formamide-formaldehyde agarose gel and blotted onto nitrocellulose paper (19). The RNA gel blot was then hybridized with nick-translated *neo* DNA fragment with a specific activity of 2×10^8 to 4×10^8 cpm/μg of DNA as described (1).

Assay for CAT activity. The preparation of the cell extract, the measurement of protein in each extract sample, and the assay for CAT activity have been described (28).

Nucleotide sequence analysis. The nucleotide sequence was determined by both the method of Maxam and Gilbert (21) and the dideoxy method of Sanger et al. (29). Analysis of the sequences was aided by the Intelli-Genetics Bionet computer program.

Primer extension. A synthetic oligonucleotide (a 23-mer, with sequence 5′-TGTTGTGCCCAGTCATAGCCGAA-3′) corresponding to the anti-sense strand nt 208–230 of the bacterial *neo* gene (2) was used as primer. The 23-mer was synthesized using a model 380A DNA synthesizer (Applied Biosystems). A 1.6-μg sample of the 23-mer was 5′-end labled with T4 polynucleotide kinase and purified through a DE-52 column. About 10^7 cpm of the DNA was hybridized with 50 μg of cytoplasmic RNA at 42°C for 16 h in a solution containing 60% formamide, 0.3 M NaCl, 7.5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0), and 1.5 mM EDTA. After alcohol precipitation, avian myeloblastosis virus reverse transcriptase was added to the RNA/DNA hybrid in 100 μl of a reaction mixture containing 10 mM $MgCl_2$, 120 mM KCl, 50 mM Tris hydrochloride (pH 8.3), 30 mM β-mercaptoethanol, and 1 mM each of unlabeled deoxyribonucleoside triphosphates. After 90 min at 37°C, the reaction was stopped by the addition of 0.2 N NaOH. The mixture was then incubated at 45°C for 60 min and subsequently neutralized with HCl. The primer-extended products were alcohol precipitated and electrophoresed on 6% polyacrylamide sequencing gels. The predicted size of the primer-extended transcript was 167 nt since the cap site for the HSV-tk promoter (23) was located 53 nt upstream from the *Bgl*II site fusions of the HSV-tk promoter and the *neo* gene in pNEO3.

RESULTS

The pI10 sequence contains a strong cellular promoter inducible by A23187 and temperature in hamster K12 cells. We have previously described the construction of a hybrid gene, pI10, which contained 1.25 kb of the 5′ flanking sequence of the rat p3C5 gene fused to the bacterial CAT gene. After transfection of this construct into hamster cells, basal CAT expression was observed at 35°C. After treatment with 7 μM A23187 for 16 h, a 15-fold increase in the CAT activity was detected (28). To test the specificity of this regulation, we used transient transfection assays to measure the level of CAT activity as driven by the rat p3C5 promoter (pI10), by the SV40 early promoter (pSV2CAT), and by the human metallothionein II_A gene promoter (phMTCAT) in hamster K12 cells, under induced and noninduced conditions.

The results (Fig. 1) demonstrated that (i) the pI10 promoter was a strong cellular promoter and it was highly active under noninduced conditions; (ii) a 10-fold increase in CAT activity after A23187 treatment was only observed in pI10

