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Regulation of the human β-actin promoter by upstream and intron domains

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ABSTRACT

We have identified three regulatory domains of the complex human β–actin gene promoter. They span a region of about 3000 bases, from not more than -2011 bases upstream of the mRNA cap site to within the 5′ intron (832 bases long). A distal upstream domain contains at least one enhancer-like element. A proximal upstream domain, with a CArG [for CC(A+T rich)6GG] motif found in all known mammalian actin genes, seems to confer serum, but not growth factor, inducibility. The third domain is within the evolutionarily conserved 3′ region of the first intron and contains a 13 base-pair sequence, identical to the upstream sequence with the CArG motif. This domain also contains sequences that are both serum and fibroblast growth factor inducible.

INTRODUCTION

The transcriptional activity of several viral promoters has been largely attributed to the presence of enhancer elements (reviewed in 1). More recently, similar elements have been identified in many cellular genes (reviewed in 2). The β–actin promoter is an active cellular promoter (3). In this study, we have used the expression of β–actin promoter-directed chloramphenicol acetyl-transferase (CAT) as an *in vivo* promoter assay (4) to detect the presence and to map the positions of regulatory domains.

Recently, we constructed a β–actin promoter expression vector (3). The promoter that directs this vector consists of over 3 kilobases of 5′ flanking sequences, a 5′ UTR, and an intron (see Figure 1). This 5′ intron contains evolutionarily conserved sequences (5) and interrupts the 5′ UTR of the β–actin gene. To test the activity of this promoter, we have inserted the bacterial CAT gene from pSV2 CAT (4) downstream of the 5′ intron. We found that this chimeric HβA CAT gene is highly active in various mammalian cell lines (3).

Transient expression of the pHβA CAT DNA and its deleted derivatives in human and rodent cell lines was used to define the 5′ boundary of the β–actin promoter as well as to detect the presence of at least three regulatory domains: a distal promoter domain that is enhancer-like, a proximal promoter domain that is orientation-dependent, and an intron domain downstream of the mRNA cap site.

Transcription of the non-muscle actin genes in quiescent fibroblasts is stimulated by serum (6,7). However, there is limited similarity in 5′ flanking region nucleotide sequences between the human β– and γ-actin genes (8) except for the CArG motif found in all known mammalian actin genes (9,10). Furthermore, the similarity between the sequence with the CArG motif in the

human γ-actin gene and the serum response elements (SREs) of the human c-*fos* gene (11,12) and the *Xenopus* cytosketetal actin gene (13) suggests that this motif could be part of a serum responsive sequence. There are three such motifs in the human β–actin gene (9,14): one in each of the three regulatory domains. We tested the serum and growth factor inducibilty of the three domains in G418 resistant substrains and found that the intron domain is the only one activated by both serum and fibroblast growth factor. In contrast, the proximal promoter domain is inducible by serum only and the distal domain is the least serum responsive region.

MATERIALS AND METHODS

Plasmid construction

The construction of pHβA CAT (pHβAPr-1-CAT) has been described (3). pHβA-51 CAT was constructed by digestion of pHβA CAT with restriction endonucleases *Aat* II and *Xho* I. The CAT gene fragment was then ligated with pBR322 DNA fragment *Aat* II-*Sal* I. pHβA-466 CAT was constructed by digestion of pHβA CAT with restriction endonucleases *Aat* II and *Sac* I. The CAT gene fragment was then ligated with pUC19 DNA fragment *Aat* II-*Sac* I. pHβA-574 CAT was constructed by digestion of pHβA CAT with restriction endonucleases *Aat* II and *Nhe* I. The CAT gene fragment was then ligated with pBR322 DNA fragment *Aat* II-*Nhe* I. pHβA-962 CAT was constructed by digestion of pHβA-962/-52 SVCAT with restriction endonucleases *Xho* I and *Hin* dIII. The CAT gene fragment was then ligated with pHβA CAT DNA fragment *Xho* I-*Hin*d III containing the first intron. pHβA-962/-52 SVCAT was constructed by blunt-end ligation of the promoter fragment *Pvu* II-*Xho* I into the *Bgl* II site of pA10 CAT (15). pHβA-2006 CAT and pHβA[-467/-2006]-466 CAT were constructed by ligation of the promoter fragment *Sac* I-*Sac* I in either orientation into the *Sac* I site of pHβA-466 CAT. pHβAM$_1$(β$_1$)-2 CAT was constructed by digestion of pHβA CAT with restriction endonucleases *Aat* II and *Xho* I. The CAT gene fragment was then ligated with pM$_1$(β$_1$)-2 (16) DNA fragment *Aat* II-*Xho* I. pHβAΔ52-910 (ΔIVS) CAT was constructed by digestion of pHβA CAT with restriction endonucleases *Bst* EII and *Hin*d III. The CAT gene fragment was then ligated with pHβAPr-9Δ fragment *Bst* EII-*Hin*d III. pHβAPr-9Δ was constructed by digestion of pHβAPr-9 (3) with restriction endonucleases *Xho* I and *Bam* HI. The larger DNA fragment was then ligated with the 102 bp pM$_1$(β$_1$)-2 (16) DNA fragment *Xho* I-*Sau* 3AI (*Sau* 3AI site is at +52 in the 5′ UTR). pHβA-51Δ52-910 (ΔIVS) CAT was constructed by digestion of pHβAΔ52-910 (ΔIVS) CAT with restriction endonucleases *Aat* II and *Xho*I. The CAT gene fragment was then ligated with pBR322 DNA fragment *Aat* II-*Sal* I. pHβA-466Δ52-910 (ΔIVS) CAT was constructed by digestion of pHβAΔ52-910 (ΔIVS) CAT with restriction endonucleases *Aat* II and *Sac* I. The CAT gene fragment was then ligated with pUC19 DNA fragment *Aat* II-*Sac* I. pHβA-574Δ52-910 (ΔIVS) CAT was constructed by digestion of pHβAΔ52-910 (ΔIVS) CAT with restriction endonucleases *Aat* II and *Nhe* I. The CAT gene fragment was then ligated with pBR322 DNA fragment *Aat* II-*Nhe* I. pHβAM$_1$(β$_1$)-2 Δ52-910 (ΔIVS) CAT was constructed by digestion of pHβAΔ52-910 (ΔIVS) CAT with restriction endonucleases *Aat* II and *Xho* I. The CAT gene fragment was then ligated with pM$_1$(β$_1$)-2 (16) fragment *Aat* II-*Xho* I.

