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# Delimitation and Characterization of *cis*-Acting DNA Sequences Required for the Regulated Expression and Transcriptional Control of the Chicken Skeletal α-Actin Gene

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**We have previously observed that DNA sequences within the 5′-flanking region of the chicken skeletal α-actin gene harbor a *cis*-acting regulatory element that influences cell type and developmental stage-specific expression (J. M. Grichnik, D. J. Bergsma, and R. J. Schwartz, Nucleic Acids Res. 14:1683–1701, 1986). In this report we have constructed unidirectional 5′-deletion and region-specific deletion-insertion mutations of the chicken skeletal α-actin upstream region and inserted these into the chloramphenicol acetyltransferase expression vector pSV0CAT. These constructions were used to locate DNA sequences that are required for developmental modulation of expression when transfected into differentiating myoblasts. With this assay we have delimited the 5′ boundary of a *cis*-acting regulatory element to ca. 200 base pairs upstream of the mRNA cap site. In addition, we have preliminarily identified DNA sequences that may be important subcomponents within this element. A second major focus of this study was to identify those DNA signals within the regulatory element that control transcription. Toward this end, the expression phenotypes of progressive 5′-deletion and deletion-insertion mutants of the 5′-flanking region of the chicken skeletal α-actin gene were assayed in microinjected *Xenopus laevis* oocytes. These experiments defined a *cis*-acting transcriptional control region having a 5′ border 107 base pairs preceding the α-actin RNA cap site. Proximal and distal functionally important regions of DNA were identified within this element. These DNA signals included within their DNA sequences the "CCAAT" and "TATA" box homologies.**

The actin genes represent a multigene family whose individual members are expressed in a developmentally regulated and tissue-specific manner (6, 16, 17, 38, 43, 47, 49, 51, 52). Of particular interest in our laboratory have been the molecular mechanisms underlying the selective induction of the chicken skeletal (striated muscle-specific) α-actin gene family member in the course of muscle cell differentiation.

During the terminal development of skeletal muscle cells, proliferating mononucleated myoblasts stop dividing and fuse to form multinucleated myotubes. Coincident with this transition, the relative level of skeletal α-actin mRNA is induced at least 25-fold over its level in prefusion myoblasts both in ovo and in vitro (26a). In a recent report we have demonstrated that *cis*-acting DNA sequences 11 to 422 base pairs (bp) upstream of the skeletal α-actin structural gene are at least in part responsible for this developmental modulation of expression (22). In that study, portions of the 5′-flanking region of the chicken skeletal α-actin gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) structural gene of the pSV0CAT expression vector of Gorman et al. (19) and transfected into proliferating myoblasts. The transiently expressed level of CAT enzymatic activity was measured at advancing stages of myoblast cell development to assess promoter function. The amount of CAT activity was found to be low in transfected prefusion myoblasts, but as the cells withdrew from the cell cycle and fused, the relative level of CAT activity was concomitantly induced ca. 9- to 15-fold. In contrast, skeletal α-actin promoter-CAT chimeras manifested markedly restricted CAT expression when transfected into nonmyogenic cells. This demonstration that developmentally regulated transient expression was achieved when a cloned gene was transfected into cultured cells indicated that this was an ideal system for a more detailed analysis of the template DNA requirements for *cis*-acting developmental modulation of expression of the skeletal α-actin gene.

A critical component of the regulatory element of a gene is the *cis*-acting DNA sequences which are required for accurate and efficient initiation of mRNA transcription. Comparison of the DNA sequences immediately upstream from the mRNA start site of many eucaryotic genes transcribed by RNA polymerase II reveals two regions of DNA which are highly conserved in both sequence and position. These include the Goldberg-Hogness "TATA" box (5) and the "CCAAT" box (2, 12) which are located ca. 30 and 80 bp upstream of the mRNA cap site, respectively. The TATA box appears to function in vivo and in vitro as the dominant signal responsible for determining the precise start site of mRNA initiation (5, 50), although it may influence the efficiency of transcription in vivo (9, 24, 25). The canonical CCAAT box has been shown to influence the efficiency of mRNA synthesis of some eucaryotic genes (8, 9, 25, 28). A DNA binding protein has been identified that specifically interacts with the CCAAT motif of the herpes simplex virus thymidine kinase (TK) promoter to potentiate transcription (28). The chicken skeletal α-actin DNA sequences upstream of the structural gene contain the CCAAT and TATA homologous sequences 5′-CCAAAT-3′ and 5′-ATAAAA-3′ (15).

Other less ubiquitous positive transcription control elements have been identified upstream of some eucaryotic genes. One of these signals is a guanine-cytosine (G-C)-rich hexanucleotide, 5′-CCGCCC-3′, that was first recognized as two repeats in the 5′-promoter region of the herpesvirus TK gene (35, 36). A cellular transcription factor, termed Sp1, was shown to activate the initiation of RNA transcription

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when it bound to these G-C-rich elements (28) as well as to the G-C-rich motifs repeated six times in the upstream region of the simian virus 40 (SV40) early gene promoter (10, 11, 18). Interestingly, we have noticed two previously unrecognized copies of the CCGCCC hexanucleotide immediately preceding the chicken skeletal α-actin TATA box. The requirement of these and other potential elements for the *cis*-acting transcriptional control of the chicken skeletal α-actin gene has not been previously investigated.

The primary goals of the experiments described in this study were to delimit the 5′ boundary and examine the functional properties of the *cis*-acting DNA sequences required for developmentally regulated induction of the chicken skeletal α-actin gene during terminal muscle cell differentiation. Toward these ends, we constructed chimeric genes that contain a series of 5′ deletions and one region-specific deletion-insertion mutation of the upstream flanking region of the α-actin gene, fused to the coding sequences of the CAT gene within the expression vector pSV0CAT. These recombinants were transfected into cultured primary chicken myoblasts and assayed for the accumulation of CAT enzymatic activity at three different developmental stages of muscle cell differentiation. With this approach we found that the 5′ boundary of the *cis*-acting regulatory element required for maximal activation of the α-actin gene resides ca. 200 bp upstream from the authentic mRNA cap site. Furthermore, we have preliminarily identified DNA signals that may be critical subcomponents of this regulatory element.

In conjunction with our analysis of the 5′ regulatory element of the chicken skeletal α-actin gene, we sought to identify those DNA signals within this region that are directly required for accurate and efficient initiation of mRNA transcription independent of putative regulatory factors that may modulate the level of transcription. For this purpose, the 5′-deletion and deletion-insertion mutants used in the transfection studies were maintained adjacent to the α-actin structural gene, and their relative levels of expression were assayed in microinjected *Xenopus laevis* oocytes. Steady-state levels of RNA having the same 5′ terminus as authentic skeletal α-actin mRNA were measured by S1 protection analysis. These experiments established that a much more limited *cis*-acting region of DNA was required for maximal expression of the α-actin gene in *Xenopus* oocytes than that which was critical for regulation during development in myoblasts. The rough 5′ boundary of the transcription control region was positioned 107 bp upstream from the α-actin mRNA cap site. Within this element, proximal and distal regions of DNA were identified which almost entirely accounted for the positive control of RNA expression in injected *Xenopus* oocytes. These DNA signals include within their sequences the canonical CCAAT and TATA boxes, but not the G-C-rich motifs.

## MATERIALS AND METHODS

**Construction of 5′-deletion mutant skeletal α-actin-CAT chimeric plasmids.** Plasmid pAc3.6 was constructed by subcloning a *Hin*dIII-*Bam*HI DNA fragment, encompassing 1.6 kilobases of the 5′ portion of the skeletal α-actin structural gene and 2.0 kilobases of upstream flanking sequences, into the *Hin*dIII-*Bam*HI site of plasmid pBR322 DNA (W. Zimmer, unpublished data); the plasmid DNA sequences between the *Hin*dIII and *Bam*HI sites were replaced in this recombination. This chimeric plasmid served as the progenitor of all of the 5′-deletion mutants used in this study. The 5′-deletion fragments of the skeletal α-actin DNA sequences cloned into plasmids pBR322 or pSV0CAT are illustrated diagrammatically in Fig. 1. All plasmid constructions were cloned into competent *Escherichia coli* RR1 cells. Three of the 5′ deletions of DNA sequences were generated from the unique restriction enzyme *Pvu*II, *Sma*I, and *Xma*III sites at nucleotide positions 422, 200, and 76 bp upstream of the structural gene, respectively. Plasmid pAc3.6 was digested with one of these enzymes, the 5′ protruding termini were filled with deoxynucleotide triphosphates (dNTPs) by using