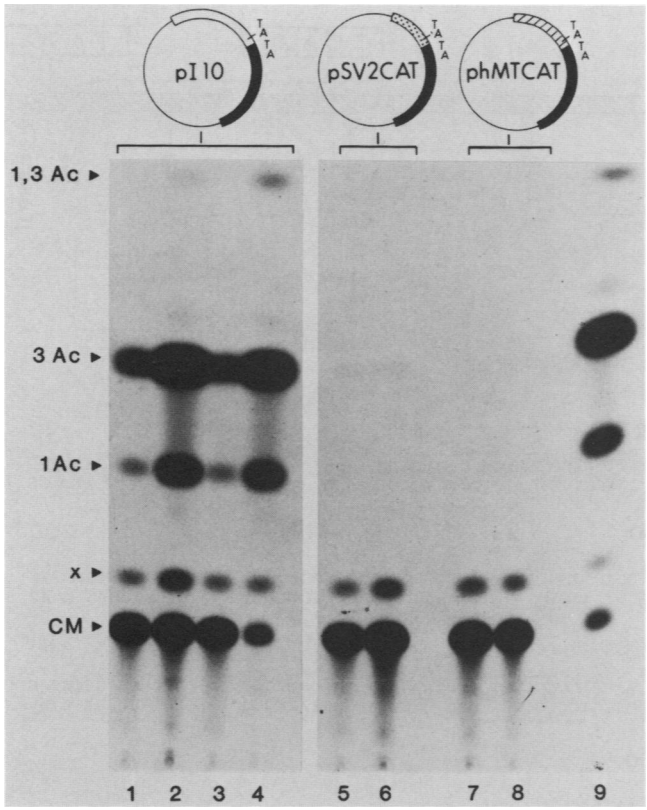


FIG. 1. Comparison of promoter activities in K12 cells. K12 cells were transfected with 3 μg of pI10 (lanes 1 through 4), pSV2CAT (lanes 5 and 6), or phMTCAT (lanes 7 and 8). These plasmids contained the promoters from the rat p3C5 gene (pI10), the SV40 early gene (pSV2CAT), and the human metallothionein II_A gene (phMTCAT). In lanes 1 and 7, the cells were continuously incubated at 35°C in normal medium. In the other samples, 32 h after transfection, the cells were either shifted to 40.5°C (lanes 2 and 5), changed to glucose-free medium (lane 3), or treated with 7 μM A23187 (lanes 4, 6, and 8). Total cell extract was prepared from all samples 48 h after transfection. Equal portions (10 μl) of each sample were assayed for CAT activity. The autoradiogram is shown. The assay performed with 0.1 U of CAT (P-L Biochemicals) is shown in lane 9. The positions of chloramphenicol (CM) and its acetylated forms (1,3-Ac, 3-Ac, and 1-Ac) are indicated. The spot marked x represents an impurity from the [^{14}C]chloramphenicol used.

transformants; (iii) an 8-fold increase in CAT activity was only observed in pI10 transformants when the transfected K12 cells were shifted to the nonpermissive temperature, 40.5°C. Since neither the SV40 early promoter nor the metallothionein promoter showed any substantial response to the calcium ionophore or temperature induction, the regulatory sequences for A23187 and temperature induction contained in pI10 were distinct from the viral gene or heavy metal control elements associated with metallothionein genes.

In the case of stimulation by glucose starvation, we consistently observed a twofold increase in CAT activity in pI10 transformants in transient assays. Because of the relatively low level of stimulation in these assays, induction by glucose starvation is being studied in stable transformants, where the effect is more substantial.

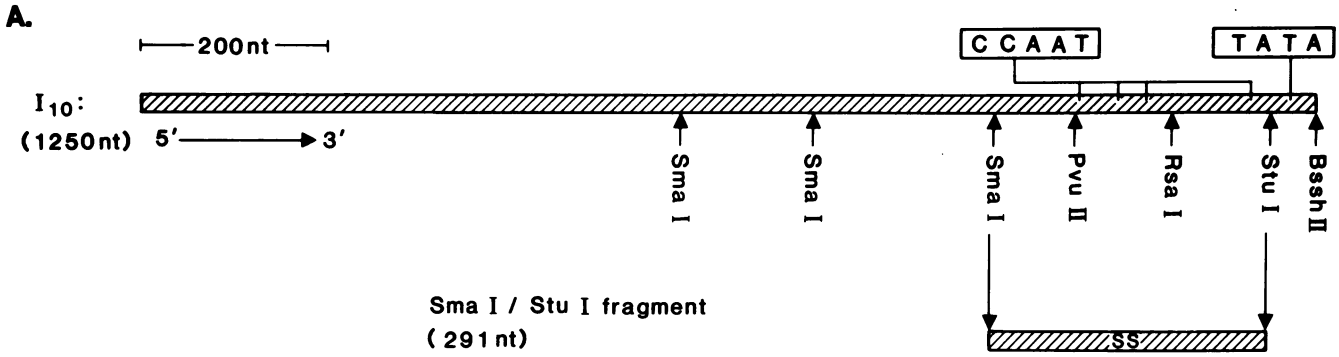
Sequence organization of the rat p3C5 promoter. To characterize the control elements present in pI10, the 1.25-kb fragment was restriction mapped (Fig. 2A), and the nucleotide sequence immediately 5′ to the rat p3C5 structural gene was determined (Fig. 2B). This sequence, when compared with other eucaryotic promoters, shared similar features such as the presence of a TATA sequence and, 30 nt upstream from it, the presence of a CCAAT sequence. Interestingly, three other CCAAT sequences, in either direct or inverted orientations, were located further upstream. A search for inverted repeats yielded four perfect pairs ranging from 7 to 10 nt. Interspersed within this promoter sequence were islands of guanine plus cytosine (GC)-rich regions. The overall GC content of this sequence is 64%, and the ratio of CpG to GpC is about 1.