pHβA-2006/-52 SVCAT was constructed by digestion of pHβA-2006 CAT with restriction

endonucleases *Xho* I and *Hind* III. The CAT gene fragment was then ligated with pHβA-962 SVCAT fragment *Xho* I-*Hind* III. pHβA[-467/-2006]-466/-52 SVCAT was constructed by digestion of pHβA[-467/-2006]-466 CAT with restriction endonucleases *Xho* I and *Hind* III. The CAT gene fragment was then ligated with pHβA-962 SVCAT fragment *Xho* I-*Hind* III. pHβA-962/-52 SVCAT and pHβA-52/-962 SVCAT were constructed by blunt-end ligation of the promoter fragment *Pvu* II-*Xho* I in either orientation into the *Bgl* II site of pA10 CAT (15). pHβA -2006/-575 SVCAT was constructed by digestion of pHβA-2006/-52 SVCAT with restriction endonucleases *Nhe* I and *Xho* I. The CAT gene fragment was then ligated with pBR322 DNA fragment *Nhe* I-*Sal* I. pHβA144-639 (IVS) SVCAT was constructed by blunt-end ligation of the intron fragment *Bgl* I-*Bgl* I into the *Bgl* II site of pA10 CAT (15). pHβA107-656 SVCAT was constructed by ligation of the intron fragment *Sph* I-*Tth* 111I into the *Sph* I site of pSV2 CAT (4). pHβA657-910 SVCAT was constructed by ligation of the promoter fragment *Tth* 111I-*Bam* HI into the *Bgl* II site of pA10 CAT (15).

Cell culture and transfection

Rat-2 and mouse NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. Human Hut-12 cells (3) were grown in DMEM with 20% fetal calf serum. Cells cultured in 60mm dishes at about 50% confluency were transfected with 10 μg of plasmid DNA using the calcium phosphate coprecipitation procedure (17).

Transient CAT expression

Cells were collected 2 days after transfection, and CAT activity was determined as described (4).

Stable CAT expression

Various chimeric CAT plasmids were cotransfected with pSV2neo (18). Cells containing stably integrated copies of transfected DNA were selected by adding geneticin (G418) to the medium at a concentration of 600 μg/ml as described (18). Colonies of G418 resistant cells were collected and expanded into mass culture. CAT activity of individual stable substrains was determined as described (4). Stable substrains that are active in CAT expression were serum starved as described (7,11,12,19). Typically, subconfluent cultures were serum starved for 3 days in media containing 0.5% serum. Cells were collected after 10 hours in media containing 0.5% or 15% serum for CAT assays to assess inducibility by serum, or in media containing 0.5% serum with or without EGF (50ng/ml), FGF (50ng/ml), or PDGF (5 half-maximal units/ml). Growth factors were purchased from Collaborative Research. CAT activity of individual extracts was determined as described (4). CAT assays for each stable transfectant were carried out with the same amount of protein in each reaction. Protein concentration was estimated by BioRad protein assay, with bovine serum albumin as standard. Quantitation of CAT assays was obtained by scintillation counting in BetaBlend (WestChem).

Nuclear runoff transcription

Nuclei were isolated from about $5X10^7$ cells as described (6). NIH-3T3 derived transfectant substrains were serum starved as described above. Serum stimulated cells were collected after 30 minutes in media containing 15% serum. Runoff transcription reactions, RNA purification and hybridization were carried out as described (6). $4X10^5$ to 10^6 cpm of ^{32}P-labeled RNA was