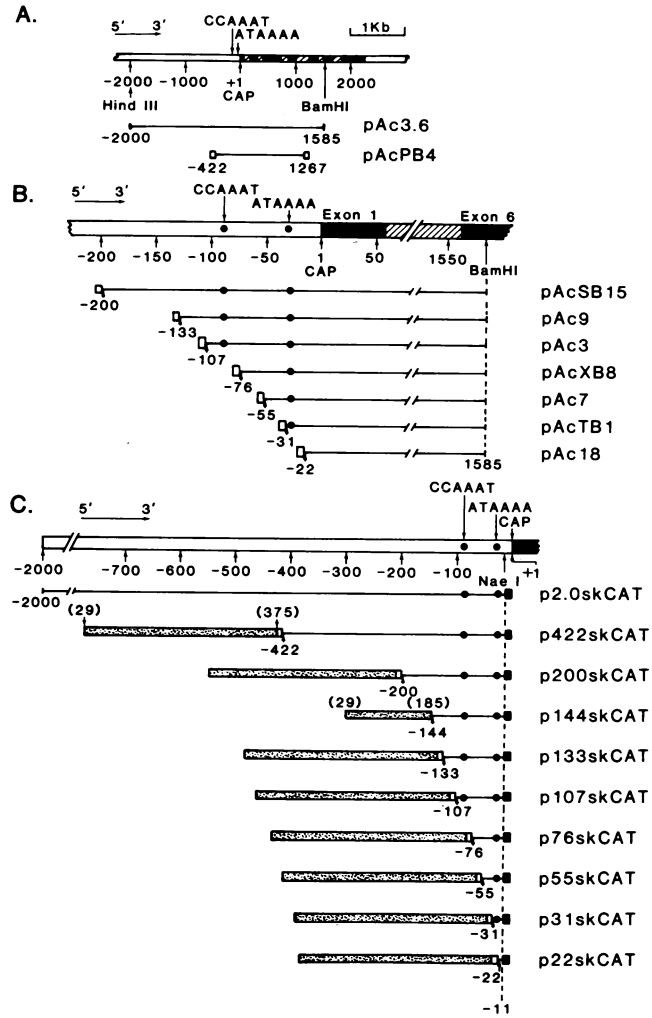


FIG. 1. 5′ Deletions in the upstream flanking region of the chicken skeletal α-actin gene. Details of the unidirectional deletion mutagenesis are described in Materials and Methods. All or part of the wild-type physical map of the chicken α-actin gene is diagrammed at the top of each panel. Open bars, Flanking DNA; solid black bars, α-actin exons; cross-hatched bars, α-actin introns. Coordinates refer to bp of DNA sequence relative to the mRNA cap site (CAP) at position +1. The positions of the α-actin CCAAT and TATA box homologies, CCAAAT and ATAAAA, are indicated by arrows above bars and solid circles within bars. Below each α-actin sequence representation, the regions of the deletion mutants cloned into pBR322 (A and B) or pSV0CAT (C) are illustrated by thin lines. Open boxes, *Bam*HI linkers; solid boxes, *Hin*dIII linkers; solid circles, CCAAAT or ATAAAA boxes; stippled bars, pBR322 sequence. The nucleotide positions of the endpoints of the deletion mutants are shown below the thin lines; the nucleotide positions of pBR322 sequences are shown above the stippled bars in parentheses. The names of the CAT recombinant clones are shown at the right of the figure.

DNA polymerase I large fragment when necessary, and synthetic *Bam*HI linkers were ligated to these termini. The ligation products were then digested with *Bam*HI and fractionated by gel electrophoresis. The appropriate DNA fragment was purified and cloned into the *Bam*HI site of plasmid pBR322 DNA. Recombinants with insert DNA oriented in the appropriate direction were denoted pAcPB4, pAcSB15, and pAcXB8. Other 5′ deletions were generated by sequential exonuclease III and S1 nuclease digestions from an unique *Sac*II site occurring 147 bp upstream from the RNA cap site. Potential protruding termini were filled with dNTPs by using DNA polymerase I large fragment, and synthetic *Bam*HI linkers were ligated to deletion endpoints. The ligation products were digested with *Bam*HI and cloned into the *Bam*HI site of pBR322 plasmid DNA as described above. The resulting chimeric plasmids were named pAc9, pAc3, pAc7, and pAc18. These had 133, 107, 55, and 22 bp, respectively, of 5′-flanking sequence adjacent to the 1.6 kilobases of skeletal α-actin structural gene. An eighth 5′-deletion mutant harboring 32 bp of upstream DNA was created from the pAc7 chimeric plasmid DNA, since this mutant DNA lacked most upstream skeletal α-actin gene. Plasmid pAc7 DNA was digested with *Taq*I restriction enzyme, the 5′ protruding termini were filled with dNTPs with DNA polymerase I large fragment, and the terminus was ligated to a *Bam*HI linker molecule. The ligation products were digested with *Bam*HI, and the appropriate DNA fragment was cloned into the *Bam*HI site of plasmid pBR322 DNA. The product recombinant was called pAcTB1. The endpoint of each deletion mutant was determined by the chemical DNA sequence analysis of Maxam and Gilbert (31).

The constructions of p2.0skCAT and p422skCAT were described previously (22). The upstream flanking sequences of the eight other deletion-recombinant DNAs were linked to the CAT gene of the plasmid expression vector pSV0CAT of Gorman et al. (19), as follows. Each chimeric plasmid DNA was digested with *Nae*I, which had total of six restriction sites: two sites in skeletal α-actin DNA sequences at nucleotide positions −12 and +358, and four sites within plasmid DNA sequences. Appropriate DNA fragments were ligated to synthetic *Hin*dIII linker molecules and cloned. The resulting recombinants contained solely skeletal α-actin 5′-flanking sequence upstream from nucleotide residue −11 that was juxtaposed to a *Hin*dIII linker attached to plasmid DNA sequences (Fig. 1C). These 5′-deletion mutants were named according to the distances of the deletion endpoints from the authentic skeletal α-actin mRNA cap site. These recombinant DNAs were digested with *Hin*dIII, and the fragments containing α-actin 5′-flanking sequences together with 346 bp of plasmid DNA (except for the 2.0-kb fragment; see Fig. 1C) were cloned into the *Hin*dIII site of plasmid pSV0CAT DNA. These 5′ deletions were named in the same manner as the previous deletion clones above. A final 5′-deletion mutant was constructed from plasmid p422skCAT, one of the skeletal α-actin promoter-CAT recombinants. Sequences between the unique *Sac*II and *Eco*RV sites were deleted from p422skCAT DNA via *Sac*II and S1 nuclease digestions followed by *Eco*RV digestion. Potential 5′ protruding termini were filled with dNTPs with DNA polymerase I large fragment, the resulting ends were joined by ligation, and the DNA products were cloned. The resulting plasmid, p144skCAT, lacked 278 bp of α-actin upstream sequences, a *Bam*HI linker molecule, and 190 bp of plasmid pBR322 DNA sequences as compared with its parent plasmid, p422skCAT. The α-actin flanking region-CAT sequence junctions were all confirmed by the Maxam and Gilbert sequencing procedure (31).

**Construction of deletion-insertion skeletal α-actin-CAT chimeric plasmids.** The strategy used to construct the deletion-insertion mutants is diagrammatically illustrated in Fig. 2. Plasmid pAc3.6 DNA was digested with *Xma*III and recloned. As a consequence, all α-actin DNA sequences 3′ to the nucleotide position −76, together with 564 bp of pBR322 DNA, were removed in the resulting pX2.0-8 plasmid chimera. An unique *Nru*I site present in plasmid sequences 33 bp away from the reconstituted *Xma*III site was utilized for the insertion of a *Bam*HI linker molecule. The resulting chimera was named pXN2.0-1. A *Bam*HI DNA fragment derived from the recombinant pAcTB1 DNA was inserted by ligation into the *Bam*HI-linearized and dephosphorylated pXN2.0-1 DNA. The cloned product recombinant DNA, termed pTBNr2.0-1, was identical to the progenitor plasmid pAc3.6 DNA except it contained plasmid pBR322 DNA in the place of α-actin DNA sequences between the canonical CCAAT and TATA box homologies. This chimeric plasmid DNA was digested with the restriction enzyme *Nae*I, and synthetic *Hin*dIII linkers were ligated to the termini and recut with *Hin*dIII. The digestion fragments were fractionated by gel electrophoresis, and the appropriate DNA fragment was purified and then cloned into the *Hin*dIII site of the pSV0CAT vector DNA. Relevant regions of the resulting recombinant plasmid DNA, including the plasmid DNA insert, its 5′ and 3′ junctions, and the α-actin-CAT fusion joint, were analyzed in two directions by the Maxam and Gilbert sequencing procedure (31).

The strategy of construction described above was expected to result in the precise replacement of α-actin DNA with plasmid DNA sequences without altering the distance between the CCAAT and TATA motifs. However, four additional nucleotide residues appeared in the course of the construction of this mutant plasmid, designated p2.0di+4skCAT. A 4-bp stretch of DNA was removed from the intervening region by *Xma*III cleavage followed by S1 nuclease digestion. Possible protruding termini were filled with dNTPs by DNA polymerase I large fragment, the termini were fused by ligation, and the products were cloned into competent *E. coli* RR1 cells. The intervening insert DNA from the resulting chimeric plasmid, p2.0di · skCAT, was sequenced by the procedure of Maxam and Gilbert (31).