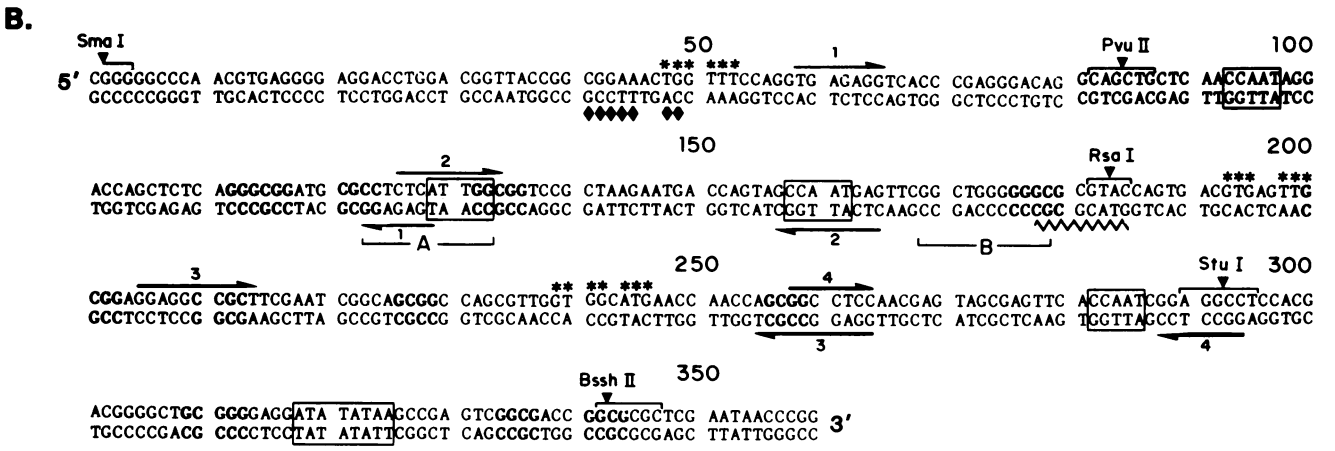
The p3C5 gene is not a major heat shock gene, as it is only induced two- to threefold by heat in wild-type cells (1). However, p3C5 can be viewed as a stress-inducible gene since it is transcriptionally activated by calcium shock (28) and by deprivation of glucose (20). In this context, it is interesting that two blocks of homologous sequence (A and B, Fig. 2B), 11 and 12 nt long, are shared by the promoters of the human heat shock *hsp70* gene (B. Wu, R. Kingston, and R. Morimoto, Proc. Natl. Acad. Sci. USA, in press) and that of the p3C5 gene.

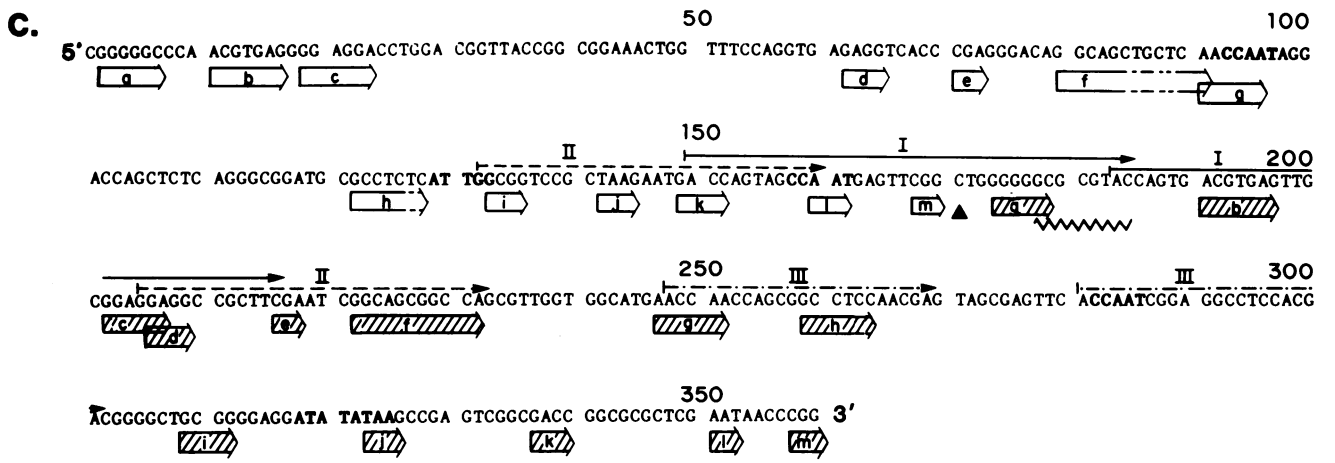
There are many short direct repeats within this relatively compact region, some of which are indicated in Fig. 2C. One notable feature of the sequence is that the direct repeats (a through m) in the entire sequence can be arranged into two units of 170 nt, each containing the exact array of 13 direct repeats. Near the junction of the two untis, there is a stretch of eight alternating purine/pyrimidines characteristic of Z DNA. This tandem repeat arrangement is similar to the shorter, but more perfect repeats commonly observed in viral enhancers. In addition, sequences partially homologous to the viral core enhancer elements (12, 36) can be located within this sequence (Fig. 2B).

Further computer analysis of the sequence yielded three long stretches (22 to 37 nt) of direct repeats (71 to 80% homology), two of which are partially overlapping (Fig. 2D). Deletion analysis of the promoter region suggested that the *Sma/Stu* fragment contributed significantly to basal level transcription as well as to specific induction by A23187 and the K12 ts mutation (S. C. Chang et al., manuscript in preparation).