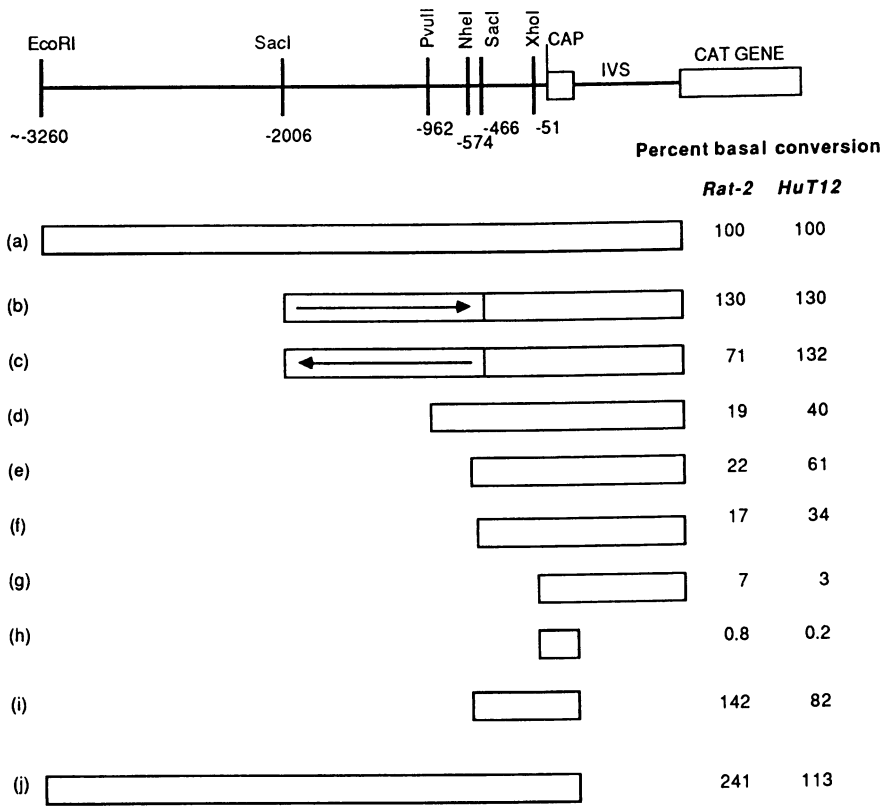


Figure 1. *Human β-actin promoter contains multiple regulatory domains.*
The various promoter segments are shown in relation to a restriction map of the human β-actin gene sequence from pHβAPr-1-CAT (3). The positions of restriction sites are based on published DNA sequence (14). The chimeric DNAs are designated HβA CAT (lane a), HβA-2006 CAT (lane b), HβA[-467/-2006]-466 CAT (lane c), HβA-962 CAT (lane d), HβA-574 CAT (lane e), HβA-466 CAT (lane f), HβA-51 CAT (lane g), HβA-51Δ52-910(ΔIVS) CAT (lane h), HβA-574Δ52-910(ΔIVS) CAT (lane i), HβAΔ52-910 (ΔIVS) CAT (lane j). CAT activity of $H\beta AM_1(\beta_1)$-2 CAT relative to HβA CAT is 106% in rat-2 cells and 150% in HuT-12 cells. CAT activity of $H\beta AM_1$ (β_1)-2Δ52-910 (ΔIVS) CAT relative to HβA CAT is 248% in rat-2 cells and 173% in HuT-12 cells. CAT activity shown is average of 2-8 independent experiments expressed as a fraction of the average CAT activity for HβA CAT in rat-2 or HuT-12 cells. Average of standard deviation is 13%. The higher CAT activity for HβAΔ52-910 (ΔIVS) CAT versus HβA CAT was observed in 21 independent experiments with 3 different preparations of each plasmid DNA.

hybridized to these immobilized DNAs: pCAT, with only CAT coding sequences; pHFβA-3′UT, with 3′ UTR sequences that are β-actin specific (5); pGH with human c-*fos* first exon sequences (27); and $p\beta_2M$, containing β_2-microglobulin sequences as a cell-cycle independent probe (R. Baserga, personal communication).

RESULTS

Deletion analysis of the human β–actin promoter

As a first step in our analysis of this promoter, we have defined the essential regions for full promoter activity. Using unique restriction endonuclease sites in the 5′ flanking region, we progressively deleted sequences between an *Eco* RI site at about -3260 and an *Xho* I site at -51 up-stream of the mRNA cap site in a 5′ to 3′ direction. The resulting CAT activity from transient expression in rat-2 and human cells directed by these residual upstream segments is shown in Figure 1.

Full promoter activity is retained after deleting the 5′ most ~1300 base pairs between the *Eco* R1 and *Sac* I sites (see Figure 1, lane b). Therefore, the human β–actin promoter requires no more than about 2 kilobases of 5′ flanking sequences for maximum activity. A second β–actin gene clone, $M_1(\beta_1)$-2(16), has a 5′ flanking region of about 2 kilobases and can direct the expression of the CAT gene to levels similar to those directed by the 3.3 kilobase long segment (see Figure 1, legend).

Further deletions to the *Pvu* II site at -962 (see lane d), to the *Nhe* I site at -574 (see lane e) or to the *Sac* I site at -466 (see lane f) reduce promoter activity to similar extents. Thus it seems that this promoter has at least two major transcriptional domains upstream of the mRNA cap site. A distal region is located upstream of -962 and a proximal region downstream of -466. This proximal region includes the ~180 bp upstream segment that is evolutionarily conserved (5) and contains the CCAAT and TATA sequences. The proximal domain retains about 20% of full promoter activity in rat-2 cells and about 40% in human cells (see Figure 1).

We tested several β–actin 5′ flanking segments for their ability to act as orientation-independent enhancers, upstream of the SV40 early promoter in pA10 CAT (15) and compared them to pSV2 CAT (4). The 911 base-pair DNA segment between *Pvu* II site (at -962) and the *Xho* I site (at -51), which includes the proximal domain, is one-fifth as active as pSV2 CAT (data not shown) but the same fragment, in an inverted orientation, is significantly less active than the normal orientation (4% of pSV2 CAT activity). Thus it seems that the proximal transcriptional domain of this promoter does not contain an orientation independent regulatory element.

In contrast, when we inverted a ~1.5 kilobase *Sac* I fragment containing the distal transcriptional domain (*Sac* I sites at -2006 and -466) at least 70% of full promoter activity remained whether upstream of the β–actin promoter (see Figure 1, lane c) or upstream of the SV40 early promoter (depicted in Figure 4A lane c, data not shown). Therefore the distal promoter region appears to contain enhancer-like control element(s).

