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A 29-Nucleotide DNA Segment Containing an Evolutionarily Conserved Motif Is Required in *cis* for Cell-Type-Restricted Repression of the Chicken α-Smooth Muscle Actin Gene Core Promoter

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A series of 5′ deletion mutations of the upstream flanking sequences of the chicken α-smooth muscle (aortic) actin gene was prepared and inserted into the chloramphenicol acetyltransferase expression vector pSV0CAT. Deletion recombinants were transfected into fibroblasts, which actively express the α-smooth muscle actin gene, and primary myoblast cultures, which accumulate much lower quantities of α-smooth muscle actin mRNAs. The first 122 nucleotides of 5′-flanking DNA were found to contain a "core" promoter capable of accurately directing high levels of transcription in both fibroblasts and myotubes. The activity of this core promoter is modulated in fibroblasts by a "governor" element(s) located at least in part between nucleotides −257 and −123. This region contains sequences potentially conserved between mammalian and avian α-smooth muscle actin genes as well as one of a pair of 16-base-pair inverted CCAAT box-associated repeats which are conserved among all chordate muscle actin genes examined to date. A smaller DNA segment (−151 to −123) containing these upstream CCAAT box-associated repeats was sufficient to suppress expression of the core promoter in muscle cultures, suggesting that the upstream CCAAT box-associated repeats play a negative role in the α-smooth muscle actin gene promoter.

Cellular differentiation is established and maintained by selective expression of sets of genes. One of the primary mechanisms by which this is achieved is alteration of the transcriptional activity of appropriate genes (9). Tissue-specific transcriptional control is presumably mediated by signals embedded within protein-coding genes; indeed, DNA segments which perform this function have been identified in several instances (4, 8, 10, 12, 13, 27, 30, 43). *Cis*-acting tissue-restrictive regulatory regions within individual members of multigene families are of particular interest, since, even though these loci presumably arose from common progenitor genes, they may be transcribed with distinctly different patterns of expression. Multigene families are therefore ideal model systems for characterizing mechanisms of differential gene expression and the evolutionary processes leading to regulatory specialization.

In most eucaryotic species, individual members of actin multigene families are regulated with distinct patterns of both tissue (6, 14, 39) and developmental stage (14, 16, 22, 29, 34, 35, 37) specificity. Mammalian and avian species express four muscle actin isotypes which are separable into striated and smooth muscle isoforms (40). As a first step towards defining the regulatory mechanisms controlling the smooth muscle-specific genes, we characterized the organizational and functional features of the chicken α-smooth muscle actin locus, which encodes the major actin expressed in aortic smooth muscle (7). The complete nucleotide sequence of this gene, including its flanking sequences, was determined. Examination of the 5′-flanking DNA of this gene revealed the presence of a pair of inverted repeats which included a 16-base-pair (bp) DNA motif termed the "CCAAT" box-associated repeat (CBAR) (7; D. J. Bergsma, S. L. Carroll, and R. J. Schwartz, UCLA Symp. Mol. Cell. Biol., in press). Remarkably, this CBAR element was found to be evolutionarily conserved as repeats within the 5′-flanking region of all known muscle-specific vertebrate actin genes. In addition, hybridization analyses suggested the presence of additional 5′-flanking nucleotides which are conserved between the human and chicken α-smooth muscle actin genes (7).

Our earlier studies, however, provided no information on the functional significance of the conserved 5′ upstream elements. In this communication, we report that we have delimited regions within the 5′-flanking DNA of the α-smooth muscle actin gene which are necessary in *cis* for maximal expression in fibroblasts as well as sequences which dictate cell-type restriction of transcription. The 5′-flanking region contains a segment of ca. 100 nucleotides which functions as a "governor" element regulating the level of expression of a core promoter that is highly active in cells normally expressing the α-smooth muscle actin gene. Interestingly, this core promoter also appears to direct a high level of transcription in cells not expressing their endogenous α-smooth muscle actin gene; this transcription can be quite efficiently repressed via the linkage in *cis* of a 29-bp segment containing one of the pair of evolutionarily conserved inverted repetitive motifs.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, Bio-Rad Laboratories, and Miles-Yeda and used according to the recommendations of the manufacturer.

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Oligonucleotide linkers (octamers) were obtained from New England BioLabs. Cell culture reagents were from GIBCO Laboratories. Purified *Escherichia coli* chloramphenicol acetyltransferase (CAT) and acetyl coenzyme A (lithium salt) were purchased from Pharmacia. Purified *E. coli* β-galactosidase (grade VIII) and *o*-nitrophenyl-β-D-galactopyranoside were bought from Sigma Chemical Co. [^{14}C]chloramphenicol was purchased from New England Nuclear Corp., and ^{32}P-labeled nucleotides were from ICN Radiochemicals. Plasmids pRSVβgal and HSVtk5′Δ105 were kind gifts from Grant MacGregor (Howard Hughes Medical Institute, Baylor College of Medicine) and from Steven McKnight (Carniege Institution of Washington). All other chemicals used were of the highest purity available.

Plasmid constructions. To facilitate manipulation of the 5′-flanking sequences of the chicken α-smooth muscle actin gene, plasmid pAC3-7AcE5 was constructed. This clone, which encompasses 1,012 bp of 5′-flanking sequence, the first exon (45 bp), and 157 bp of the first intron, was derived by isolating an *Eco*RI-*Acc*I fragment from the α-smooth muscle actin genomic clone pAC377 (7), filling in the ends with DNA polymerase I (Klenow fragment), and cloning the fragment into the *Sma*I site of pUC8 (41). A *Hin*dIII fragment from pAC3-7AcE5 which includes nucleotides −690 to +202 (relative to the α-smooth muscle actin gene mRNAcap site) was digested with *Fnu*4HI, the ends were filled with DNA polymerase I (Klenow fragment), and synthetic *Hin*dIII linkers were ligated to the deletion endpoints. The linkered fragments were redigested with *Hin*dIII, after which a linkered fragment spanning nucleotides −257 to +19 was isolated and cloned into the *Hin*dIII site of pUC8 to produce pMOM257; the insert orientation selected was such that the *Pst*I site corresponding to position −238 of the α-smooth muscle actin gene was adjacent to the *Pst*I site of the pUC8 polylinker. Plasmid pMOM870 was derived from pMOM257 by digesting pMOM257 with *Pst*I, removing a small *Pst*I fragment which included the 5′-most *Hin*dIII linker, and replacing the small *Pst*I fragment with a *Pst*I fragment from pAC3-7AcE5, which spans −870 to −239 of the α-smooth muscle actin gene 5′-flanking sequence. The construction of pMOM870 restored the sequences from −870 to +19 to their original state, while retaining the *Hin*dIII linker at the *Fnu*4HI site (+19) in the first exon sequences.