The *Sma/Stu* element increases transformation efficiencies. To determine whether the promoter described above contained an enhancerlike element, we isolated a *Sma*I/*Stu*I fragment from the 1.25-kb fragment originally contained in pI10 (Fig. 2A). This fragment was 291 nt long and was dissociated from the TATA sequence of the rat promoter since the *Stu*I site was located 25 nt 5′ to the TATA sequence. It retained the four CCAAT sequences and most of the direct and inverted repeats. This fragment was inserted into pNEO3, which contained the HSV-tk promoter sequence fused to the neomycin resistance gene, *neo* (1). The *Sma/Stu* element was inserted at the 5′ or 3′ side of this transcription unit (Fig. 3). In addition, two orientations of insertions were generated for the 5′ insertions. These recombinants were used to transfect K12 cells under normal culture conditions, and the transformation efficiencies are shown in Fig. 3. The transformation efficiencies observed in the recombinants containing the *Sma/Stu* fragment were about 10- to 20-fold higher than those in the parental plasmid pNEO3. The insertion at the 3′ end (pNELK1) was about as effective as the 5′ insertions (pNESS41 and pNESS17), even though the distance of the *Sma/Stu* fragment from the heterologous HSV-tk TATA sequence varied from 680 nt for







D.

I.	150	ACCAGTAGCCAATGAGTT-CGGCTGGGGGGCGCGTAC	185
	184	ACCAGT-GAC-GTGAGTTGCGG-AGGAGGCCGC-TTC	216
II.	132	GGCGGTCCGCTAAGAAT-GACCAGTAGCCA	160
	205	GGAGG-CCGCTTCGAATCGGC-AGCGGCCA	232
III.	248	ACCAACCAGCCGCCTCCAACGA	269
	281	ACCAATCCGAGGCCTCCA-CGA	301

FIG. 2. Sequence organization of the promoter region of the rat p3C5 gene. (A) Schematic representation of the 1.25-kb rat sequence contained in pI10 and the 291-nt *Sma*/*Stu* fragment derived from pI10. (B) Nucleotide sequence of the rat promoter. The sequence was numbered starting from the *Sma*I site, which defines the 5′ end of the *Sma*/*Stu* fragment in panel A. The positions of four pairs of inverted

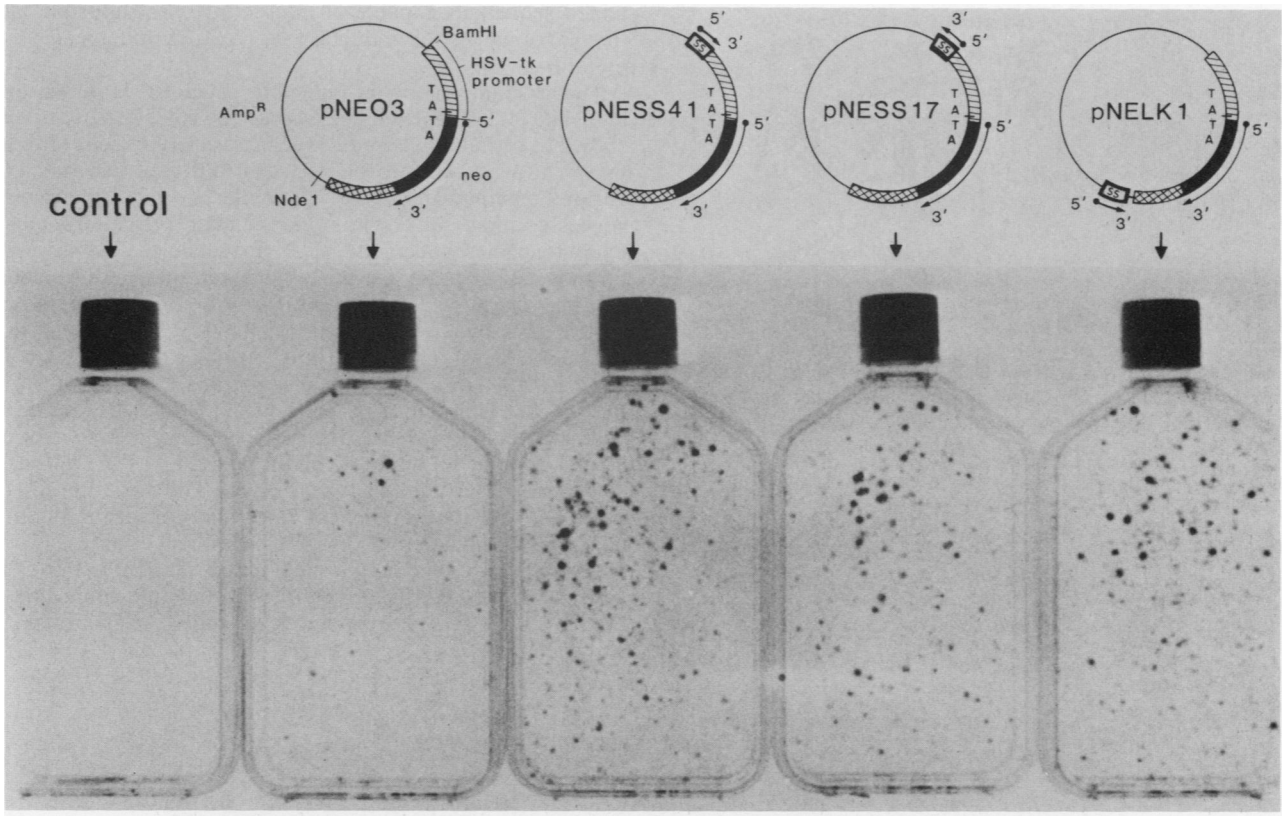


FIG. 3. Effect of the *Sma/Stu* fragment on transformation efficiency. The structures of pNEO3, pNESS41, pNESS17, and pNELK1 are shown in the top panel. The relative location and orientation of the *Sma/Stu* fragment (SS) with respect to the HSV-tk promoter (▨), *neo* coding sequence (■), poly(A) addition site (▨), and pBR322 (——) sequence in each construct are as indicated. Except for the mock control, K12 cells were transfected with 5 μg of each plasmid. Two weeks after the transfection, G418-resistant colonies were stained with 1% crystal violet in methanol-water (25:75, vol/vol).