In addition to the upstream transcriptional domains, we tested for possible downstream regulatory domain(s) since we found evolutionarily conserved sequences in the first intron (5). First we removed intron sequences from various β–actin promoter plasmids and tested the effects on transient CAT expression. In the presence of upstream promoter domains, deletion of the first intron leads to higher CAT activities, especially in rat-2 cells (see Figure1, lanes i and j as examples). Similarly, we have isolated a number of stable transfectants expressing HβAΔ52-910(ΔIVS) CAT (depicted in Figure 1 lane j) which are more active than those expressing the

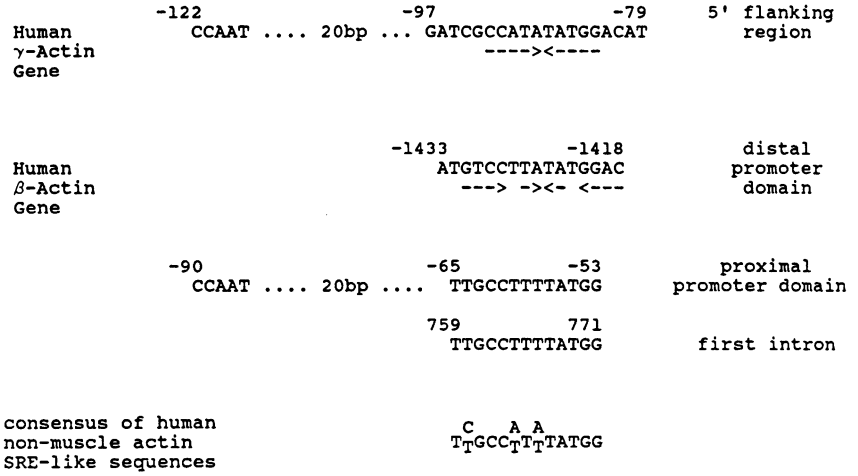


Figure 2. *Comparison of the c-fos SRE-related sequences in the human* β- *and* γ*-actin genes.* Base positions for human β–actin gene are according to ref. 5 and 14. Base positions for human γ-actin gene are according to ref.8. The 19 bp human γ-actin gene putative SRE has 15 bases matching (shown underlined) with the human c-*fos* SRE GATGTCCATATTAGGACAT (11,12) and 13 bases matching (shown underlined) with a *Xenopus* cytoskeletal actin SRE GATGCCCATATTTGGCG AT (13).

intact HβA CAT. These effects are not likely due to abberant RNA processing since the intron deletion plasmids still have a fully functional intron in the 3′ UTR (4).

Although the increase in CAT activity following intron deletion suggests that the intron sequences might have an inhibitory effect on CAT expression, when we tested these sequences directly for their ability to modulate the expression of the heterologous SV40 early promoter, they showed significant stimulatory activity. Both the 5′ and 3′ conserved regions (5) of this intron (depicted in Figure 4A lanes e and f) were found to stimulate the heterologous promoter (from pA10 CAT) to levels about one-tenth as active as pSV2 CAT (Figure 4 B and 4D, data not shown). This result suggests that TG element-like sequences (20) and viral enhancer-like sequences (5) located in this intron may be functional.

We then tested the ability of these intron sequences to effect transcription from the β–actin promoter in the absence of the upstream promoter domains. The residual promoter activity directed by only 51 base pairs of 5′ flanking sequences (see Figure 1, lane h) was comparable to that of pA10 CAT. This residual promoter activity was increased tenfold with the addition of the 5′ intron segment (see lane g). Thus, in the absence of the upstream domains, putative control elements in this intron appear to contribute to promoter activity.

Growth regulatory domains of the human β–actin promoter

Transcription of actin and several cellular genes are induced by growth factors present in serum (6,7). Serum response elements (SRE) with similar sequences have been identified in the 5′ flanking region of the c-*fos* proto-oncogene (11) and in the 5′ flanking region of a *Xenopus*