Plasmid p690asmCAT was constructed by digesting pMOM870 with *Hin*dIII, isolating the appropriate fragment (−690 to +19), and cloning it into the *Hin*dIII site of pSV0CAT (18). Plasmid p257asmCAT was constructed by digesting pMOM257 with *Hin*dIII and cloning the derived fragment (−257 to +19) into the *Hin*dIII site of pSV0CAT. Plasmid p257asmCAT was digested with *Pvu*II, and the fragment spanning −219 of the α-smooth muscle actin gene sequence to nucleotide 150 of the CAT structural gene was purified for redigestion with *Hae*III. *Hin*dIII linkers were added to the fragment encompassing nucleotides −151 to +19 and cloned into the *Hin*dIII site of pSV0CAT to yield p151asmCAT. Plasmid p122asmCAT was produced by digesting p257asmCAT with *Ava*II, adding *Hin*dIII linkers, and then cloning the fragment (−122 to +19) into the *Hin*dIII site of pSV0CAT. The insert orientation and junctional sequences of all of these constructions, as well as the sequence of the internal junction in p690asmCAT, were verified by Maxam-Gilbert sequencing (25).

Plasmid pB105*tk*CAT is a plasmid vector we constructed in which the transcription of the CAT structural gene is controlled by the herpes simplex virus thymidine kinase (*tk*) gene promoter (nucleotides −105 to +51; 26). A *Bgl*II linker flanked on either side by *Bam*HI sites was positioned immediately 5′ of the *tk* promoter, with a fragment from pBR322 (nucleotides 29 to 375) inserted 5′ of the *Bgl*II linker.

Plasmids were propagated in *E. coli* K-12 RR1. Plasmid DNAs were prepared by using an alkaline lysis procedure (3) and two successive CsCl-ethidium bromide gradient centrifugations. Prior to transfection, the morphology of all plasmid preparations was examined by electrophoresis in 1.4% agarose gels containing TEA (160 mM Tris hydrochloride [pH 7.5], 8 mM EDTA, 80 mM sodium acetate, 70 mM NaCl) buffer. Preparations were judged acceptable if >50% of the DNA was supercoiled (form I).

Establishment, maintenance, and transfection of cell cultures. Fertilized White Leghorn eggs were obtained from the Department of Poultry Science, Texas A&M University, and incubated at 37°C. Primary cultures of chicken embryo fibroblasts were established by trypsinizing tissue from the backs of 10- to 11-day chicken embryos (42). Fibroblasts were incubated in a 5% CO_2 atmosphere at 37°C while growing in minimal essential medium supplemented with 5% tryptose phosphate broth, 5% heat-inactivated calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were trypsinized and passaged every 5 to 7 days. All experiments were performed with fibroblasts between passages 4 and 8.

Fibroblasts were seeded into 100-mm culture dishes 24 h prior to transfection at a density of 2.0×10^6 to 2.5×10^6 cells per plate. Transfections were performed with supercoiled plasmid DNAs in calcium phosphate precipitates (44) prepared as described earlier (2). Five micrograms each of pRSVβgal and the appropriate CAT vector were transfected per 100-mm dish. Cells were then incubated for 4 h at 37°C, at which time the medium was removed, the cells were subjected to a 30-s glycerol shock at room temperature (17), plates were rinsed with Hanks balanced salt solution, and fresh medium was added. Fibroblasts were refed 48 h posttransfection.

Primary myoblast cultures were established by mechanically dissociating tissue from the thighs of 11-day chicken embryos as described previously (22). Following preplating, the myoblast-enriched cell populations were seeded into collagen-coated 100-mm dishes at a density of 2.5×10^6 to 3.5×10^6 cells per plate in minimal essential medium supplemented with 10% horse serum, 5% chicken embryo extract, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were incubated in 5% CO_2 at 37°C. At 24 h postplating, primary myoblast cultures were transfected as described above. Following the glycerol shock and saline rinse, cultures were fed with minimal essential medium supplemented with 10% horse serum, 2% chicken embryo extract, 100 U of penicillin per ml, and 100 μg of streptomycin per ml and maintained in this medium until collection.

Four to eight individual transfection experiments were performed with each chimera and control vector. At least three independent preparations of every plasmid DNA were used to minimize potential variability resulting from unexpected dissimilarities between DNAs. To further reduce deviations which might arise from unforseen diversity in individual cultures, DNAs were transfected into two to six distinct batches of each cell type.

Enzymatic assays. Cells were harvested by scraping 72 h posttransfection and suspended in 50 μl of CAT buffer (250 mM Tris [pH 7.5]). Cell extracts were prepared by three freeze-thaw cycles followed by centrifugation to remove cellular debris, after which the volume of the cell extracts was adjusted to 100 μl with CAT buffer. A 40-μl portion of

each extract was then assayed for CAT activity as described by Gorman et al. (18). Reactions performed in parallel with purified *E. coli* CAT enzyme demonstrated that the upper limit of linearity under these conditions was approximately 50% conversion. Samples showing >40% conversion were routinely reassayed by using a smaller quantity of cell extract. Background activity was determined by assaying extracts of cultures cotransfected with pRSVβgal and pBR322.

Assays of β-galactosidase activity were performed with 10 μl of the same cell extracts used for CAT assays under the conditions described by Miller (28), except that our Z buffer contained 50 mM Tris (pH 7.4) rather than 100 mM phosphate. The linear range of this assay was determined with purified *E. coli* β-galactosidase. Samples exceeding 80% of the upper limits of linearity were reassayed with a smaller amount of cell extract. Background activity in both fibroblast and muscle cultures was found to be essentially zero.

Isolation, blotting, and hybridization of total cellular RNA. Frozen cultures were scraped into extraction buffer (25 mM EDTA [pH 8.2], 0.1 M NaCl, 0.5% sodium dodecyl sulfate) and then extracted sequentially with extraction buffer-saturated phenol, a 25:24:1 mix of phenol-chloroform-isoamyl alcohol, and a 24:1 mix of chloroform-isoamyl alcohol. Genomic DNA was selectively removed by a series of 3 M NaAc (pH 6.0) washes (33). Samples of the resulting total cytoplasmic RNAs were denatured with glyoxal (38) and dotted (under the conditions recommended by the manufacturer) onto Biotrans nylon membrane (ICN Radiochemicals), using a Minifold apparatus (Bio-Rad Laboratories). Dot blots were baked for 2 h at 80°C and then boiled in 20 mM Tris (pH 8.0) (38). Following prehybridization, filters were hybridized under the conditions we have previously described (7) to single-stranded ^{32}P-labeled DNA probes prepared from M13 templates (2). Blots were washed four times at 68°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate and exposed to Fuji RX X-ray film at −70°C with a Cronex Lightning-Plus intensifying screen (E. I. duPont de Nemours & Co.). A graded series of exposures was prepared, and the relative hybridization of the samples was determined by scanning the autoradiograms with a BioImage image analysis computer (BioImage Corp.).