the 5′ insertions to about 2,000 nt for the 3′ insertion. Comparison of the levels of *neo* transcripts in these stable transformants by RNA blot hybridization with the *neo* probe (1) revealed that under noninduced conditions the presence of the *Sma/Stu* fragment at the 5′ or 3′ orientation increased the *neo* mRNA levels by 3- to 20-fold (Fig. 4).

Inducibility of *neo* transcripts by A23187. To further determine whether the *Sma/Stu* fragment can confer A23187 inducibility to the *neo* transcription unit in the recombinants pNESS41, pNESS17, and pNELK1, cytoplasmic RNA was extracted from individual transformants under induced and noninduced conditions. The RNA was annealed to a *neo* synthetic oligomer (23-mer) and subjected to primer extension analysis (Fig. 5). In the case of 5′ insertions, plasmids with both orientations (pNESS41 and pNESS17) responded to A23187 induction. The degree of induction varied from three- to fivefold in individual transformants. However, insertion of the *Sma/Stu* fragment 2,000 nt away from the heterologous promoter (pNELK1) resulted in a lower response to A23187 (1.5-fold), and in some cases, *neo* transcripts of other sizes were observed. In all the transformants being analyzed, including that of pNEO3, an upstream start site was preferred over the expected cap site (see discussion).

The *Sma/Stu* fragment can compete for *trans*-acting regulatory factors. To test whether the 291-nt fragment contained binding sites for the positive regulatory factors responsible for A23187 and temperature induction in K12 cells, the following competition experiments were performed. Since we have shown that pI10 can be stimulated to produce high levels of CAT activity by A23187 and temperature in transient transfection assays (Fig. 1), addition of increasing amounts of the *Sma/Stu* fragment (as contained in pUC291W) should compete away the factors from binding to pI10, thereby diminishing the inducibility of the pI10 CAT activity by A23187 and temperature in a dosage-dependent manner. The results of this set of experiments are shown in Fig. 6. When increasing amounts of the *Sma/Stu* fragment

repeats (→) and the eight alternating purine/pyrimidine bases (〰) are indicated. The four CCAAT and TATA sequences are boxed. The GGCG sequences are highlighted by boldface type. The two blocks of sequence (A and B) are homologous to sequences located similarly in the human heat shock (Hsp 70) promoter (Wu et al., in press). Symbols: ◆, sequences homologous to E1A core enhancer (A/C GGAAGTG A/C; 12); *, sequences homologous to the viral and immunoglobulin heavy-chain enhancer (GTGG A/T A/T A/T G; 8, 36). (C) Analysis of direct repeats. The direct repeats (a through m) are indicated with open and shaded boxes. The small triangle (▲) designates the apparent junction of the two repeating units. The location of three long stretches of imperfect repeats (I, II, and III) are indicated. The broken line (〰) refers to the alternating purine/pyrimidine sequence as in panel B. The four CCAAT and TATA sequences are shown in boldface type. (D) Sequence homology of long repeats (I through III). In repeat I, 26 of 37 nt is homologous; repeat II, 21 of 30 nt; and repeat III, 18 of 22 nt.

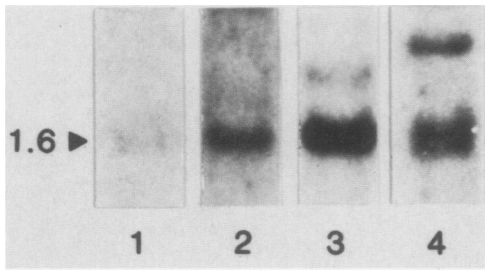


FIG. 4. Level of *neo* transcripts in stable transformants. Stable pNEO3 (lane 1), pNESS41 (lane 2), pNESS17 (lane 3), and pNELK1 (lane 4) transformants were expanded into mass culture. The cells were grown at 35°C in DMEM. Total cytoplasmic RNA was extracted and size-separated on formamide-formaldehyde gels. The RNA blot was hybridized with nick-translated *neo* DNA fragment (1). The autoradiograms are shown. The expected size of the *neo* transcript is indicated by the arrow.

were added to cotransfection mixtures, the CAT activity in response to A23187 and temperature decreased correspondingly, while the basal level was relatively unaffected. These results suggested to us that the *Sma*/*Stu* fragment contained sequences capable of interacting with diffusible factors involved in the induction by A23187 and the K12 ts mutation.