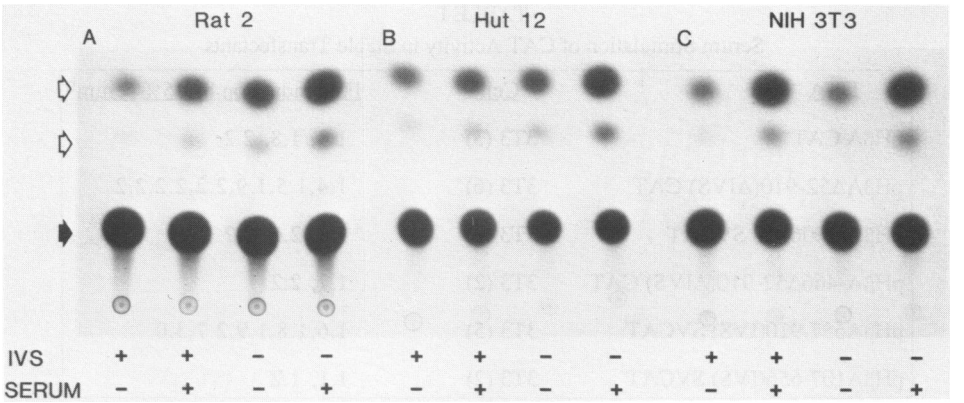


Figure 3. Serum stimulation of CAT activity during transient expression in mammalian cell lines.
DNA was transfected into Rat-2 (panel A), HuT12 (panel B) or NIH 3T3 (panel C) cells . DNAs were either HβA CAT (for plus IVS lanes) or HβAΔ52-910 (ΔIVS) CAT (for minus IVS lanes). Cells were harvested after 35-40 hours in media containing 0.5% serum and 10 hours in same media (for minus serum lanes) or 10 hours in media containing 15% serum (for plus serum lanes). Average stimulation of transient expression of $H\beta AM_1(\beta_1)$-2 CAT by serum was 2.6-fold in Rat-2 cells, 1.2-fold in HuT-12 cells, and 2.3-fold in 3T3 cells with constant amounts of protein (9-25 μg) per assay. Average stimulation of transient expression of $H\beta AM_1(\beta_1)$-2Δ52-910 (ΔIVS) CAT DNA by serum was 2.3-fold in Rat-2 cells, 1.2-fold in HuT-12 cells and 2.8-fold in 3T3 cells with constant amounts of protein (10-25 μg) per assay. The above data was based on 6-8 independent experiments. In another set of experiments, 10 μg of $pH\beta AM_1(\beta_1)$-2Δ52-910 (ΔIVS) CAT DNA was cotransfected with 20 μg of pCH110 DNA (Pharmacia) into about 1.5 x 10^6 NIH 3T3 cells in 100 mm dishes. Average stimulation by serum was 3.2-fold in 3T3 cells after normalization using β-galactosidase activities as internal controls. Solid arrow indicates the position of chloramphenicol and hollow arrows indicate the positions of mono-acetylated-chloramphenicol.

cytoskeletal actin gene (13). A DNA sequence that resembles those two SRE sequences is found in the 5′ flanking region of the human γ-actin gene (see Figure 2). These three DNA sequences all contain the CArG motif found in mammalian actin genes (9,10). The human β-actin gene has three CArG sequences (9,14). One such sequence is located, like the human γ-actin SRE-like sequence, between the CCAAT and TATA sequences (see Figure 2). In addition, a second CArG sequence, identical to the one located in the proximal promoter domain, is found in the conserved region of the first intron (5). The third CArG sequence, like the human γ-actin SRE-like sequence and the c-*fos* SRE (11,12), has dyad symmetry and is located in the distal promoter domain(see Figure 2).

Although two of three human β-actin CArG motif containing sequences do not, like the known SREs, have dyad symmetry (11,12), their potential as regulatory elements remains plausible since both are evolutionarily conserved (5). Thus we tested the various β-actin promoter domains for serum inducibility to find out if it correlates with the presence of the SRE-like sequence. We first tested for serum inducibility of chimeric CAT genes, either with or

TABLE I
Serum Stimulation of CAT Activity in Stable Transfectants

DNA	Cell[a]	Fold induction by 15% serum[b]
pHβA CAT	3T3 (3)	1.8, 1.8, 2.2
pHβAΔ52-910(ΔIVS) CAT	3T3 (6)	1.4,1.5,1.9,2.2,2.2,2.2
pHβA-2006/-52 SVCAT	3T3 (3)	1.8, 2.6, 2.7
pHβA-466Δ52-910(ΔIVS) CAT	3T3 (2)	1.7, 2.2
pHβA657-910(IVS) SVCAT	3T3 (5)	1.6,1.8,1.9,2.7,3.0
pHβA107-656(IVS) SVCAT	3T3 (2)	1.1, 1.2

[a]The number in brackets indicates the number of stable transfectants expressing each CAT gene tested for serum induction.

[b]CAT assays for each stable transfectant were carried out with the same amounts of protein in each reaction. Results were obtained using 2 to 6 sets of independent cell extract preparations.

without the first intron, during transient expression in either rat-2, human or mouse 3T3 cells. We found, as shown in Figure 3, that both chimeric CAT genes are serum inducible to varying degrees in all three cell lines. Thus the 5′ flanking region should contain serum responsive sequence(s). Interestingly, the serum stimulation of CAT activity is higher in the two rodent cell lines than in human HuT 12 fibroblasts. This is reminiscent of the results from serum stimulation of the c-*fos* gene in mouse 3T3 versus human HeLa cells (21).

To map the location of such serum responsive sequence(s), we next tested for serum inducibility of CAT activity in a total of 30 quiescent stable transfectants expressing various chimeric CAT genes. These stable substrains were isolated by using pSV2neo co-transfection and G418 selection (18). After serum starvation, the various substrains expressing these CAT genes became quiescent (7,11,12,19). Parallel dishes of cells were refed with media, with or without supplemental serum as described in Materials and Methods. After 10 hours the cells were harvested for CAT assay and the results reported in Table 1 represent the fold-induction of CAT activities in the serum stimulated cells over values of the non-stimulated cells. Several transfectant substrains that were not induced by serum serve as controls for this type of comparison, including a pSV2 CAT transfectant (1.02-fold induction). The results from such uninduced transfectant substrains (also see below) suggest that the CAT assay values of cells that are induced are not related to general effects on cellular transcription or on the turnover of CAT RNA or enzyme.