**Cell culture and transient expression assay.** Tissue from the hind limbs of day-11 white Leghorn chicken embryos (Department of Poultry Science, Texas A&M, College Station) was dissected free of skin and cartilage and mechanically dissociated by the method of Fischbach (14). The cell suspension was passed through double layers of both sterile cheesecloth and lens paper to further separate individual cells and to remove cellular debris, then preplated at a density of $1 \times 10^8$ to $2 \times 10^8$ cells per 100-mm plastic culture dish for 20 min. Cell populations that remained in suspension were enriched in myoblasts. These cells were then plated at a density of $2 \times 10^6$ to $3 \times 10^6$ cells per 100-mm plastic dish (previously coated with rat tail collagen) and incubated at 37°C in a 5% $CO_2$ environment. Cells were incubated before transfection in Eagle minimal essential medium containing Earle salts, L-glutamine (GIBCO Laboratories, Grand Island, N.Y.), 8.6% horse serum (GIBCO), 4.7% chicken embryo extract, and 100 U each of penicillin and streptomycin per ml. Proliferating myoblasts were transfected with supercoiled plasmid DNA in calcium phosphate precipitates (20, 54) ca. 24 h after plating as reported elsewhere (22). Briefly, 4 μg of supercoiled plasmid DNA was added as a

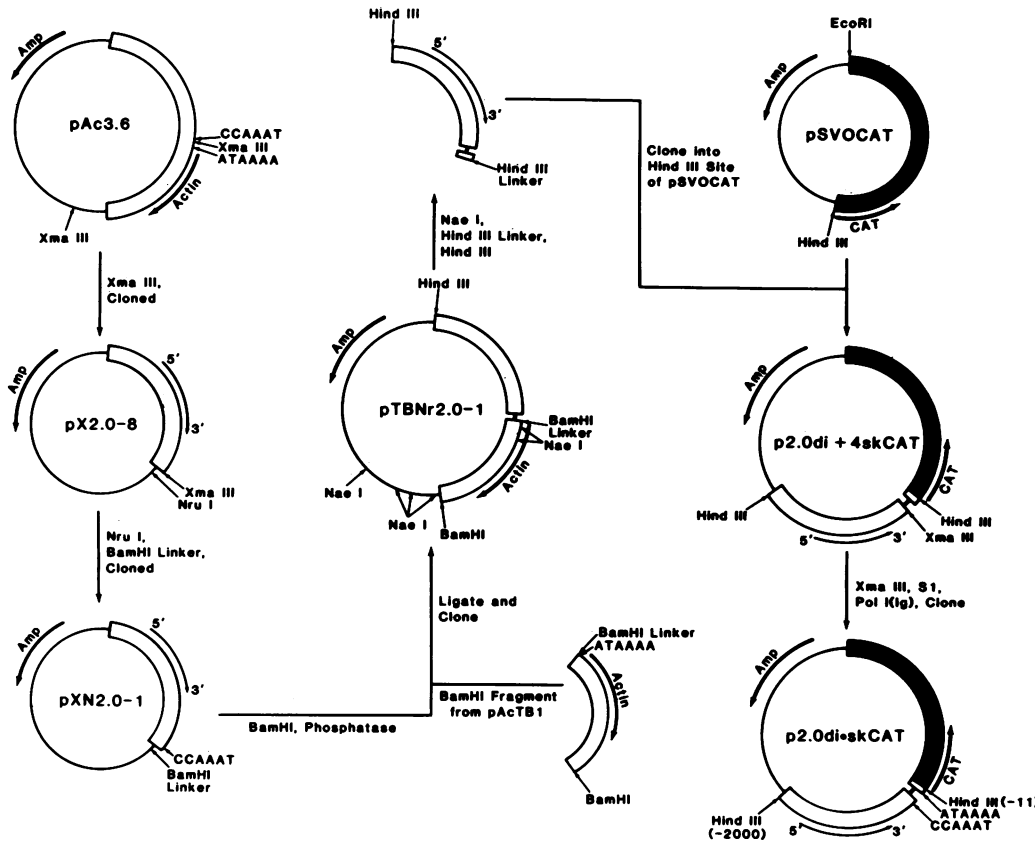


FIG. 2. Schematic outline of the strategy used to construct α-actin deletion-insertion mutant-CAT recombinants. Details of the region-specific mutagenesis are given in Materials and Methods. Circles, Recombinant plasmid DNA: thin lines, pBR322 vector DNA; open boxes, α-actin gene 5′-flanking region; solid black boxes, CAT gene and 3′ flanking region. Arrows around circumference of circles show position and 5′-to-3′ direction of α-actin, β-lactamase (Amp), and CAT genes and, when appropriate, the 5′-to-3′ orientation of the α-actin 5′-flanking region. The names of chimeras are indicated in the centers of the representative recombinants. The nucleotide positions of the endpoints of the α-actin 5′-flanking region are indicated in parentheses. When appropriate for reference purposes, the positions of the α-actin CCAAT and TATA box homologies, CCAAAT and ATAAA, are indicated.

calcium phosphate precipitate to each 100-mm culture plate of cells. After 4 h of incubation, the medium was removed from the cultures, and the cells were subjected to a ca. 1-min glycerol shock, washed with 5 ml of Hanks buffer, and then incubated further with complete Eagle medium. Transfections were performed on multiple occasions with different plasmid DNA preparations to account for possible unrecognized dissimilarities in DNA morphology as well as potential disparity in the timing of development of the cells.

**CAT assay.** At three separate time points posttransfection, cells were harvested, and cell extracts were prepared and used in CAT assays as previously described by Gorman et al. (19). These three time points coincided with three stages of myoblast differentiation: prefusion, fusion, and postfusion. These stages of development were roughly achieved at 48, 72, and 96 h after the initial plating of the cells. Briefly, cells were scraped from culture dishes, pelleted by centrifugation, suspended in 50 μl of CAT buffer (250 mM Tris hydrochloride, pH 7.5), and frozen and thawed at least three times. Cell debris was removed by centrifugation and saved for DNA quantitation by the method of Labarca and Paigen (29). The CAT content of the supernatant fluid of each sample was determined in a reaction mixture consisting of 50 μl of the cell extract, 20 μl of a 4 mM solution of acetyl coenzyme A (Pharmacia), and 0.5 μCi of [$^{14}$C]chloramphenicol (New England Nuclear Corp.). After 45 min at 37°C, an additional 20 μl of a 4 mM solution of acetyl coenzyme A was added to the reaction, and the mixture was incubated at 37°C for another 45 min, after which the reaction was terminated and extracted with ethyl acetate. The organic phase was dried, suspended in 15 μl of ethyl acetate, and spotted onto a silica gel thin-layer plate. Chromatography was performed with a chloroform-methanol mixture (95:5) as the solvent. Acetylated and unacetylated forms of chloramphenicol were visualized by autoradiography using XAR-5 X-ray film (Kodak). To quantitate experiments, radioactive spots were excised from the silica plates and their $^{14}$C content was measured by liquid scintillation counting. The amount of CAT enzyme was initially determined as picomoles of protein per minute per microgram of DNA per sample. To compare the results of several independent experiments, the levels of CAT activity in cells transfected with recombinant plasmid DNAs were calculated relative to the level of CAT activity produced in cells transfected in parallel with pSV2CAT plasmid DNA, a recombinant plasmid whose CAT gene is under the transcriptional control of the SV40 early gene promoter (19) and not under development regulatory control in differentiating myoblasts (22). These values were averaged.

***Xenopus* oocyte injection and RNA preparation.** Ovarian tissue was surgically removed from gravid *X. laevis* female frogs, and oocytes were dissected away from follicular tissue

in modified Barth solution (26). For each sample, an average of 20 oocytes were microinjected with 3 ng (in a volume of ca. 15 nl) of supercoiled plasmid DNA per oocyte which had been centrifuged at 1,000 × *g* for 10 min to visualize nuclei. Oocytes were incubated at 18°C for 24 h and then frozen at −80°C. Total RNAs were extracted from each set of oocytes essentially as described by Probst et al. (45). RNA was redissolved in 50 μl of water and precipitated with 150 μl of 4 M sodium acetate (pH 6.0) (44). The RNA pellets were rinsed with 70% ethanol and redissolved in water. At least two separate plasmid DNA preparations were used for the injection experiments.

**S1 nuclease mapping.** RNA was analyzed by the S1 nuclease mapping procedure of Berk and Sharp (4). Single-stranded, uniformly $^{32}$P-labeled S1 DNA probes were prepared from an M13 DNA recombinant as previously described (3). The skeletal α-actin insert DNA within the cloning cassette of the M13 chimera encompassed the DNA sequences flanking the skeletal α-actin RNA capsite, including nucleotide residues between −106 and +48. Briefly, single-stranded chimeric M13 DNA was hybridized with a 17-mer sequencing primer (Pharmacia P-L Biochemicals) from which mixed, $^{32}$P-labeled and unlabeled dNTPs were extended with DNA polymerase I large fragment across the insert DNA. The extended $^{32}$P-labeled DNA was then digested with *Eco*RI, which has a single recognition site within the M13 DNA sequences upstream of the insert DNA. The digestion products were heat denatured at 90°C in 30% dimethyl sulfoxide, quick chilled, and fractionated in a 5% polyacrylamide gel. The $^{32}$P-labeled fragment DNA was located by autoradiography and recovered from the gel as described by Maxam and Gilbert (31). This purified S1 probe DNA was then precipitated in probe excess with 20 μg of total oocyte RNA from each sample, along with 30 μg of bacterial tRNA, and pelleted by centrifugation. The conditions of the hybridization and subsequent S1 nuclease digestions are presented in detail in a previous report (3). To verify that the conditions of hybridization were in DNA probe excess, parallel S1 experiments were performed using a dilution series of chicken breast muscle RNA. The products of the S1 nuclease mapping were analyzed by electrophoresis on polyacrylamide-urea sequencing gels (31). The $^{32}$P-labeled DNA product bands were visualized by autoradiography with XAR-5 X-ray film. Radioactive bands corresponding to DNA protected by RNA from S1 nuclease digestions (which would be expected to result in a protected fragment ca. 48 nucleotides in length) were excised from the gel and measured by scintillation counting.