The calcium ionophore-inducible promoter is active and inducible in different cell lines. Since the p3C5 gene has been shown to be inducible by A23187 in a variety of cells (28), its promoter was likely to be active in different cell types of various mammalian species. However, recent experiments suggested that some viral and cellular promoters might exhibit species specificity. For instance, the SV40 early promoter was more active in hamster than mouse cells, whereas the mouse metallothionein promoter was active in mouse cells but not in hamster cells (22). To examine this issue, we transfected pI10 into various cell lines such as mouse LA9, NIH 3T3, human hepatoma HepG2, rat fibroblast NRK, and monkey COS cells (Table 1). As a comparison, pSV2CAT was used in parallel transient transfection assays. Similar to the viral enhancer, pI10 was ubiquitously expressed in all the cell types tested. The variation in the promoter activities among cell lines may reflect the relative competency of different cell lines in transfection assays as well as species specificity. With the exception of the COS cells, pI10 was consistently three- to ninefold more active than pSV2CAT in the noninduced state. After treatment

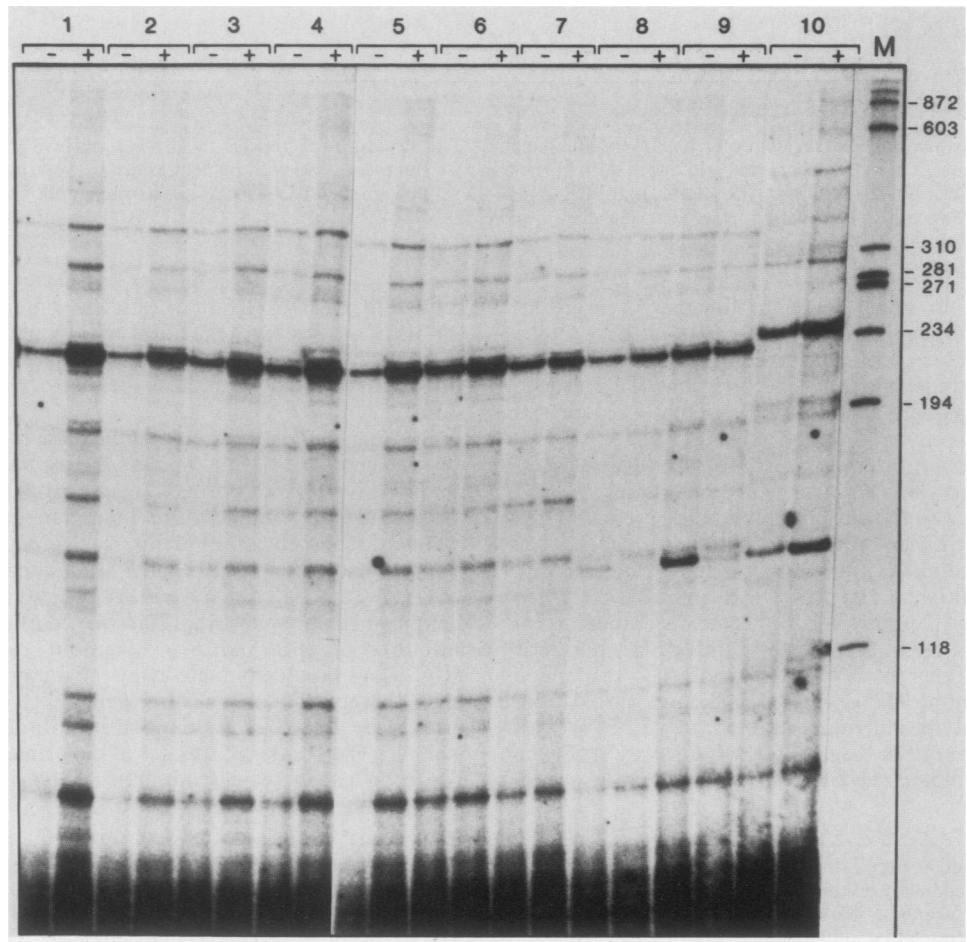


FIG. 5. Inducibility of *neo* transcripts by A23187. Total cytoplasmic RNA was extracted from pNESS41 stable transformants (1 through 4), pNESS17 stable transformants (5 through 7), and pNELK1 stable transformants (8 through 10). The RNA was reannealed to a *neo* synthetic oligomer (see Materials and Methods). The primer-extended *neo* transcripts were analyzed on a 6% polyacrylamide-urea gel. The autoradiograms are shown. −, Normal culture conditions; +, A23187 added to culture medium; M, ϕX174 *Hae*III digest size marker.