The expression of the HβA CAT gene is serum inducible by about two fold in all three of our NIH-3T3 derived substrains (data not shown). Thus the level of serum stimulation of CAT activity is not significantly lower in stable transfectants than is found in transient expression experiments. Like the transient expression results, expression of CAT genes without the 5′ intron (HβAΔ52-910 CAT) are serum inducible in six such substrains (see Table 1). We also tested the ability of the upstream flanking regions to respond to serum with a heterologous

promoter. The β–actin DNA segment from -2006 to -52 inserted upstream of the SV40 early promoter (depicted in Figure 4A, lane c) imbues it with 1.8 to 2.7 fold serum inducibility in three 3T3 substrains (see HβA-2006/-52 SVCAT in Table 1). Thus one or more putative β–actin gene SRE are located in the upstream promoter sequences. Since the two upstream CArG sequences are located in distinct promoter domains, we tested the serum inducibility of CAT genes independently driven by either the proximal or the distal promoter domain. Two 3T3 substrains expressing a chimeric gene with the proximal domain, HβA-466Δ52-910(ΔIVS) CAT, (depicted in Figure 4A, lane b) are serum inducible in CAT activity (see Table I and Figure 4C), thus suggesting that the proximal promoter domain contains SRE sequence(s). In contrast, three 3T3 substrains expressing a chimeric gene with the distal domain, HβA-2006/-575 SVCAT, (depicted in Figure 4A, lane d), with the distal promoter domain upstream of the SV40 early promoter, are only weakly serum inducible (see Table 1). Thus the distal CArG sequence is apparently not part of an active SRE. Alternatively, its activity could be modulated by growth inhibitory sequences (22).

To test the possible serum responsiveness of sequences in the first intron, we isolated stable transfectants expressing HβA-51 CAT (Figure 1, lane g) with an intact intron I and 51 base pairs of 5′ flanking sequences. The expression of this CAT gene is reproducibly but weakly serum inducible (see Table I). Since there are two evolutionarily conserved regions within the first intron (5), we tested whether one or both of these regions contain serum responsive sequence(s). Two overlapping first intron derived segments, one spanning bases 107 to 656 and another spanning bases 144 to 639 (refer to ref. 5 for base pair position) containing TG element-like sequences (20) were tested in pA10 CAT (15). A third intron segment spans bases 657 to 910 and contains a 30 base-pair conserved sequence (5) including a CArG motif was also tested in pA10 CAT (14). The CAT gene driven by the latter intron region is serum inducible (see pHβA637-910(IVS) SVCAT in Table I and Figure 4B), but those driven by the other intron regions are not serum inducible (see Table I and Figure 4D). Therefore the 30 bp conserved sequence, containing the CArG sequence, may also be part of a serum inducible control element.

To further substantiate that this β–actin intron contains SRE sequence(s), we compared serum inducibility of intron domain directed CAT genes and the endogenous β–actin genes in isolated nuclei. Transcription of the endogenous β–actin genes and the transfected CAT genes driven by the β–actin 3′ intron domain was strongly stimulated soon after addition of serum (see Figure 5, lanes 1 and 2). However, transcription of the transfected CAT genes driven by the β–actin 5′ intron domain and the endogenous β2-microglobulin genes, unlike the endogenous β–actin genes, was not significantly serum inducible (see Figure 5, lanes 3 and 4). Thus the SRE(s) in the 3′ intron domain can respond to serum stimulation at the transcriptional level. Furthermore, the rapid stimulation in CAT transcription parallels not only the response of the endogenous β–actin (lanes 1-4) and c-*fos* (lanes 5 and 6) genes but also that of the transfected CAT genes directed by the c-*fos* SRE (lanes 5 and 6). In general, results from these nuclei runoff transcription reactions substantiate our conclusion based on CAT activity measurements. However, these data also suggests that the low inducibility of CAT expression by serum could be due to high background of accumulated CAT enzymatic activities.

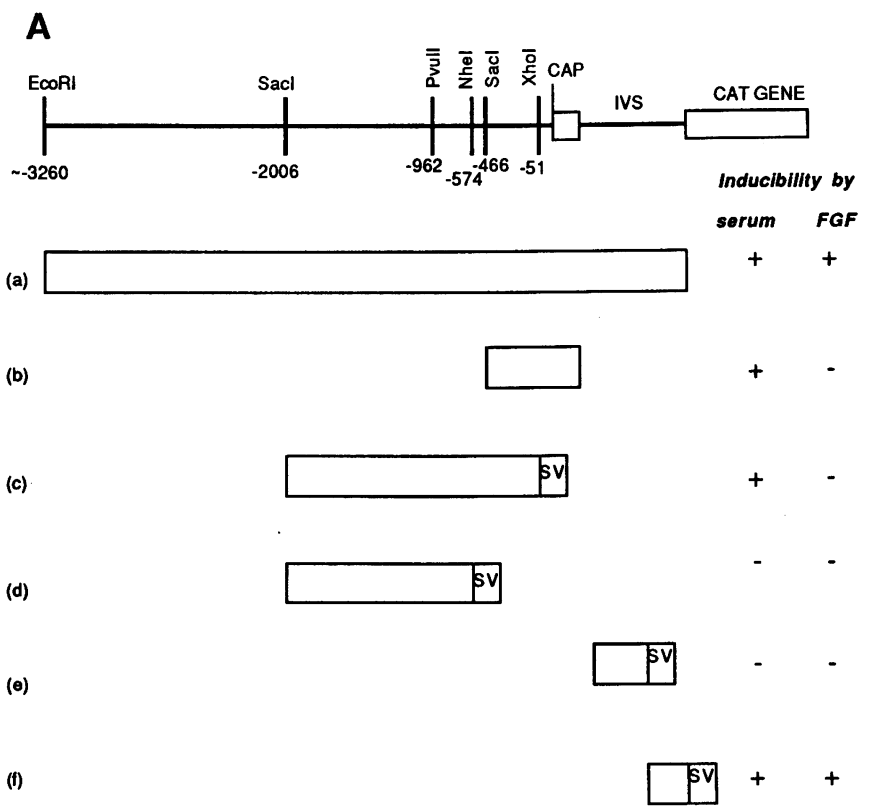
The growth factors in serum are responsible for inducing quiescent serum-starved cells to progress through the cell cycle (reviewed in 23). Both epidermal growth factor (EGF) and

platelet derived growth factor (PDGF) have been reported to induce β–actin gene transcription early in the G_0/G_1 transition (6,7,24). Therefore we tested the ability of three growth factors, EGF, PDGF, and fibroblast growth factor (FGF) to induce the expression of CAT genes driven by either the upstream transcriptional domains or intron segments in stable transfectants (see Figure 4).