## RESULTS

**Construction of 5′-deletion mutants.** To identify DNA sequences within the 5′-flanking region of the chicken skeletal α-actin gene that regulate its activation during myoblast terminal differentiation, deletion mutants of the upstream sequences were constructed and joined in vitro to the promoterless bacterial CAT gene in the expression vector pSV0CAT (Fig. 1C). The strategy used for deletion mutagenesis is explained in detail in Materials and Methods. The 10 α-actin 5′-flanking region-CAT recombinants were denoted pXskCAT, where X indicates the base-pair position (or the kilobase position for p2.0skCAT) of the 5′ endpoint of the deletion relative to the authentic α-actin RNA cap site.

**Transient expression phenotypes of 5′-deletion recombinants in transfected myoblasts.** We recently developed and characterized a transient expression assay to measure the CAT activities of chicken skeletal α-actin 5′-flanking region-CAT recombinant genes in differentiating myoblasts (22). A limitation of this system is that only those regulatory control signals contained within a portion of the upstream DNA sequence of the α-actin gene are examined. The identification of other potential contributors towards the regulation of the α-actin gene, such as DNA sequences within or 3′ to the structural gene, RNA stability, chromosomal environment, or methylation effects, are forfeited when using this expression assay. Also, the interpretation of the expression phenotypes of unidirectional deletion mutants may be complicated by the positioning of foreign DNA sequences juxtaposed to the remaining DNA. Nevertheless, we have shown this system to be useful for identifying a *cis*-acting developmental control element that, at least in part, regulates the expression of the skeletal α-actin gene during myogenesis. We have also previously demonstrated by S1 mapping that the fusion gene CAT mRNA transcripts are under the transcriptional control of the α-actin 5′-flanking region and are accurately initiated at a surrogate start site 32 bp upstream of the CAT AUG translation initiation site (22).

The 10 α-actin promoter-CAT chimeric plasmid DNAs were transfected as calcium phosphate precipitates into proliferating cultured chicken myoblasts ca. 24 h after the cells were plated. As controls, cells were transfected in parallel with plasmid DNA from both pSV2CAT, an expression vector in which the CAT gene is under the transcriptional control of the SV40 early gene promoter (19) and not under developmental control in differentiating myoblasts (22), and pSV0CAT, a vector which lacks a defined eucaryotic promoter or enhancer element (19). Transfected cells were harvested ca. 48, 72, and 96 h after the initial plating of the cells. At 48 h the cells were predominantly replicative, prefusion myoblasts; at 72 h a majority of the cells were withdrawing irreversibly from the cell cycle and fusing to form multinucleated myotubes; and at 96 h the cells were mostly in a postfusion, terminally differentiated state. The assay of a CAT gene product was used as a measure of promoter activity at each of the three stages of myoblast cell development examined.

The autoradiograph films of the CAT assays of one representative experiment are shown in Fig. 3. At the prefusion stage, pSV2CAT-transfected myoblasts contained high levels of CAT activity; this activity remained approximately at that level at the fusion and postfusion stages of cell development. In contrast, pSV0CAT-transfected cells produced barely detectable levels of CAT activity at all three time points examined. The α-actin promoter-CAT plasmids p2.0skCAT, p422skCAT, p200skCAT, p144skCAT, p133skCAT, and p107skCAT exhibited a profile of CAT expression in transfected myoblasts that was distinct from those of the positive and negative controls. In prefusion myoblasts (48 h), these recombinant plasmid DNAs directed the production of a level of CAT activity that was substantially less than that accumulated in pSV2CAT-transfected cells, but those levels increased with time (72 and 96 h). This correlation of the induction of CAT activity with cell development was most notable in cells that were transfected with p2.0skCAT, p422skCAT, and p200skCAT plasmid DNA. The 5′-deletion recombinants containing α-actin upstream DNA sequences with endpoints 3′ to the CCAAT motif exhibited little to no accumulation of CAT activity in transfected cells above the basal level found in pSV0CAT-transfected cells.

At least four separate transfection experiments were per-

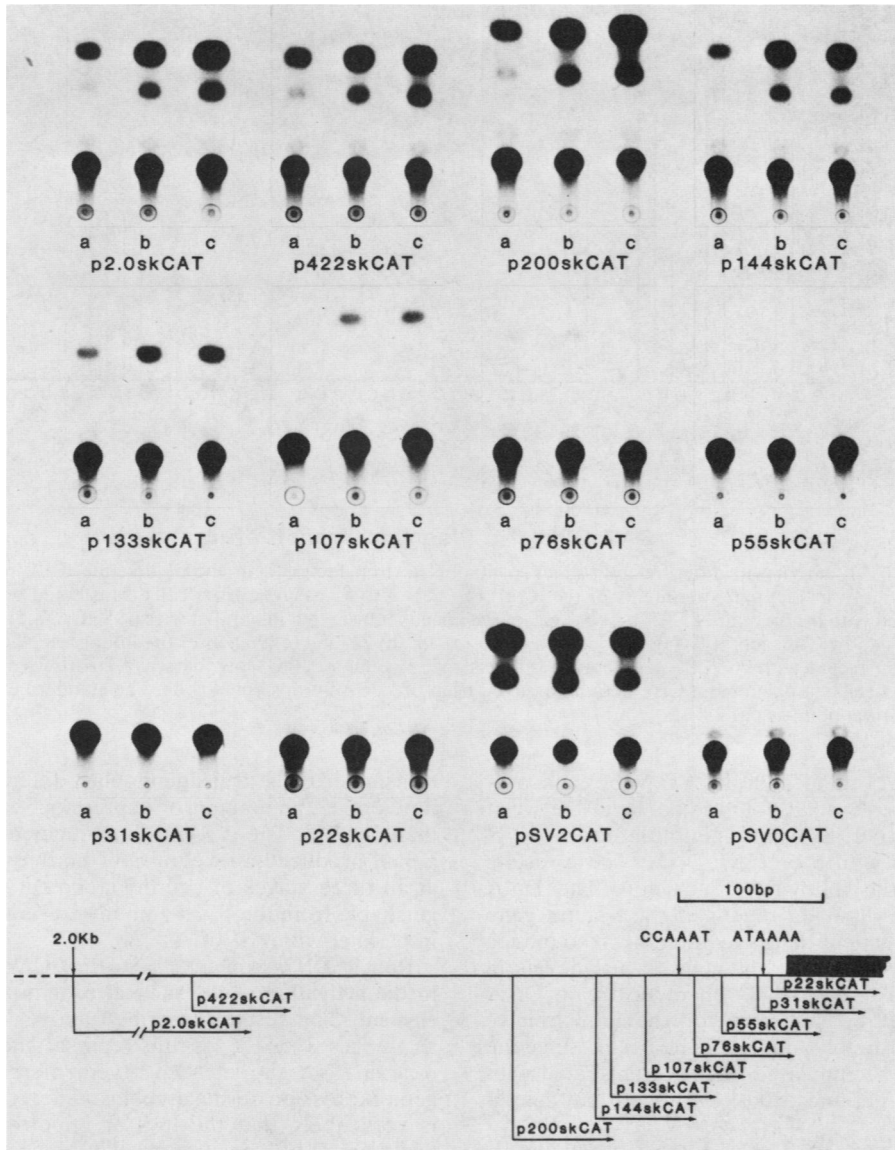


FIG. 3. Transient expression analysis of the chicken skeletal α-actin 5′-flanking deletion-CAT recombinants in developing chicken myoblast cultures. Proliferating myoblast cultures were transfected with the indicated recombinants, and CAT activity was determined ca. (a) 48, (b) 72, and (c) 92 h after the initial cell plating as described in the text. The upper portion of the figure shows the autoradiograms of the acetylated chloramphenicol derivatives obtained from one transfection experiment with each of these recombinants. CAT activity was measured as described by Gorman et al. (19). At the bottom is a diagram showing the relative positions of the 5′-deletion endpoints of each of the recombinants. Thin line, α-actin 5′-flanking upstream sequence; solid box, CAT structural gene. The positions of the α-actin CCAAT and TATA box homologies, CCAAAT and ATAAAA, are indicated.

formed using two different preparations of plasmid DNA. To minimize differences within experiments and also to compare several independent experiments, the amounts of CAT activity that accumulated in cells transfected with the 5′-deletion recombinant plasmid DNAs were calculated relative to the level of CAT activity present in cells transfected in parallel with pSV2CAT plasmid DNA. The arithmetic mean of the relative levels of CAT activity of at least four trials with each of the 5′-deletion chimeric plasmids is illustrated diagramatically as a histogram in Fig. 4. The level of CAT activity accumulated in cells tranfected with pSV2CAT DNA, with which all the other levels are compared, was arbitrarily set at a value of 100 for each time point of myoblast differentiation examined. The standard error of each individual sample average was calculated to be ca. 25%. Although subtle differences in the relative levels of CAT expression in transfected cells could not be evaluated, this figure was well within an acceptable range of deviation for the purpose of the studies in this report.