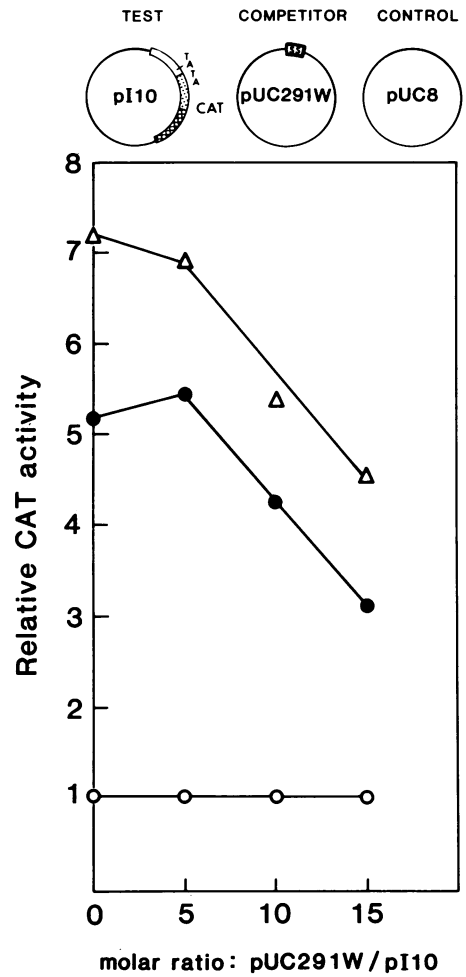


FIG. 6. Competition for *trans* regulatory factors by the *Sma*I/*Stu* fragment. K12 cells grown in 10-cm-diameter dishes were cotransfected with 1 μg of test plasmid (pI10; size, 5.7 kb) and increasing amounts of competitor plasmid (pUC291W; size, 3.0 kb). To maintain a constant amount of DNA used in each experiment, control plasmid (pUC8; size, 2.7 kb) was added to adjust the total plasmid DNA at 11 μg. At 48 to 50 h after transfection, cell extract was prepared from the transfected cells treated for 16 h with 7 μM A23187 at 35°C (△), from transfected cells treated for 16 h at 39.5°C (●), or from control cells incubated in normal medium at 35°C (○). Each extract was assayed for protein concentration, and the CAT activity was determined using 75 μg of protein. The relative CAT activity, expressed as fold increase over that of the control cells, was plotted against the molar ratio of competitor to test DNA.

with 7 μM A23187, the pI10 promoter was 10- to 25-fold more active than the SV40 early promoter.

Since in COS cells the CAT activity expressed by pSV2CAT was considerably higher than in other cell lines, we assayed for the copy number of the plasmids in these transformants just before cell extracts were prepared (Fig. 7). As expected, pSV2CAT replicated to high copy numbers in the COS cells which were permissive for SV40 replication. Although pI10 was not able to replicate in the COS cells, the CAT activity driven by its promoter was about equal to that of the amplified pSV2CAT (Table 1).

DISCUSSION

The discovery of viral enhancer elements and tissue-specific enhancer elements such as those found in the immunoglobulin genes led to the expectation that other cellular genes would also contain upstream promoter elements with enhancerlike properties. Indeed, such elements are implied for the tissue-specific expression of insulin and chymotrypsin genes (34), for the RNA polymerase I promoter activity of ribosomal genes (17), for viral or poly(I)-poly(C) activation of β-interferon genes (10), and for the basal level transcription of the metallothionein gene (Haslinger and Karin, in press). Our studies on the calcium ionophore regulatory/promoter sequence provide another example and allow us to make some comparisons of this new class of non-tissue-specific cellular regulatory sequences with some of the better-characterized enhancer elements.

The sequence and structural organization of the rat promoter shares both similarities and differences with known enhancer elements. First, several sequence elements homologous to the viral and immunoglobulin heavy-chain core enhancer sequence can be located. These, together with the occurrence of four CCAAT sequences, might explain the very high basal level activity of this promoter sequence under noninduced conditions. Second, the organization of the rat regulatory sequence into two direct repeat domains resembles the tandem repeats found in activator sequences of a number of DNA viruses and cellular gene, although these sequences are in general shorter and are more precisely matched. Thus, it has been proposed that duplication of upstream promoter elements may be associated with the involvement of some enhancer activities (Haslinger and Karin, in press). The occurrence of a stretch of eight alternating purine/pyrimidine residues near the junction of the two direct repeat domains is intriguing, as similar features have been found in other viral and cellular promoter sequences (25; Haslinger and Karin, in press).

This promoter sequence is GC rich, but the striking feature is the high abundance of CpG residues in this promoter region (CpG/GpC = 1.0). To maintain the CpG bases, these residues must have persisted as nonmethylated forms. It would be interesting to determine whether this gene system, like a few other housekeeping genes, is located within islands of nonmethylated CpG-rich DNA found in mammalian genomes (3, 37).

Most of the cellular enhancers described so far act to increase transcriptional activities in a conditional manner. For example, the immunoglobulin enhancer only functions in lymphoid cells, and the β-interferon enhancer requires induction for its activity. If one compares some of these enhancers with the SV40 early enhancer, they have similar activities to the viral element (24). In contrast, the rat regulatory sequence we have cloned in pI10 is more efficient than the SV40 enhancer in many cell lines, before and after A23187 induction. The differential promoter activities are not due to replication or stability of the transfected pI10 DNA, as compared to pSV2CAT (Fig. 7; unpublished data). When the 291-nt fragment dissected from pI10 is fused to the HSV-tk promoter, it is capable of significantly increasing the mRNA level of a covalently linked heterologous gene. Like other enhancers, it can act in an orientation-independent manner over a distance of 2,000 nt from the heterologous gene unit. Notably, even in the absence of A23187 induction, the enhancing property of the 291-nt fragment towards the HSV-tk promoter activity is substantial.