RESULTS

Actin mRNA expression in cultured chicken embryo fibroblasts and myoblasts. The α-smooth muscle actin protein was originally identified in vascular smooth muscle (15) and

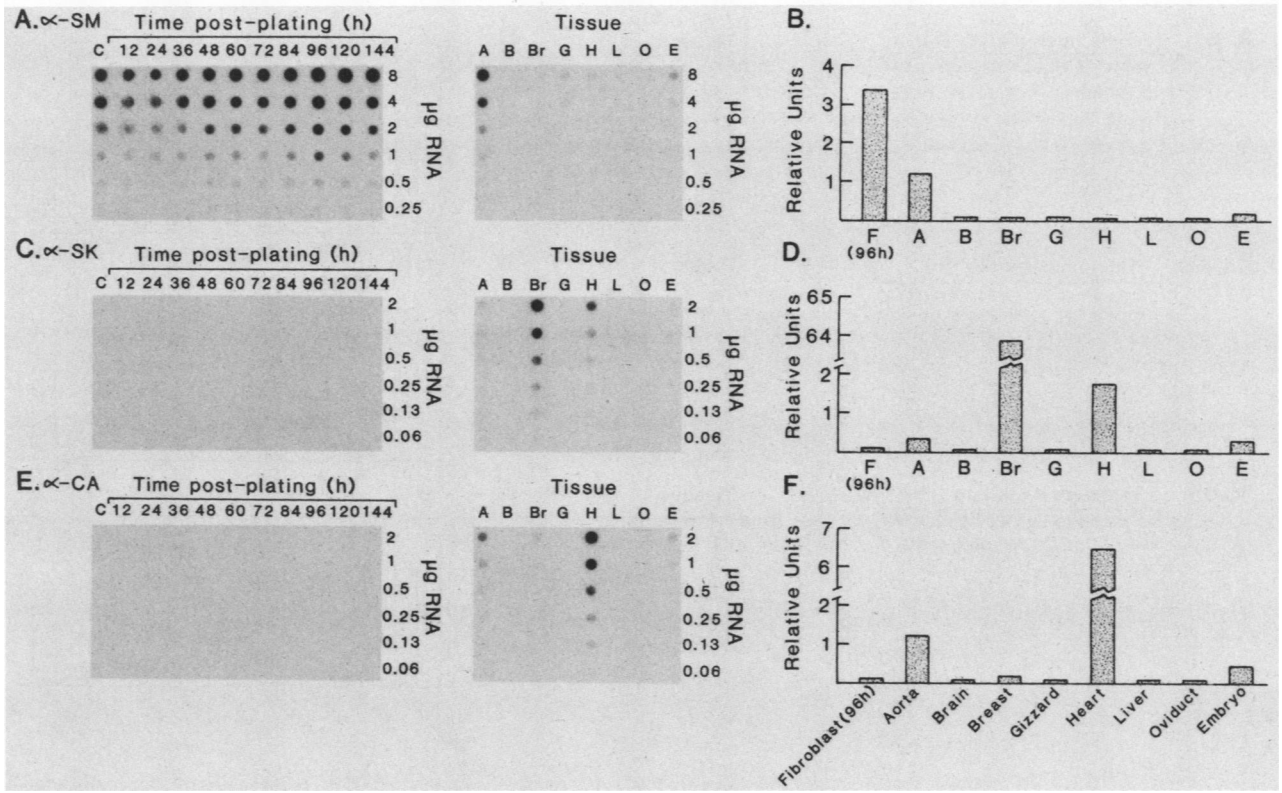

FIG. 1. Steady-state levels of muscle actin mRNAs in proliferating fibroblasts relative to levels in adult tissues and 10-day embryo. Successive twofold dilutions of each RNA were dotted onto nylon filters. Filters carrying total cytoplasmic RNA isolated from passage 5 fibroblast cultures at the specified times postplating (12 to 144 h) or confluent passage 4 cultures (c) were hybridized in the same bag with standard filters carrying total cytoplasmic RNA isolated from adult tissues or 10-day embryo. Each pair of filters was hybridized with a 3′-untranslated region probe for α-smooth (α-SM; A) (7), α-skeletal (α-SK; C) (22), or α-cardiac (α-CA; E) (22) muscle actin mRNA. Relative levels of hybridization of individual messengers were determined by scanning representative autoradiograms with a BioImage image analysis computer; units are arbitrary and are not intended to be used for the comparison of relative levels of different actin mRNAs. The levels obtained in fibroblast cultures 96 h postplating relative to those of adult tissues are indicated graphically in panels B (α-smooth), D (α-skeletal), and F (α-cardiac). In panels A, B, C, D, and E, the following abbreviations are used: A, aorta; B, brain; Br, breast muscle; G, gizzard; H, heart; L, liver; O, oviduct; E, embryo; F, fibroblast. It should be noted that the amount of breast RNA used in panel C is 10-fold less than that used for other RNAs.

subsequently detected in primary and passaged cultures of chicken embryo fibroblasts (39). These observations indicated that fibroblast cultures might be useful in the definition of *cis*-acting regulatory elements within the α-smooth muscle actin gene. It was essential that our chimeric plasmids be introduced into cells actively transcribing their endogenous α-smooth muscle actin gene. Constancy of expression of this locus is, however, by no means assured, since the steady-state levels and rate of biosynthesis of the α-smooth muscle actin protein fluctuate in cultured vascular smooth muscle cells, apparently as a function of the cellular growth state (32). To examine the possibility that a similar phenomenon involving alterations in mRNA levels was operative in fibroblasts, passaged chicken embryo fibroblasts were trypsinized and plated at a density of 1.75×10^4 cells per cm^2. At specified times postplating, dishes were taken for RNA isolation and determination of cell density. Total cytoplasmic RNAs were isolated, glyoxalated, and, after being serially diluted, used to prepare replicate dot blots. Total cytoplasmic RNAs from several adult chicken tissues (aorta, brain, breast, gizzard, heart, liver, and oviduct) and 10-day chicken embryos were similarly treated to provide a standard of comparison. These sets of dot blots were hybridized to isotype-specific actin mRNA probes as described in Materials and Methods.