Those serum inducible 3T3 substrains expressing HβA CAT (Figure 4A, lane a) and HβA657-910(IVS) SVCAT (lane f) are also inducible by FGF and PDGF, but not EGF (see, for example, Figure 4B). Similarly, FGF stimulates transient expression of HβA657-910(IVS) SVCAT in 3T3 cells (average induction of 2.5 fold).

We also tested 3T3 substrains carrying the upstream serum responsive segments for FGF induction. Neither HβA-466Δ52-910(ΔIVS) CAT (Figure 4A, lane b) nor HβA-2006/-52 SVCAT (lane c), which are serum inducible, are inducible with EGF or FGF (see, for example, Figure 4C). Furthermore, we were able to confirm this result with nuclear runoff transcription (data not shown). Thus, only sequences within the first intron appear to confer FGF inducibility.

Finally, β–actin promoter derived segments that are not inducible with serum are also un-



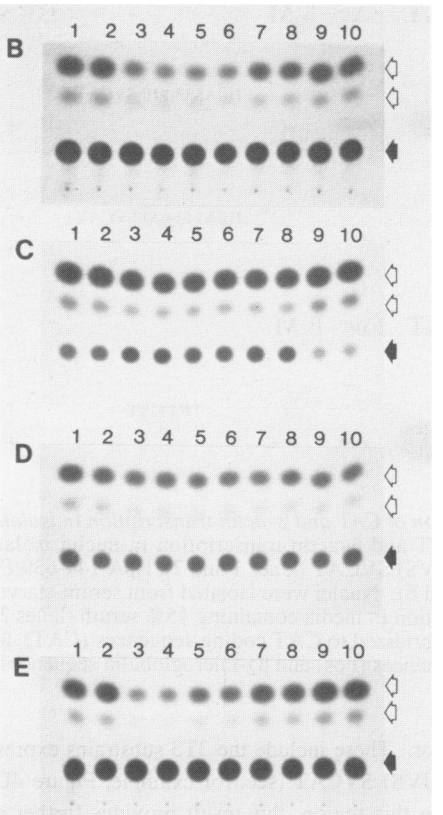


Figure 4. Human β-actin promoter contains upstream and downstream serum/growth factors responsive elements.

Panel A: Summary of serum and growth factors responsiveness of CAT activities in some of the stable transfectants listed in Table I. NIH3T3 derived stable transfectants are expressing either HβA CAT (lane a), HβA-466Δ52-910 (ΔIVS) CAT (lane b), HβA-2006/-52 SVCAT (lane c), HβA-2006/-575 SVCAT (lane d), HβA144-639 (IVS) SVCAT (lane e) or HβA657-910 (IVS) SVCAT (lane f). Stable transfectants expressing HβA CAT (lane a) is inducible by serum (1.9-2.9 fold), FGF (1.8-2.0 fold), and PDGF (2-fold). Stable transfectants expressing HβA657-910(IVS) SVCAT (lane f) is inducible by serum (1.6-1.8 fold), FGF (1.4-2.2 fold), and PDGF (1.3-fold). See Table I for serum inducibility of the other substrains.

Panels B-E: Stimulation of CAT activity in quiescent cultures of 3T3 substrains expressing HβA657-910 (IVS) SVCAT (panel B), HβA-466Δ52-910 (ΔIVS) CAT (panel C), HβA 144-639 (IVS) SVCAT (panel D) or TF3 (panel E) with EGF (50 ng/ml), or FGF (50 ng/ml) or 15% serum. CAT activities are from unstarved cells (lanes 1 and 2), starved cells without addition (lanes 3 and 4), starved cell supplemented with EGF (lanes 5 and 6), starved cells supplemented with FGF (lanes 7 and 8), starved cells supplemented with serum (lanes 9 and 10). Similar results were obtained using 4-10 independent cell extract preparations. Each reaction contained 30 μg protein (panel B), 20 μg protein (panel C), 25 μg protein (panel D), 15 μg protein (panel E). CAT assays were incubated for 15 minutes (panel C), 30 minutes (panel E), or 2 hours (panels B and D). Solid arrows indicate the position of chloramphenicol and hollow arrows indicate the positions of mono-acetylated-chloramphenicol.

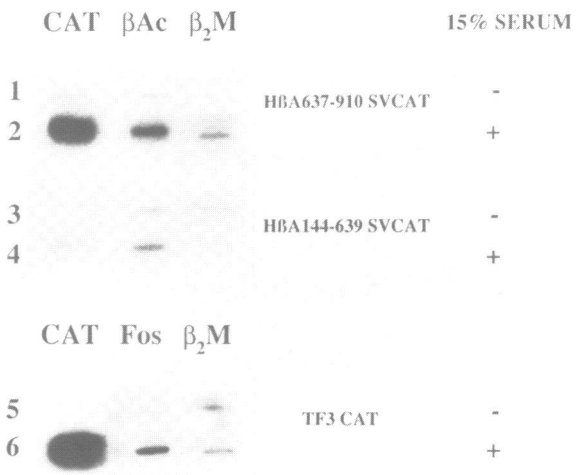


Figure 5. *Serum stimulation of CAT and β–actin transcription in isolated nuclei*
Serum stimulation of CAT and β–actin transcription in nuclei isolated from 3T3 substrains expressing HβA637-910(IVS) SVCAT (lanes 1 and 2), HβA 144-639(IVS) SVCAT (lanes 3 and 4) or TF3 (27) (lanes 5 and 6). Nuclei were isolated from serum-starved cells (lanes 1, 3 and 5) or after 30 minutes incubation in media containing 15% serum (lanes 2, 4 and 6). Radiolabeled runoff transcripts were hybridized to CAT coding sequences (CAT), β–actin 3′UTR sequences (βAc), c-*fos* first exon sequences (Fos) and β2-microglobulin sequences (β2M).