However, evidence is accumulating that the positioning of the enhancer element and the promoter sequences may modulate the enhancer activities. For example, the SV40 enhancer will act on the human β-globin gene containing an intact promoter but not on a β-globin gene with only a TATA

TABLE 1. promoter activites in various cell lines[a]

Plasmid	A23187	% Conversion of [^{14}C]chloramphenicol[b]				
		LA9	NIH 3T3	HepG2	NRK	COS 7
pSV2CAT	−	0.8 (1.0)	0.3 (1.0)	1.6 (1.0)	0.8 (1.0)	69.5 (1.0)
	+	0.6 (0.7)	0.5 (1.7)	2.2 (1.4)	2.5 (3.1)	82.9 (1.2)
pI10	−	2.4 (3.0)	0.9 (3.0)	12.9 (8.1)	7.5 (9.4)	37.9 (0.5)
	+	7.8 (9.8)	4.8 (16.0)	39.4 (24.6)	16.4 (20.5)	89.4 (1.3)

[a] CAT activity was assayed from cells transfected for 48 to 50 h with 3 μg of either pSV2CAT or pI10 and expressed as percent conversion of [^{14}C]chloramphenicol to its acetylated forms.
[b] Numbers in parentheses indicate the fold increase over the basal activity of pSV2CAT, set as unity in each of the cell lines.

box unless the two elements are very close (32). Similar observations were made for the interferon gene regulatory element and the β-interferon TATA box (10). To test for such effects, we fused the 291-nt fragment directly 5′ to the SV40 promoter with most of the 72-base-pair repeats removed (pSV1BCAT; 4). We found that although the 291-nt fragment was as effective as the SV40 72-base-pair repeats it replaced, the element no longer responded to A23187 induction (data not shown). In another set of experiments, when this enhancer was placed at the 3′ end of the intact SV40 promoter in pSV2neo (31), it could enhance the transformation efficiency (A. Y. Lin and A. S. Lee, unpublished data). Therefore, in using enhancers for activating heterologous gene units, the choice of downstream promoters and the proximity of the enhancer and the promoter may be critical. The effect of species specificity is also an important consideration.

While analyzing the initiation site for the HSV-tk/*neo* fusion transcripts using the *neo* synthetic primer, we noted that in all the pNEO3 recombinants tested, including the parental plasmid pNEO3, the major primer extended band was slightly larger than the predicted size initiating from the normal cap site for the HSV-tk promoter (23). When another synthetic oligonucleotide was used which was complementary to the 5′ untranslated tk region (+34 to +50) of the tk/*neo* fusion transcript, the major start site was more precisely mapped at about 100 nt upstream of the normal site (unpublished data). Since this result was obtained also in the case of the parental plasmid with no enhancer, the selective use of the upstream site is an unique property of this tk/*neo* fusion gene and is not due to the insertion of an enhancer. It is possible that the fusion of the HSV-tk promoter to the *neo* gene in this construct caused secondary structure changes resulting in the utilizaiton of some cryptic initiation signals which were present upstream of the normal start site. For example, a CAAT- and TATA-like sequence can be located 90 and 40 nt upstream of the start site we observed. In other HSV-tk fusion genes, major transcripts originating upstream from the normal tk start site have also been observed (6, 10).

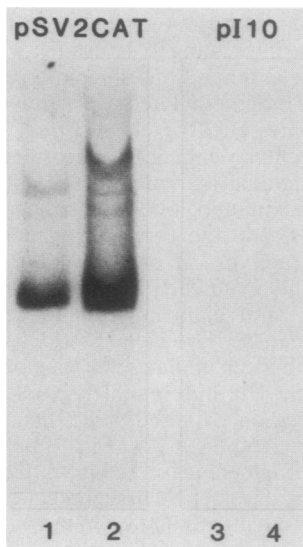


FIG. 7. Analysis of transfected DNA in COS cells. COS 7 cells grown in 15-cm-diameter dishes were transfected with 6 μg of pSV2CAT (lanes 1 and 2) or pI10 (lanes 3 and 4). At 48 h after transfection, plasmid DNA was isolated from the cells (13). The DNA was suspended in 100 μl of 0.01 M Tris (pH 7.4)–0.001 M EDTA and subjected to electrophoresis on a 1% agarose gel. Portions of 20 μl (lanes 1 and 3) or 40 μl (lanes 2 and 4) from each sample were applied to the gel. After electrophoresis, the gel was blotted (30) and hybridized with nick-translated pBR322 DNA (specific activity, 5×10^7 cpm/μg). Autoradiograms are shown.

Transcriptional regulatory factors with the ability to enhance transcription in vitro and to bind to defined stretches of promoter DNA have recently been purified from cellular extracts (5, 7, 26). The 291-nt sequence we have isolated has the ability to compete for *trans*-acting factors involved in A23187 induction and those defined by the K12 ts mutation. More detailed analyses of the molecular interaction between these factors and DNA will provide information on this new class of inducers which is specifically generated when cultured cells are treated with A23187.

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