Figure 1 presents representative autoradiograms of filters hybridized to probes derived from the 3′-untranslated regions of the α-smooth (panel A), α-skeletal (panel C), and α-cardiac (panel E) muscle actin genes. Examination of the levels of α-smooth muscle actin mRNAs found in fibroblasts indicates that these messengers accumulate variably during the course of this experiment, with maximum and minimum levels achieved 96 and 24 h postplating, respectively. Maximum expression of α-smooth muscle actin mRNAs was ca. threefold higher in fibroblasts than in the intact adult aorta, the tissue containing the greatest quantity of these same messengers. This result is not necessarily indicative of the level of α-smooth muscle actin mRNAs found in fibroblasts relative to vascular smooth muscle cells, since the intact aorta contains a variety of cell types other than smooth myocytes. Nonetheless, fibroblasts express significant quantities of α-smooth muscle actin mRNAs and therefore were judged useful for our experimental purposes. It is also notable that α-skeletal and α-cardiac muscle actin mRNAs are undetectable in these cultured cells, which indicates that our fibroblast cultures are virtually free of contaminating myoblasts, a cell type which accumulates large quantities of striated muscle actin mRNAs (22).

Figure 2 presents representative autoradiograms of dot blots hybridized to cytoplasmic actin mRNA isotype-specific

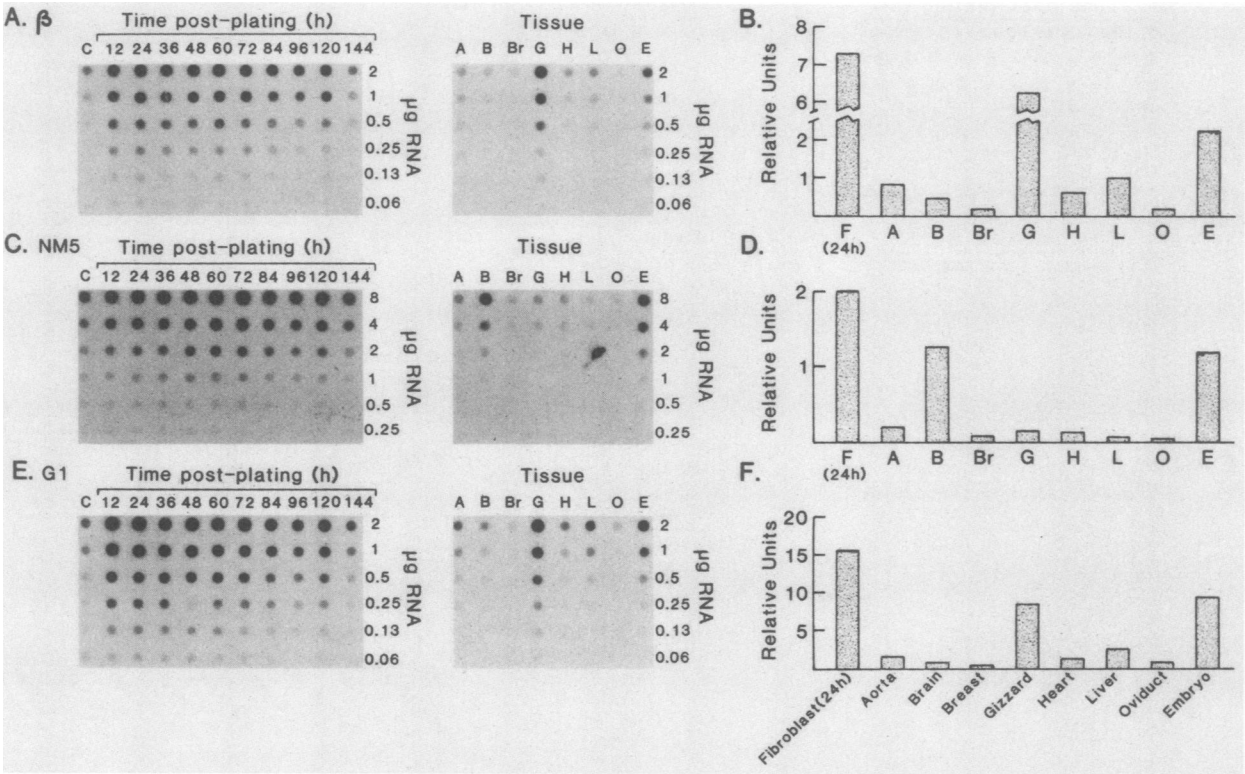

FIG. 2. Steady-state levels of cytoplasmic actin mRNAs in proliferating fibroblasts relative to levels in adult tissues and 10-day embryo. Successive twofold dilutions of each RNA were dotted onto nylon filters. Filters carrying total cytoplasmic RNA isolated from passage 5 fibroblasts at specified times postplating (12 to 144 h) or confluent passage 4 cultures (c) were hybridized in the same bag with standard filters carrying total cytoplasmic RNA isolated from adult tissues or 10-day embryo. Each pair of filters was hybridized with a 3′-untranslated region probe for either β (A) (22), nonmuscle type 5 (NM5; C) (1), or G1 (E); G1 is a cytoplasmic actin gene distinct from β and nonmuscle type 5 (our unpublished results) cytoplasmic actin mRNAs. Relative levels of hybridization were determined by scanning representative autoradiograms with a BioImage image analysis computer; as in Fig. 1, units are arbitrary. The levels obtained in fibroblast cultures 24 h postplating relative to those of adult tissues are indicated graphically in panels B (β), D (nonmuscle type 5), and F (G1). In panels A, B, C, D, and E, the following abbreviations are used: A, aorta; B, brain; BR, breast muscle; G, gizzard; H, heart; L, liver; O, oviduct; E, embryo; F, fibroblast. Following this experiment, the filter shown in panel A was stripped and reprobed with the α-smooth muscle actin mRNA 3′-untranslated region probe; the pattern obtained was identical to that shown in Fig. 1A.

probes. As above, both blots and histograms are presented to describe the results obtained with probes derived from the 3'-untranslated regions of the β-cytoplasmic (panels A and B), nonmuscle type 5 (panels C and D), and G1 (panels E and F) actin genes. Messenger RNAs for all three of these cytoplasmic actins are easily detectable in cultured fibroblasts and in general are found at levels higher than those found in either adult tissues or the 10-day embryo. Interestingly, the levels of these mRNAs also fluctuate during the course of the growth curve, with the lowest levels apparent in the confluent plates from which the fibroblasts were trypsinized and in cultures 144 h postplating. The highest levels of the β-cytoplasmic and G1 actin messengers were achieved 12 to 24 h postplating, with a distinct peak being less well defined for the nonmuscle type 5 mRNA. Figure 3 presents curves exhibiting the relationship among the time postplating, relative muscle actin mRNA levels (panel A), relative cytoplasmic actin mRNA levels (panel B), and cell density (panel C). This comparison demonstrates distinctly different patterns of expression for the α-smooth muscle actin mRNAs and the cytoplasmic messengers, with the lowest accumulation of α-smooth muscle actin mRNAs found in subconfluent, actively proliferating cultures and gradually increasing as these cells approach confluence. These data suggest, therefore, that expression of the α-smooth muscle actin locus may show some as yet undefined relationship with the growth state of the fibroblasts. Since the α-smooth muscle actin messenger levels are continually increasing over the period corresponding to our transfections, these data further suggest that the cognate gene is transcriptionally active during our transfection experiments.