The rough 5′ boundary of the upstream region of the α-actin gene required for the induction of CAT expression during myoblast differentiation was indicated from the experimental data summarized in Fig. 4. Deletion recombinants that retained 200 bp or more of 5′-flanking region were expressed at ca. 20% of the relative level of activity found in pSV2CAT-transfected cells at the 48-h prefusion myoblast time point. With advancing cell development, the relative levels of CAT activity expressed in cells transfected with

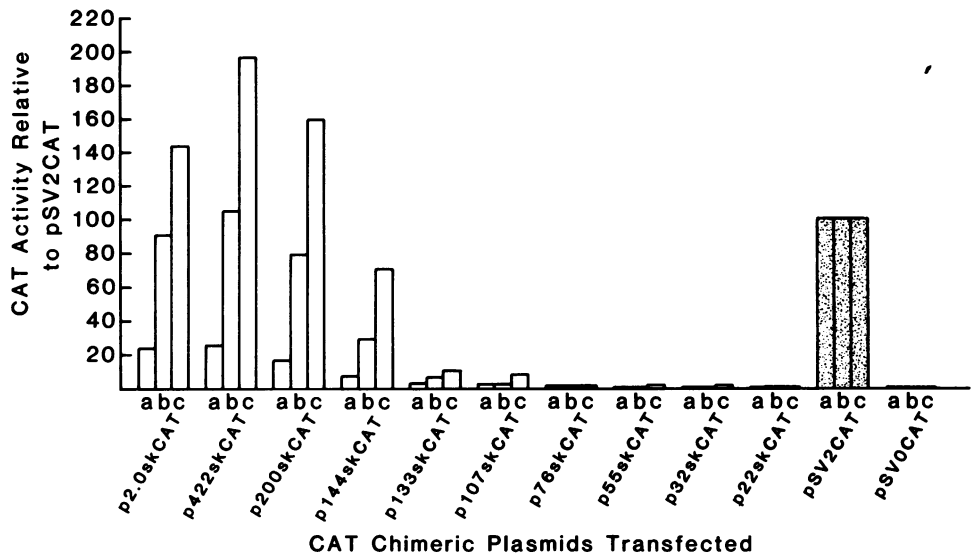


FIG. 4. Induction of CAT activity in differentiating chicken myoblasts transfected with the 5′ deletion-CAT recombinants: average obtained from four separate transfection experiments of the levels of CAT activity assayed from cells transfected with the indicated CAT chimeric DNAs, compared with the amount of CAT activity measured in cells transfected in parallel with pSV3CAT. Transfected cell cultures were harvested and assayed for CAT activity as described in the text (a) 48, (b) 72, and (c) 96 h after the initial cell plating. The level of CAT activity found in cells transfected with pSV2CAT was arbitrarily assigned a value of 100. Open bars, CAT activity assayed from indicated CAT chimeric-transfected cells; stippled bars, CAT activity assayed from pSV2CAT-transfected cells. The standard error of each individual sample average for each time point is ca. 25%.

p2.0skCAT, p422skCAT, and p200skCAT were each progressively induced up to 6- to 10-fold to final levels between 140 and 200% relative to that accumulated in cells transfected in parallel with pSV2CAT DNA. These results are consistent with the following conclusion. The DNA template within the 5′-flanking region of the α-actin gene harbors a regulatory element that is required in *cis* to induce the expression of the adjacent CAT gene to high levels in differentiating myoblasts. Only 200 bp of upstream DNA sequence appeared to be sufficient for the induction of maximal levels of CAT activity in transfected cells, since the relative levels of that accumulated in cells transfected with p2.0skCAT, p422skCAT, and p200skCAT were not significantly different.

Deletion recombinants with 5′ endpoints 3′ to 200 bp of upstream α-actin sequence exhibited reduced levels of CAT activity in transfected cells (Fig. 4). Plasmid p144skCAT-transfected cells accumulated a level of CAT activity ca. 8% of the amount present in pSV2CAT-transfected cells at the 48-h (prefusion) stage. During the course of cell development to the 96-h time point, this level was induced ca. ninefold to about 70% of the level of CAT activity that occured in pSV2CAT-transfected cells. Myoblasts transfected with p133skCAT and p107skCAT DNA displayed a more drastic reduction in the expression of CAT activity. Cells transfected with these chimeras and harvested at the 48-h stage showed CAT activity which was ca. 3% of that in pSV2CAT-transfected cells. However, in contrast to the α-actin-CAT chimeras retaining additional upstream DNA sequences, p133skCAT and p107skCAT induced the expression of CAT activity only three- to fourfold in developing myoblasts to a level in 96-h cells of ca. 9 to 12% compared to the amount of CAT accumulated in cells transfected in parallel with pSV2CAT. The deletion recombinants p76skCAT, p55skCAT, and p32skCAT directed very little expression of CAT activity in 48-h myoblasts, resulting in ca. 1% the level of CAT activity accumulated in pSV2CAT-transfected cells. During myoblast differentiation to the 96-h time point, the amount of expression was induced no more than onefold. The ATAAA box-deficient p22skCAT recombinant produced a level of CAT activity in transfected cells at all three stages of cell development which was indistinguishable from the basal amount present in cells transfected in parallel with pSV0CAT.

**Role of DNA sequences between CCAAT and TATA boxes in the activation of the skeletal α-actin *cis*-acting regulatory element.** The results described above indicated that DNA sequences 3′ to 76 bp upstream of the authentic skeletal α-actin RNA cap site had a very minor influence on CAT gene expression during myoblast differentiation. However, it is very likely that the loss of important DNA sequences upstream of this position masked the contribution of downstream DNA sequences. To test this possibility, an α-actin promoter-CAT plasmid recombinant was constructed in which the region between the CCAAT and TATA boxes was removed and precisely replaced by plasmid pBR322 DNA sequences. The strategy used to construct this chimeric plasmid is schematically illustrated in Fig. 2 and described in detail in Materials and Methods. The initial deletion-insertion CAT plasmid, termed p2.0di+4skCAT, had an additional 4 bp between the CCAAT and TATAA motifs compared to wild-type p2.0skCAT. To duplicate the wild-type spatial relationship between the CCAAT and TATA boxes, 4 bp of DNA sequences was removed by *Xma*III and S1 digestion to construct the chimera p2.0di · skCAT (Fig. 2). The DNA sequences of the plasmid inserts and its junction termini are shown in Fig. 5.

The expression in differentiating myoblasts of the two deletion-insertion chimeras was tested in the same manner as were the 5′-deletion recombinants. The deletion-insertion chimeric DNAs were transfected in parallel with p2.0skCAT, p32skCAT, pSV2CAT, and pSV0CAT plasmid DNAs. The autoradiographic films of one set of experiments are shown at the top of Fig. 5. At the bottom of this figure is