responsive to growth factor. These include the 3T3 substrains expressing HβA144-639(IVS) SVCAT or HβA107-656(IVS) SVCAT (see, for example, Figure 4D). Since a SRE-related sequence is not present in this region, this result provides further evidence that our serum inducible substrains are dependent on transfected human β–actin promoter segments containing SRE-related sequences.

Our inability to induce CAT activities in our 3T3 substrains expressing HβA CAT or HβA 657-910(IVS) SVCAT with EGF contrasts with the results of Elder *et al.* (24) who have stimulated actin gene transcription with EGF in quiescent AKR2B cells. One possibility is that 3T3 cells may be less responsive to EGF than AKR2B cells. Another cellular gene that is inducible with EGF is the proto-oncogene c-*fos* (6,25,26,27). We have isolated two 3T3 substrains expressing a c-*fos* SRE-containing CAT gene, pTF3(27). The CAT activity in these two 3T3 substrains are inducible by serum to 3.3 and 3.6 fold, respectively. The former substrain is weakly inducible with EGF (see Figure 4E). Since the transcription of the c-*fos* gene is highly inducible by EGF in A431 cells which have high levels of EGF receptor (25,26), our results suggest that EGF inducibility is dependent on cell type and may reflect differences in receptor levels.

DISCUSSION

The human β–actin promoter appears to be a complex cellular promoter. It encompasses about 3 kilobases of noncoding sequences. We have identified three regulatory domains for this

promoter. The 5′ distal promoter region has an enhancer-like activity. Upstream of the mRNA cap site, the proximal promoter domain contains the classical CCAAT and TATA sequences. The third domain resides in an intron containing evolutionarily conserved sequences (5). These three domains each contain one CArG sequence which is also found in other mammalian actin genes (9,10).

The β–actin gene is one of the first genes induced by serum at the G_0 /G_1 cell cycle transition (6,7). We have shown that the β–actin promoter directed CAT expression is serum dependent and thus this promoter likely contains serum-responsive sequences. Furthermore, we have localized one serum responsive segment to a 254 base-pair region within the first intron and another to the 466 base-pair proximal promoter domain. These two serum responsive domains have identical CArG sequences (see Figure 2) which are evolutionarily conserved (5). In contrast, the distal promoter domain, containing a SRE-like CArG sequence with dyad symmetry, is not serum inducible in 3T3 substrains expressing HβA-2006/-575 SVCAT. If the distal SRE-like sequences does in fact interact with SRE binding proteins, then there may be a growth inhibitory sequence (22) located between the two upstream promoter regions. Experiments to further delineate the locations and activities of positive and negative cellular growth control elements in the human β–actin promoter are in progress.

The 30 bp sequence in the β–actin first intron is one of the most evolutionarily conserved noncoding sequences (5). A 254 bp intron domain, containing this sequence, is both serum and FGF inducible. Thus this domain, like the c-*fos* promoter, could contain distinct sites for interaction with nuclear proteins (11,19,21,26,28,29,30). We have detected, using DNA mobility shift assays, interaction of nuclear proteins with the CArG motif (S. Ng, unpublished data). Moreover, this interaction is sensitive to competition by oligonucleotides containing the γ-actin or the c-*fos* CArG motif. Further evidence for the intron domain as an "open" site for interaction with multiple regulatory factors came from localization of DNaseI-hypersensitive sites in chromatin (J.T. Elder, manuscript in preparation). Thus, the serum responsive region in the human β–actin gene first intron, like the c-*fos* SRE, are both located in DNase I-hypersensitive regions (31).

All known mammalian muscle actin genes have CArG motifs (9,10). The SRE-like sequences of human β– and γ-actin genes also contain CArG motifs (see Figure 2). This motif seems to be one of the major control element of muscle actin genes (10,32,33,34) but is recognized by DNA-binding protein(s) from both muscle cells (35,36) and non-muscle cells (37). The binding contacts of both the muscle and non-muscle nuclear CArG binding factors are identical and the physical characteristics of the factors are indistinguishable (L. Boxer, T. Miwa, and L. Kedes, in press). However, despite its similarity to an SRE element, this motif in the muscle actin genes is apparently inactive in fibroblasts (3) and thus implies that certain CArG-associated protein(s) are muscle-specific transcription factor(s). In contrast, the β–actin promoter is equally active in proliferating myoblast cultures as in differentiating myotube cultures (3). One possible explanation would be that the function of the muscle actin CArG motif but not the β–actin CArG motif requires the presence of muscle-specific auxiliary factors. Alternatively, the CArG motifs of muscle actin genes may be unable to interact with one or more of the SRE-binding/associated

factor(s) due to sequence divergence adjacent to the CArG motifs. Similarly, the SRE-like elements of the β-actin gene may be unable to interact with one or more of the CArG-binding factors.

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