Our laboratory has previously documented the patterns of expression of the α-skeletal, α-cardiac, and β-cytoplasmic actin genes in myoblast cultures (22) as well as the utility of these cells for transient expression assays with the α-skeletal muscle actin gene promoter (20). Myoblast cultures were subsequently found to contain small quantities of α-smooth muscle actin mRNAs (our unpublished observations), which is the result of low-level contamination with fibroblasts (initially <10% of the total cell population). These observations suggested that myoblast cultures could be useful in the identification of cell-type-restricted transcriptional elements within the α-smooth muscle actin gene promoter.

Construction of 5' deletion mutants. For the purpose of identifying the approximate location of the 5' boundaries of *cis*-acting regulatory sequences necessary for transcription in chicken cells, varying lengths of the 5'-flanking regions of the chicken α-smooth muscle actin gene were fused in vitro to the 5' end of the promoterless *E. coli* CAT gene in plasmid vector pSV0CAT (18). Figure 4 displays some of the general features of the 5'-flanking DNA of this actin gene as well as the regions which were utilized for the construction of the various actin-CAT chimeric plasmids. Each chimera within the α-smooth muscle actin gene deletion series was derived for the purpose of testing the regulatory contributions of specific elements. Plasmid p257asmCAT contained 5'-flanking DNA through the region of isotype-specific evolutionarily conserved sequences, while p686asmCAT was utilized to examine the role of sequences immediately upstream from this conserved region. Plasmid p151asmCAT retained the 5'-flanking sequences to a point just 5' of the upstream CBAR; a small region containing this repeat was removed in the construction of p122asmCAT.

S1 nuclease mapping of the transcriptional initiation site of plasmid p122asmCAT. Since it was possible that an alternative promoter had been artifactually generated in our recombinant plasmids, we decided to determine whether the transcription initiation site of these chimeras was equivalent to the native transcription initiation site of the intact gene. Twenty micrograms of either p122asmCAT (which is active in both fibroblasts and myoblasts [see below]) or pSV0CAT per plate was transfected into dishes of nonpreplated myoblast cultures. Cells were harvested 48 h posttransfection and used to prepare total cytoplasmic RNA; these RNAs

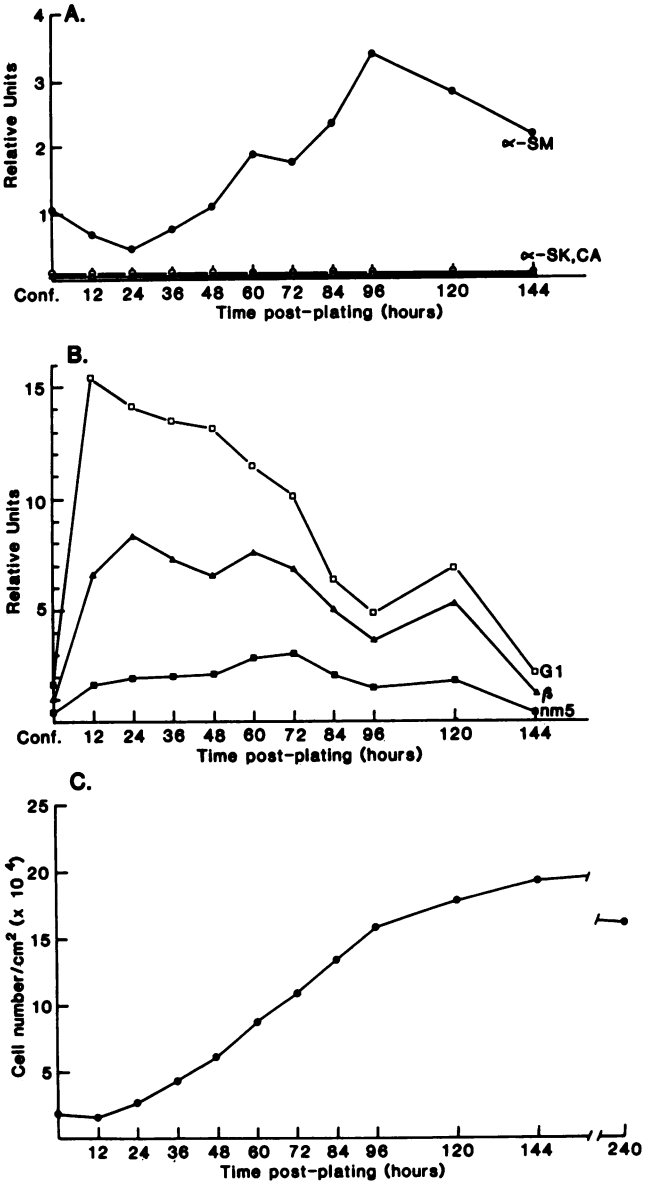

FIG. 3. Relationship of actin mRNA steady-state levels in fibroblast cultures to the growth state of the cells. (A and B) The relative quantities of actin mRNAs determined from blots presented in Fig. 1 and 2, respectively, were plotted as a function of time postplating. The 3'-untranslated region probes utilized are indicated for each curve: α-SM, α-smooth; α-SK, α-skeletal; α-CA, α-cardiac; β, β-cytoplasmic; nm5, nonmuscle type 5; G1, G1 cytoplasmic. (C) Cells were trypsinized at the indicated times postplating, diluted, and counted with a Coulter counter. Cell number per square centimeter was plotted versus time postplating. Reported cell numbers reflect counts from three plates at each time point; three different dilutions were prepared from each plate and counted in duplicate.