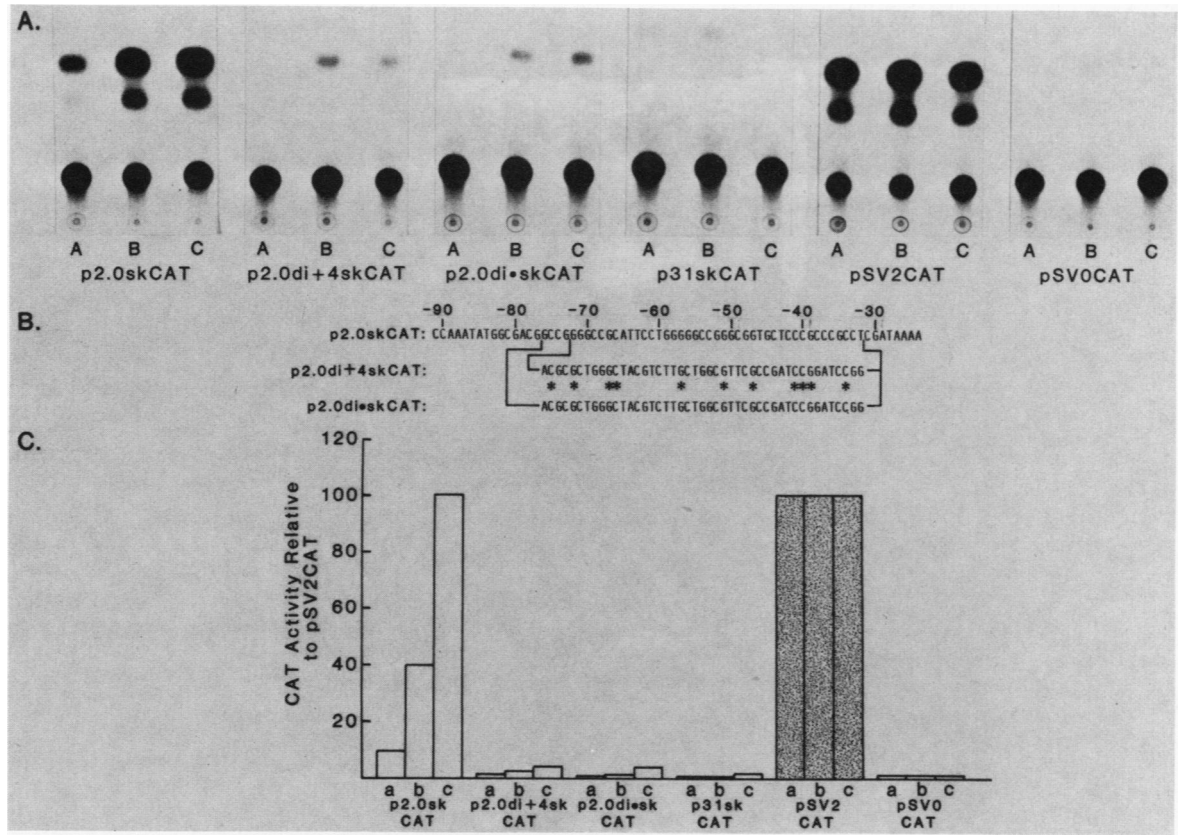


FIG. 5. Induction of CAT activity in differentiating chicken myoblasts transfected with the deletion-insertion-CAT recombinants. Proliferating cultured chicken myoblasts were transfected with the indicated recombinants and assayed for CAT activity as described in the text. (A) Autoradiographic films of the acetylated products separated by thin-layer chromatography from one CAT transient expression assay; small A, B, and C below the autoradiograms indicate CAT activity found in cells harvested ca. 48, 72, and 96 h after initial cell plating, respectively. (B) Comparison of the nucleotide sequences of the region between the CCAAAT and ATAAAA boxes of the wild-type recombinant p2.0skCAT and the deletion-insertion recombinants p2.0di+4skCAT and p2.0di · skCAT. The inserted plasmid DNA sequences are shown below the wild-type sequence (p2.0skCAT): insert pBR322–α-actin sequence junctions are indicated by lines; asterisks indicate nucleotide sequence homologies between insert fragment and wild-type sequence. (C) Histogram depicts the average obtained from three separate transfection experiments of the levels of CAT activity assayed in cells transfected with the indicated recombinants (open bars) compared with the amount measured in cells transfected in parallel with pSV2CAT (stippled bars). Transfected cells were harvested (a) 48, (b) 72, and (c) 96 h after the initial plating. Values of CAT activity are standardized relative to the amount of CAT activity measured in cells transfected in parallel with pSV2CAT (stippled bars); the level which accumulated in pSV2CAT-transfected cells was arbitrarily assigned a value of 100. Standard error for each individual figure was ca. 25%.

a histogram illustration of the average of several trials of these experiments.

A strikingly low level of CAT activity was expressed in the deletion-insertion recombinant-transfected myoblasts at the prefusion stage (Fig. 5). This CAT activity was only three- to fourfold induced during myoblast differentiation, to a level ca. 4% of that which accumulated in pSV2CAT-transfected cells. Similar deletion-insertion α-actin CAT recombinants, with 1 additional and 3 fewer bp at the 5′ junction of the bacterial DNA insert, yielded identical results (data not shown). The simplest interpretation of these results is that the region of α-actin DNA replaced by the pBR322 DNA insert forms an integral part of the *cis*-acting regulatory element. Alternatively, the plasmid DNA of the insert fragment may not (as assumed) have a neutral role in the transfection assay but rather may have a negative effect on the regulatory control region. One way in which this could be caused is if the insert DNA somehow disrupts the transcriptional control region. This possibility was tested using *Xenopus* oocytes in an in vivo transcription assay.

**Transcription of the 5′-deletion and deletion-insertion mutants in *Xenopus* oocytes.** The transcription activities directed by the upstream α-actin DNA sequences of the recombinants used in this study were examined in *X. laevis* oocytes; however, the 5′-flanking α-actin sequences were assayed in their natural configuration adjacent to the 5′ portion of the α-actin structural gene so that accurate initiation could be assessed. DNAs from the chimeric plasmids pAc3.6, pAcPB4, pAcSB15, pAc9, pAc3, pAcXB8, pAc7, pAcTB1, pAc18, and pTBNr2.0-1 (Fig. 1A and B and 2) were microinjected into *Xenopus* oocyte nuclei. The steady-state level of RNA transcripts was measured by S1 hybridization mapping using uniformly $^{32}$P-labeled single-strand probe DNA in excess. An autoradiograph exposure of the sequencing gel of one set of S1 experiments is shown in Fig. 6A. As expected, total chicken breast RNA protected ca. 48 nucleotide residues of the S1 probe DNA (Fig. 6B). The protected bands immediately flanking this fragment were most likely the result of S1 "nibbling" (4). The RNAs produced in oocytes injected with each recombinant plasmid DNA (except pAc18, which lacks a TATA box) showed a similar cluster of S1-protected fragments. This established that

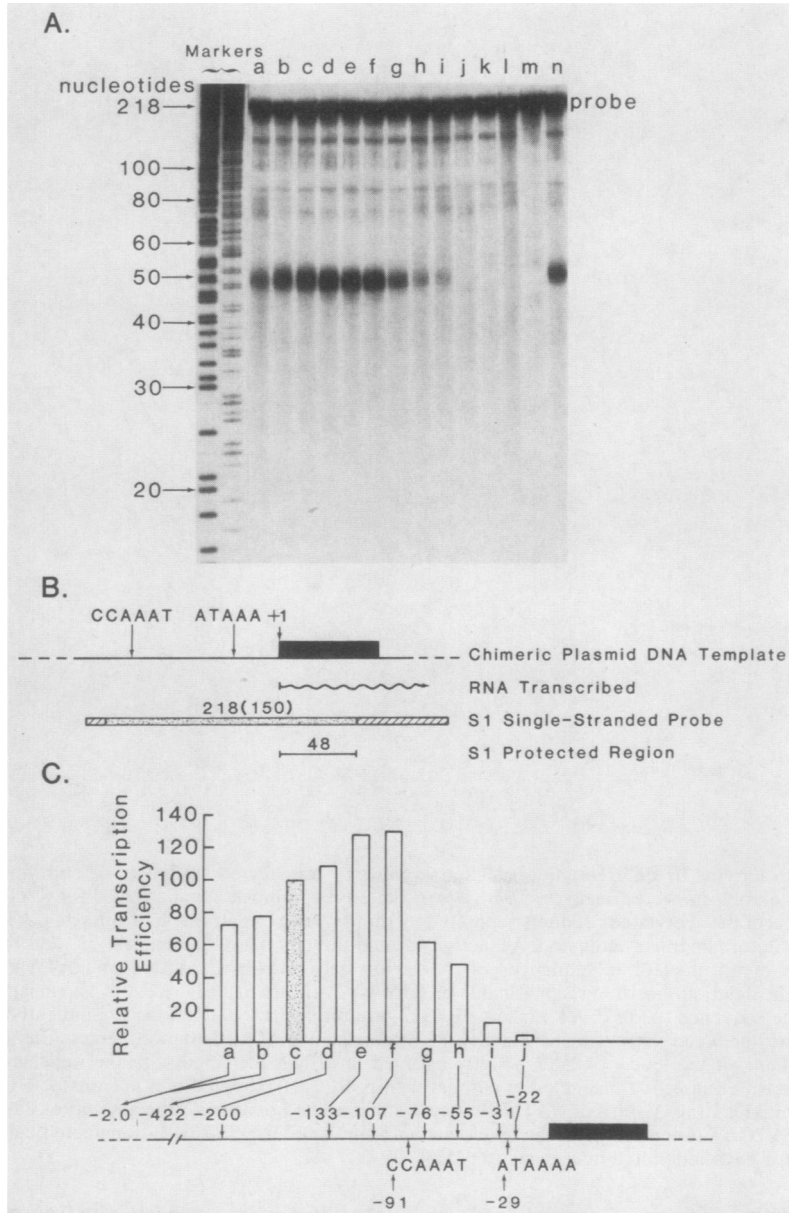


FIG. 6. Nuclease S1 mapping of RNA transcribed in *Xenopus* oocytes from the 5′-deletion and deletion-insertion mutants of the chicken skeletal α-actin gene. Supercoiled plasmid DNA was injected into oocytes (3 ng per oocyte), total RNA was purified from each set of oocytes after 24 h, and 20 μg of this RNA was assayed for S1 nuclease protection as described in the text. Accurately initiated RNA was expected to protect 48 nucleotides of the S1 DNA probe. (A) Autoradiograph of a 6% polyacrylamide sequencing gel that was used to size DNA fragments generated by S1 digestion of RNAs synthesized in oocytes. The S1 exposure signal in each lane is from approximately five oocytes. The two left lanes contained a known Maxam and Gilbert sequence pattern of a marker DNA fragment; the nucleotide positions of some of the fragments are numbered next to the arrows. The position of the undigested probe is indicated on the right. Oocytes were injected with (a) pTBNr2.0-1, (b) pAc3.6, (c) pAcPB4, (d) pAcSB15, (e) pAc9, (f) pAc3, (g) pAcXB8, (h) pAc7, (i) pAcTB1, and (j) pAc18. The other lanes contained the following controls: (k) uninjected oocyte RNA, (l) tRNA, (m) S1 probe undigested with S1 nuclease, and (n) 1 μg of total chicken breast RNA. (B) Schematic diagram of 5′ portion of the chicken skeletal α-actin gene from which the S1 probe was derived. Upper portion of illustration represents the genomic sequences encoding the mRNA 5′ untranslated region of the α-actin gene (black box), flanking sequence (thin lines, solid and dashed), and α-actin CCAAT box and TATA box homologies (CCAAAT and ATAAA), which are indicated with arrows as is the mRNA cap site (+1). Below this diagram is shown the region of the chimeric plasmid DNA template that is transcribed in oocytes (wavy arrows). Below this is represented the single-stranded, uniformly $^{32}$P-labeled S1 probe. The probe was a total of 218 nucleotide residues in length (as indicated above bar), of which 150 nucleotide residues (indicated in parentheses) encompassed the α-actin sequence illustrated at the top of the figure. Phage M13 sequence is represented by cross-hatched bars; α-actin sequence is indicated by a stippled bar. Beneath this diagram is shown the region of the S1 probe DNA that would be protected by accurately initiated skeletal α-actin RNA transcripts. (C) Histogram depicts the average of at least three (eight for columns a and b) separate determinations of the transcription efficiencies in oocytes of the α-actin 5′-flanking mutant recombinants. Nuclease S1-protected radioactive bands were excised from the sizing gel and measured by scintillation counting. The amount of radioactivity in each experiment was calculated relative to the amount measured in bands protected by RNA synthesized in oocytes injected in parallel with recombinant pAcPB4. Below the histogram are indicated the nucleotide positions of the 5′ endpoints of the 5′-flanking mutants and the α-actin CCAAT and TATA homologies. The α-actin genomic sequences are represented as in panel B.

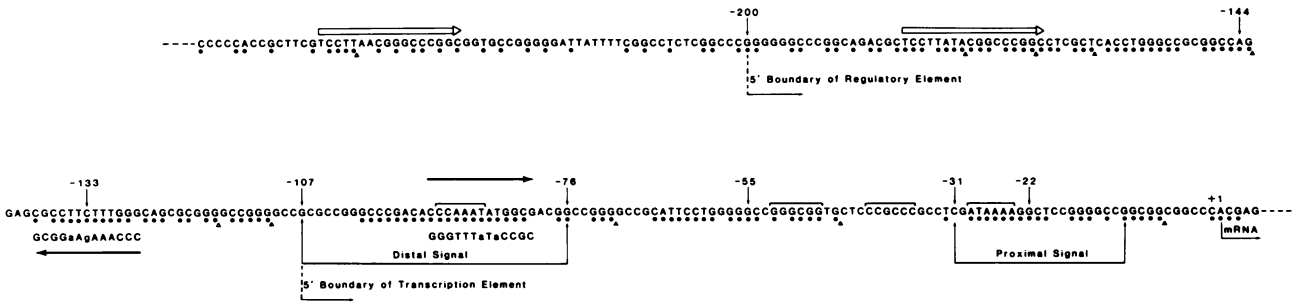


FIG. 7. Nucleotide sequence map of the chicken skeletal α-actin *cis*-acting regulatory and transcriptional control regions. Numbers above sequence are the nucleotide positions of the 5′ endpoints of the deletion recombinants and the mRNA cap site. Open arrows above sequence show the location and 5′-to-3′ orientation (arrows points in 3′ direction) of a 17-bp repeat sequence. Brackets above sequence designate the α-actin CCAAT, TATA, and G-C-rich hexanucleotide homologies. Solid arrows above and below the sequence designate the position and 5′-to-3′ orientation (arrow points in 3′ direction) of a 13-bp repeat. Below the sequence of the region designated by solid arrows is the complementary sequence of the 13-bp repeat; nucleotide homology mismatches between the inverse repeats are represented as lowercase letters. Brackets below sequence designate the approximate nucleotide boundaries of the distal and proximal transcriptional control signals. The 5′ borders of the *cis*-acting regulatory and transcriptional control regions are indicated with arrows. Dots under sequence indicate nucleotide homology with the 5′-flanking region of combined mouse and rat skeletal α-actin genes; solid triangles indicate gaps, and open triangles indicate removal of rodent sequences for alignment for maximal homology. The DNA sequence shown was repeatedly determined in two directions using the procedures of Maxam and Gilbert (31) and Sanger et al. (48).

injected α-actin chimeric plasmid DNAs that retained at least a TATA motif were accurately initiated and transcribed in oocytes at the authentic mRNA start site of the structural gene.

The average of the results of several transcription experiments is summarized in a histogram (Fig. 6C). These combined results indicate that the 5′ boundary of the region required for efficient expression of the skeletal α-actin gene is roughly 107 bp upstream from the mRNA cap site. The first 107 bp of 5′ flanking DNA was sufficient to direct the synthesis of the maximal steady-state level of RNA in oocytes. Deletion of DNA 3′ to 107 bp had a negative effect on transcription. A reduction in transcription efficiency of ca. 55, 63, 90, and 97% directly correlated with the progressive removal of nucleotide residues from 107 bp to 22 bp of upstream DNA sequences. An authentic 5′ terminus was not detected in RNA that accumulated in oocytes injected with the chimera pAc18, which retained only 22 bp of α-actin upstream sequence.

The DNA sequences from 107 bp to 2.0 kilobases upstream from the RNA cap site did not appear to play a positive role in the expression of the α-actin gene in oocytes. On the contrary, the progressive 5′-unidirectional removal of these DNA sequences resulted in a graded relative increase of up to 40% of transcription efficiency.

The relative levels of expression in oocytes of plasmids pAc3.6 and pTBNr2.0-1 were nearly identical, with pTBNr2.0-1 expressed at ca. 94% of the level of RNA accumulation in oocytes injected with pAc3.6. The very close similarity of the phenotypes of these recombinants indicated either that the α-actin DNA sequences between the CCAAT and TATA motifs have little positive effect on transcriptional efficiency or that the plasmid DNA insert in pTNBr2.0-1 had a minor negative influence on transcription efficiency in oocytes relative to the pAc3.6 expression level, or both.

## DISCUSSION

The studies described in this report were primarily directed toward delimiting the approximate 5′ boundary of the *cis*-acting DNA sequences flanking the chicken skeletal α-actin gene which are required for developmentally regulated expression in transfected differentiating myogenic cells. In addition, experiments were designed to preliminarily identify specific regions of DNA that are particularly important subcomponents within this element. The final focus of these studies was to identify those DNA signals within the regulatory element that control transcription. Toward these ends, we created sets of α-actin 5′-flanking region mutants, including 5′-deletion and deletion-insertion mutants, and analyzed their expression both in a transient expression system using cultured differentiating myoblasts and in a *Xenopus* oocyte transcription assay.

The results of the transfection experiments indicated that the rough 5′ boundary of the α-actin *cis*-acting regulatory element lies 200 bp upstream of the authentic RNA cap site. This 200 bp of DNA sequence, when fused to the coding sequences of the CAT gene within the expression vector pSV0CAT, was required and sufficient to support maximal induction of a CAT gene product during myoblast cell development. Sequences upstream of this region appeared to play no significant role in induction in the transfection system used in this study. These observations were consistent with and extended our own (22) and other (37) previous reports which established that a *cis*-acting, developmentally regulated element is contained within the 5′-flanking region of the chicken and rat skeletal α-actin genes.

We (3a) and others (27, 41) have previously identified extensive nucleotide sequence homology between the 5′-flanking region of the chicken, rat, and mouse skeletal α-actin genes. This high degree of sequence conservation between the genes of species which diverged at least 300 million years ago (46) suggests that these sequences have major biological significance. The location of the 5′ boundary of the skeletal α-actin regulatory element as positioned in this study coincides quite closely with the 5′ border of the region that comprises the greatest sequence conservation between the chicken and rodent α-actin 5′-flanking regions (Fig. 7). This agreement corroborates our experimentally derived conclusion that the nucleotide endpoint ca. 200 bp upstream from the α-actin mRNA cap site delimits the 5′ boundary of the *cis*-acting regulatory element. A 17-bp sequence immediately 3′ to this position is imperfectly (3-bp mismatch) repeated ca. 50 bp upstream (Fig. 7). Whether this reiterated sequence has a significant role in regulation

control was not indicated by the transfection assay used in this report.