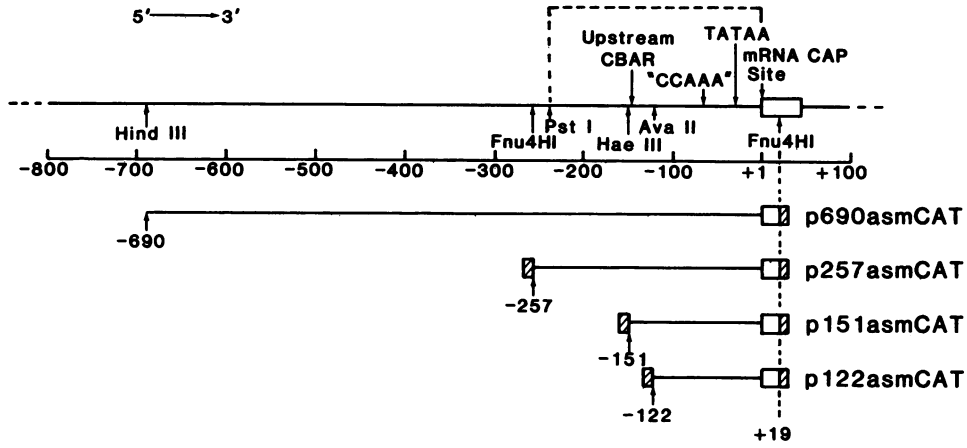

FIG. 4. 5' deletion mutations of the promoter of the chicken α-smooth muscle actin gene. Details of the unidirectional deletion mutagenesis are described in Materials and Methods. A wild-type physical map of the first exons (encoding the mRNA 5'-untranslated region) and a portion of the 5'-flanking sequence of the gene is indicated at the top. Symbols: Open bar, First exon; thin line to left of first exon, 5'-flanking DNA; dashed bracket, evolutionarily conserved sequences. Coordinates refer to base pairs of DNA sequence relative to mRNA cap site at position +1. Positions of the TATA and CCAAT homologies and upstream CBAR are indicated by arrows above the line diagram. Below the sequence representation, regions of the α-smooth muscle actin gene cloned into the *Hin*dIII site of pSV0CAT are illustrated. Symbols: Hatched boxes, *Hin*dIII linkers; open boxes, first exon sequences. The nucleotide positions of endpoints of deletion mutations are indicated below the diagram of each chimera. The names of the CAT recombinant clones are shown at the right.

were hybridized to an end-labeled probe encompassing the α-smooth muscle actin gene 5' transcription initiation site (Fig. 5B). Approximately 171 nucleotides of the α-smooth muscle actin gene chimeric probe were protected from S1 nuclease digestion by RNA from plates transfected with p122asmCAT (Fig. 5A); this fragment is precisely the size which would be expected for an mRNA initiating at the native α-smooth muscle actin gene mRNA cap site (7).

Transient expression phenotypes of α-smooth muscle actin 5' deletion recombinants in transfected chicken embryo fibroblasts and myocytes. Actin promoter-CAT chimeric plasmid DNAs were transfected into proliferating chicken embryo fibroblasts and primary myoblast cultures. For purposes of comparison, parallel transfections were performed with plasmid pSV2CAT, a vector in which the bacterial CAT gene is under the control of the simian virus 40 enhancer and early promoter region (18); plasmid pB105*tk*CAT, in which the herpes simplex virus thymidine kinase promoter (26) directs the synthesis of the CAT protein; or plasmid pSV0CAT, which lacks any defined eucaryotic promoter (18). To control for variations in both cell numbers and transfection efficiencies, all recombinant and control plasmids were cotransfected with an equal quantity of pRSVβgal, a eucaryotic expression vector in which the *E. coli* β-galactosidase (*lacZ*) structural gene is under the transcriptional control of the Rous sarcoma virus long terminal repeat (19). Transfected cells were harvested 96 h postplating (72 h after transfection). Lysates prepared from the harvested cells were assayed for both CAT activity, which was utilized as a measure of promoter efficiency, and β-galactosidase activity, which was used to normalize the CAT activity directed by each test plasmid.

Transfections into fibroblasts were performed for the purpose of determining whether the 5'-flanking sequences of the α-smooth muscle actin gene were sufficient to direct transcription in cells normally expressing this locus and, if so, which segments within this region modulate activity. We found all of the α-smooth muscle actin gene 5' deletion recombinants to be transcriptionally active in fibroblasts (data presented graphically in Fig. 6A and in numerical form in Table 1). Removal of nonconserved DNA sequences between 690 and 257 bp upstream of the mRNA cap site of this gene had no appreciable effect on the efficiency of the α-smooth muscle actin gene promoter. Deletions of nucleotides 3' to position −257, however, produced differences in the relative levels of expression of the remaining mutations, which allowed the identification of three DNA segments which function in *cis* to regulate the α-smooth muscle actin locus. Removal of the first 107 bp (nucleotides −257 to −152) within the region containing isotype-specific conserved sequences led to a 60% decrease in CAT expression, suggesting that the 5' boundary of at least one *cis*-acting regulatory element lies between these positions. Interestingly, elimination of the next 29 bp (nucleotides −151 to −123), a segment containing the upstream CBAR, resulted in a significant increase in the activity of the promoter. The transcriptional activity of p122asmCAT in these cells is in fact 250% of that found with p257asmCAT and therefore approaches the efficiency of the simian virus 40 early promoter contained in pSV2CAT.

The hypothetical *cis*-acting regulatory element(s) responsible for cell-type restriction of transcription could coincide with the DNA segments regulating expression in fibroblast cultures or they could be located elsewhere on the chromosome carrying this locus. To determine whether the 5'-flanking sequences of the α-smooth muscle actin gene function in a cell-type-restricted manner, the data obtained with transfections into fibroblasts were compared with the results of identical experiments performed in muscle cultures (Fig. 6B; Table 1). Examination of these data revealed that, whereas p690asmCAT had been quite active in fibroblast cultures, its efficiency in differentiated myotubes was not significantly different from that of pSV0CAT. Neither the removal of the 429 bp (−690 to −257) of nonconserved sequences nor the deletion of the next 107 bp (−257 to −151) of potentially evolutionarily conserved sequences altered this relationship. Elimination of nucleotides −151 to −123, however, again resulted in a dramatic increase in promoter efficiency. A comparison of the relative CAT activity directed by p122asmCAT in these myotubes and the same

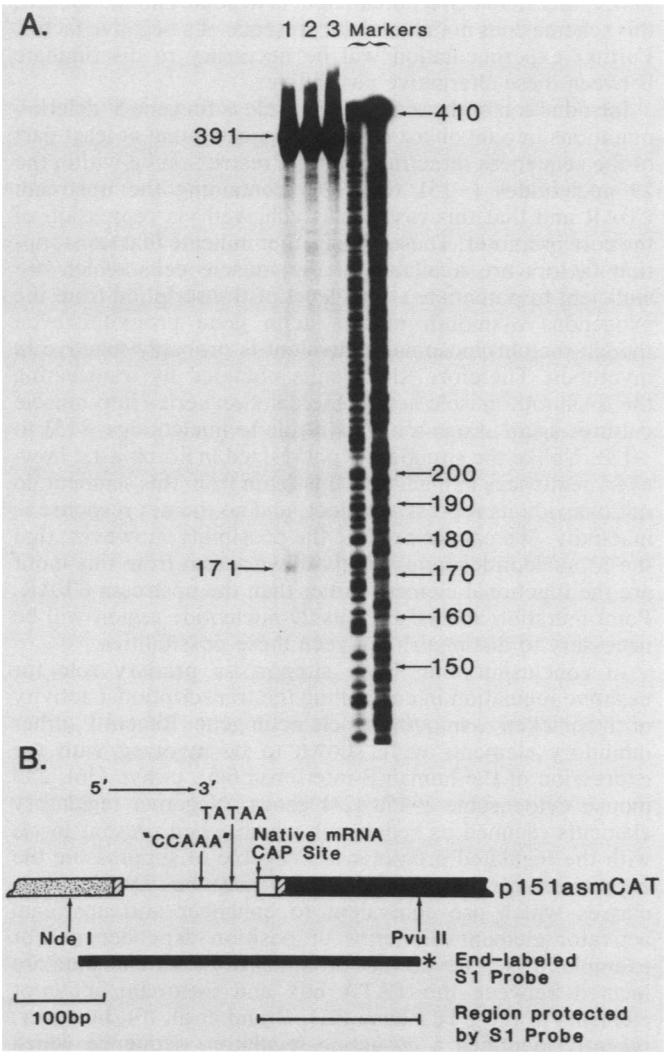

FIG. 5. S1 nuclease mapping of the transcription initiation site of p122asmCAT. Total cytoplasmic RNA from nonpreplated muscle cultures transfected with 2 μg of either p122asmCAT or pSV0CAT was hybridized at 68°C for 22 h to a ^{32}P-end-labeled *Nde*I-*Pvu*II fragment overlapping the fusion between the α-smooth muscle actin gene promoter and the CAT structural gene. Hybridization reactions were digested with S1 nuclease and fractionated on a 6% polyacrylamide sequencing gel as previously described (20). (A) Autoradiogram of fragments protected by the probe indicated in panel B. Lane 1, 100 μg of RNA from cultures transfected with p122asmCAT; lane 2, 25 μg of the same RNA used for lane 1 plus 75 μg of *E. coli* tRNA; lane 3, 100 μg of RNA from cultures transfected with pSV0CAT. In panel A, the positions of full-length probe (391 bp) and protected fragment (171 bp) are indicated by arrows to the left of the autoradiogram. The right two lanes contain a known Maxam-Gilbert sequence pattern of a marker DNA fragment; nucleotide positions of some of the fragments are denoted to the right ot the autoradiogram. (B) Schematic diagram of the plasmid from which the S1 probe was derived. Symbols: Solid bar, CAT structural gene; cross-hatched bars, *Hin*dIII linkers; open bars, α-actin first exon; thin line, α-actin 5'-flanking DNA; stippled bar, pSV0CAT sequence. The positions of the native mRNA cap site and TATA and CCAAT homologies are indicated by arrows above the diagram. Thin black bars below each schematic represent sequences spanned by the S1 probe; an asterisk denotes the ^{32}P label position. The region of S1 probe DNA which would be protected by an accurately initiated RNA transcript is exhibited below the probe.

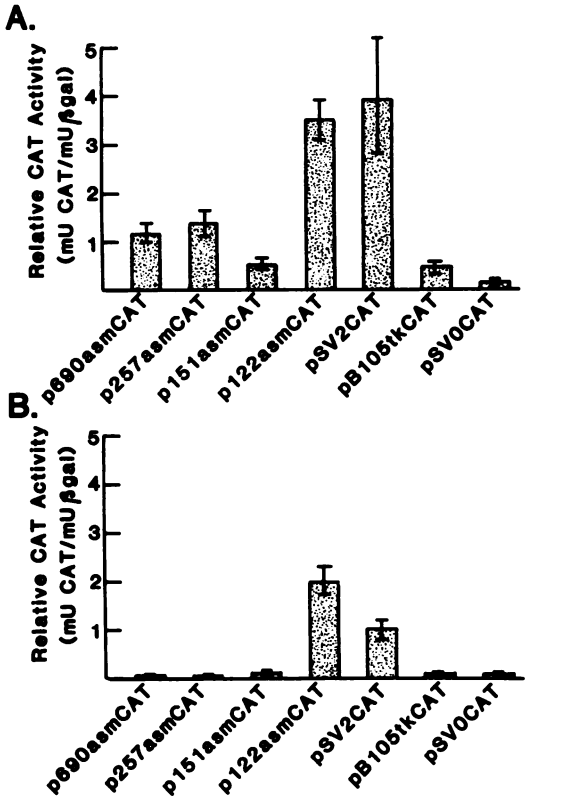

FIG. 6. Relative CAT activities directed by α-smooth muscle actin gene promoter-CAT chimeras in (A) fibroblasts and (B) muscle cultures. The 5' deletion recombinants of the α-smooth muscle actin gene promoter and control plasmids were cotransfected with pRSVβgal into passaged chicken embryo fibroblasts and primary myoblast cultures 24 h postplating as described in Materials and Methods. Transfected cells were harvested 96 h postplating and assayed for CAT and β-galactosidase activities. Values shown for relative CAT activities are the arithmetic means of at least four independent transfection experiments. Standard errors are indicated by I bars.

value for pSV2CAT shows that the efficiency of the p122asmCAT promoter is twice that of pSV2CAT.

DISCUSSION

Our earlier analyses of the structure and nucleotide sequence of the chicken α-smooth muscle actin gene identified potentially conserved sequences within the first 233 nucleo-

TABLE 1. Normalized CAT activities assayed in chicken embryo fibroblasts and myotubes 72 h after transfection with the α-smooth muscle actin gene 5' deletion mutants and control plasmids

Plasmid	mU of CAT activity/mU of β-galactosidase activity	
	Fibroblasts	Myotubes
p690asmCAT	1.19 ± 0.22	0.05 ± 0.00
p257asmCAT	1.40 ± 0.27	0.06 ± 0.01
p151asmCAT	0.55 ± 0.12	0.10 ± 0.05
p122asmCAT	3.53 ± 0.40	2.01 ± 0.32
pSV2CAT	3.96 ± 1.24	1.02 ± 0.21
pB105*tk*CAT	0.43 ± 0.16	0.03 ± 0.00
pSV0CAT	0.16 ± 0.06	0.03 ± 0.01

tides of this gene's upstream DNA and a pair of inverted CBARs within the same segment (7; Bergsma et al., in press), but did not indicate the functional significance of these elements. We now report that the 5′ boundary of the *cis*-acting regulatory elements of this locus lies between nucleotides −257 and −151 and therefore corresponds roughly to the 5′ boundary of the region potentially containing nucleotides which are conserved between the promoters of the chicken and human α-smooth muscle actin genes. Deletion mutation analysis also has allowed the location of three regulatory domains within this 257 nucleotides, one of which corresponds closely to the upstream CBAR. These results therefore support the hypothesis that the 5′-conserved region and the upstream CBAR perform regulatory functions in the α-smooth muscle actin gene promoter.

The positions of the functional elements noted by transfecting the α-smooth muscle actin gene deletion series into myoblasts and fibroblasts and their hypothetical roles are summarized in Fig. 7. Our results indicate that the first 122 nucleotides of 5′-flanking sequence are sufficient to direct maximal synthesis of CAT in fibroblasts and muscle cells and thus are not rigidly restricted in terms of cell-type specificity. For this reason, we have assigned the first 122 nucleotides of 5′-flanking sequences the role of "core promoter." We define core promoters as DNA sequences which are sufficient in *cis* to direct high-level, accurately initiated transcription when introduced into a regulated system; a core promoter may therefore coincide with the basal promoter defined in a constitutive system such as the *Xenopus* oocyte (e.g., the herpes simplex virus *tk* gene promoter [9, 20]) or may include additional functional moieties beyond those necessary for activity in a constitutive system (e.g., the chicken α-skeletal muscle actin gene promoter [2]). The activity of the α-smooth muscle actin gene core promoter appears to be modulated in fibroblasts by sequences between −257 and −123, a region we have accordingly designated the governor sequences. The results of our deletion analysis suggest that the functionality of the governor elements may be due to the combined effects of at least two *cis*-acting regulatory segments. This hypothesis predicts that the decrease in activity observed with the deletion of nucleotides −151 to −123 is indicative of the binding of a *trans*-acting negative regulatory factor to this region and that the activity or binding of this negative factor is in some way alleviated via the interaction of the region immediately upstream (−257 to −152) with a positive effector(s). However, it is possible that the removal of only a portion of the binding site for a solitary positive factor may allow the binding of this factor in such a way that transcription, rather than being potentiated, is to some extent blocked; this scheme does not invoke the presence of a negative factor. Further experimentation will be necessary to discriminate between these alternative possibilities.

Introduction of the α-smooth muscle actin gene 5′ deletion mutations into myoblast cultures suggested that at least part of the sequences directing cell-type restriction lie within the 29 nucleotides (−151 to −123) containing the upstream CBAR and that this restraint is achieved via repression of the core promoter. These data further indicate that transcription factors are available in the muscle cells which are sufficient to potentiate a high level of transcription from the exogenous α-smooth muscle actin gene promoter even though the chromosomal equivalent is probably inactive in myotubes. Therefore, the results obtained by transfecting the α-smooth muscle actin gene deletion series into muscle cultures again assign a negative role to nucleotides −151 to −123. Unlike the situation hypothesized in fibroblasts, however, sequences immediately upstream from this segment do not override its repressive effect, and so the net response is inactivity. We cannot exclude the possibility, however, that the 13 nucleotides immediately downstream from this motif are the functional element rather than the upstream CBAR. Point mutation analysis of this 29-nucleotide region will be necessary to distinguish between these possibilities.

In conclusion, our study supports a primary role for negative regulation in controlling the transcriptional activity of the chicken α-smooth muscle actin gene. Recently, other inhibitory elements were shown to be involved with the expression of the human β-interferon (45), c-myc (36), and mouse cytochrome P-450 (24) genes. Negative regulatory elements (defined as sequences which when present in *cis* with the regulated promoter are capable of suppressing the expression of the affected locus) may be divisible into classes which are equivalent to enhancer and upstream activator elements in terms of position dependence. For example, it is believed that proximal negative elements are located between the TATA box and upstream activator elements of the *CYC1* gene (21). Brand et al. (5), however, recently identified a *cis*-acting regulatory sequence which demonstrates the positional and orientational characteristics of an enhancer, but functions to repress, rather than enhance, transcription. We are therefore currently characterizing the spatial requirements and promoter specificities of the negative regulatory element(s) in the chicken α-smooth muscle actin gene.

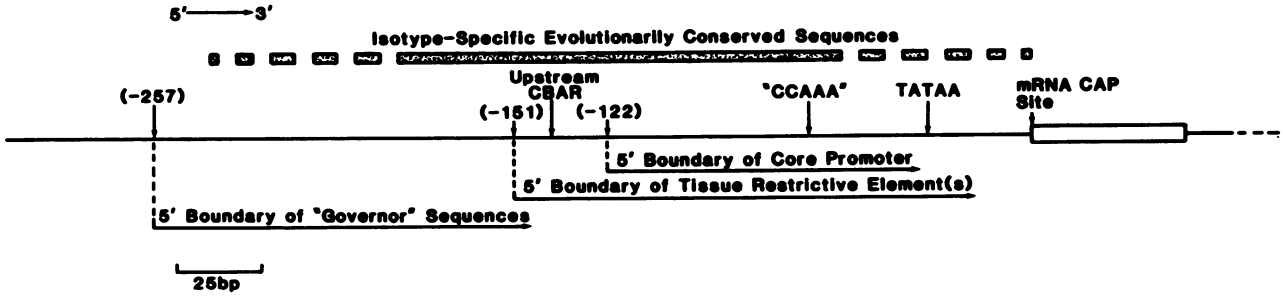

FIG. 7. *Cis*-acting regulatory elements in the 5′-flanking sequences of the chicken α-smooth muscle actin gene. Numbers in parentheses above the schematic diagram are the nucleotide positions of 5′ endpoints of the deletion recombinants. Symbols: Open bar, first exon; thin line to left of the first exon, 5′-flanking DNA. The positions of the mRNA cap site, TATAA and "CCAAA" homologies, and the upstream CBAR are indicated by arrows above the map. The broken stippled bar above the schematic denotes the region potentially containing evolutionarily conserved sequences. The 5′ borders of the governor sequences, tissue restrictive element(s), and core promoter are represented by arrows below the diagram.

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