The removal of α-actin DNA sequences 3′ to the nucleotide position −200 had a detrimental effect on activation during muscle cell development, which indicated that this region of DNA played a positive role as part of the *cis*-acting element. The removal of DNA sequences 5′ to −144 bp resulted in a ca. 55% relative decrease of the level of activation during myoblast differentiation. A drastic reduction of the level of activation (ca. 93%) occurred when only 11 bp of additional DNA sequence was deleted to the −133 bp endpoint. We believe that this severe effect implicates a particularly critical regulatory role for DNA sequences near or within the region encompassing nucleotide positions −144 to −133. Part of the oligonucleotide sequence 5′-CGCCTTCTTTGGG-3′ falls within this region. It is quite notable that the complement of this sequence reads 5′-CCCAAAGAAGGCG-3′, which matches 11 of a possible 13 bp of a sequence present ca. 35 bp downstream in the opposite orientation (Fig. 7). This downstream repeat contains the skeletal α-actin CCAAT box (15) and is conserved as part of a larger 20-bp sequence homology found between chicken, rat (42), and mouse (27) 5′-flanking skeletal α-actin genes. The upstream inverted 13-bp repeat is conserved in sequence and sequence orientation in the upstream 5′-flanking regions of both rat and mouse α-actin genes, which suggests an important biological function. The results of the deletion mapping of DNA sequences between nucleotide positions −144 and −133 are consistent with this hypothesis. It is also noteworthy that both the upstream and downstream 13-bp oligonucleotide sequences are highly conserved as an inverted repeat in approximately the same region but in different orientations in the 5′-flanking regions of the chicken smooth and cardiac α-actin genes (S. L. Carroll, D. J. Bergsma, and R. J. Schwartz, J. Biol. Chem., in press). It is conceivable that this repeated sequence may have been conserved in the 5′-flanking region of these genes due to a critical function as regulatory elements as they diverged during evolution from a presumptive primordial muscle-specific actin gene.

The DNA sequence between −107 and −76 bp upstream from the α-actin mRNA cap site appeared to have some role within the *cis*-acting regulatory element as determined from the transfection assays. This region contains a 20-bp DNA sequence which is highly conserved between chicken, rat (42), and mouse (27) genes (Fig. 7) as discussed above. These sequences may have been conserved for regulatory purposes; however, the results of the *Xenopus* transcription assay indicated that this region is required for transcriptional control. It is possible that this region may be used in concert for both regulatory and transcriptional functions.

Portions of the DNA sequence between the CCAAT and TATA motifs are conserved between chicken and rodent skeletal α-actin genes (Fig. 7) and therefore are likely to have a biologically important function. Transfection assays of the 5′-deletion-CAT recombinants were not useful in investigating this possibility since deletions lacking DNA sequences 5′ to nucleotide position −76 expressed very low levels of CAT activity in transfected myoblasts. To examine the regulatory contribution of these internal DNA sequences, we replaced them with plasmid pBR322, leaving the authentic CCAAT and TATA boxes intact along with upstream DNA sequences. These deletion-insertion-CAT chimeras were induced to a final activity ca. 25 times less than that of the wild type-CAT recombinant in the transfection assay. The simplest conclusion consistent with this result is that the region between the CCAAT and TATA boxes is a critical subcomponent of the 200-bp *cis*-acting regulatory region of the α-actin gene. Hu et al. (27) have demonstrated that a majority of this region in mouse, rat, and chicken 5′-flanking α-actin sequences is a large imperfect inverted repeat of DNA sequences immediately upstream of the CCAAT box and that these sequences can potentially form a stable hairpin loop structure with a stem of ca. 40 bp. This hypothetical structure encompasses a region between 144 and 44 bp upstream of the chicken skeletal α-actin structural gene. Hu et al. (27) have speculated that the high degree of conservation of these inverted repeat sequences indicates that they may function in the tissue-specific expression of the skeletal α-actin genes. If this proposal is valid, it is conceivable that the drastically attenuated phenotypes in the transfection assays of the deletion-insertion chimeras, together with those of the deletion recombinants retaining 133 and 107 bp of upstream α-actin sequence, may reflect the disruption of a required inverted repeat.

It is possible that the segment of plasmid pBR322 DNA chosen to replace actin sequences may not exert a neutral effect within this region but rather somehow negatively influences the regulatory element. For instance, this negative contribution could be mediated by altering the local conformation of the region so that the binding of putative *trans*-acting regulatory factors to upstream DNA sequences is somehow prevented. Alternatively, the inserted plasmid DNA may produce a final DNA template that cannot be efficiently transcribed. Although the first possibility could not be easily tested, potential transcription effects were investigated (see below) by utilizing *X. laevis* oocytes as a source for assaying transcription efficiency. This transcription system was also used to locate the position of the rough boundaries of the signals within the *cis*-acting regulatory element of the α-actin gene that were required for accurate and efficient transcription. The *Xenopus* oocyte appeared to offer an ideal transcription system for these purposes since microinjected plasmid DNAs would be transcribed in an environment that was presumably constitutive in nature in the absence of regulatory factors. A variety of genes such as the sea urchin histone (45), simian virus 40 (53), herpesvirus TK (33, 34), and human c-*myc* (40) genes, as well as the Moloney murine sarcoma virus long terminal repeat (21), have been shown to be expressed accurately and efficiently in *Xenopus* oocytes.

The results of the *Xenopus* oocyte transcription assays established the 5′ boundary of a *cis*-acting element required for efficient expression of the skeletal α-actin gene to be ca. 107 bp upstream of the mRNA cap site (Fig. 7). This border was ca. 93 bp downstream from the 5′ boundary of the *cis*-acting regulatory element. This dissimilarity most likely reflected the requirement of the oocyte transcription system for transcription-specific factors while the transfection assay probably has a sequence requirement for the coordinate interaction of putative regulatory and transcription factor binding to achieve maximal expression.

At least two regions of DNA were identified within the *cis*-acting transcription element that were required for accurate and efficient transcription in *Xenopus* oocytes. The positions of the signals are illustrated in Fig. 7. The control element closest to the RNA cap site we termed the proximal signal, and the element further 5′ was denoted as the distal signal, a notation adopted from the convention of McKnight and Kingsbury (35). The proximal element was required for accurate transcription in oocytes. This element contains the Goldberg-Hogness TATA box homology 5′-ATAAAA-3′.

The TATA box has been implicated to be the dominant element responsible for determining the precise site of initiation of RNA synthesis of eucaryotic structural genes (5, 50). The 3′ border of this element was positioned roughly 11 bp upstream from the RNA start site, since this endpoint marked the juction of the α-actin-CAT recombinants used in the transfection assays and we have shown previously that such a recombinant is accurately initiated at a surrogate start position (22). We suggest that the function of the proximal signal in the regulation of the α-actin gene is, at least in part, at the level of initiation of accurate RNA transcripts. Also, we have previously indicated that this region may be required for expression of the α-actin gene in transfected myoblasts (22).

The distal signal was found to be required for efficient expression of the α-actin gene in *Xenopus* oocytes. The phenotypes of progressive 5′-deletion recombinants in the oocyte transcription assay indicated that only 107 bp of 5′-flanking DNA was necessary for maximal expression of the α-actin gene in oocytes. The 5′ unidirectional removal of the distal signal to a endpoint 76 bp upstream of the mRNA cap site reduced the efficiency of transcription more than 50%. Therefore, this region appears to be a positive control element. The distal control signal contains the sequence 5′CCAAAT-3′, which resembles both in sequences and position the CCAAT box which is found near many eucaryotic genes (2, 12) and has been shown to be required for efficient transcription of some eucaryotic genes (8, 9, 25, 28).

Deletion recombinants that retained upstream DNA sequences with endpoints between nucleotide positions −76 and −32 exhibited attenuated expression in oocytes coincident with the graded loss of DNA sequences. These results seemingly revealed a region of DNA that played an important positive role in transcription efficiency. Indeed, this region contains two copies of the G-C-rich hexanucleotide 5′-CCGCCC-3′, which has been recogized as forming a critical part of two transcriptional control signals of the herpesvirus TK gene promoter, as elegantly established by McKnight and Kingsbury using a *Xenopus* oocyte transcription assay (35). Also, one of the α-actin upstream hexanucleotides is contained within the oligonucleotide 5′-TCCCGCCC-3′, which is repeated twice within the transcriptionally important 21-bp repeat upstream region of the SV40 early promoter (1, 7, 13, 30, 39). Moreover, the six G-C-rich hexanucleotides tandemly repeated in the SV40 early promoter and the two G-C-rich sequences of the TK promoter have been shown to bind a transcription potentiation factor, Sp1 (10, 11, 18, 28). However, the slightly reduced (ca. 6%) phenotype of expression in oocytes of the deletion-insertion recombinant compared to the wild-type expression phenotype indicated that the region between nucleotide positions −76 and −32 contributes only a minor positive role towards the efficient transcription of the α-actin gene. This minor decrease in expression may reflect the loss of the two G-C-rich hexanucleotides; however, these sequences are a far more crucial element of the herpesvirus TK promoter (35, 36) and therefore may not be functionally equivalent in the α-actin gene. Alternatively, the slight relative difference in expression level may have resulted from the 4-bp increase in the distance that separated the CCAAT and TATAA boxes in the deletion-insertion recombinant. This increase may perturb important spatial arrangements between functional elements. However, McKnight (32) has demonstrated that expansions in a similar region of the herpesvirus TK promoter of up to 10 bp have no detectable effect on expression in oocytes. Possibly, the plasmid insert DNA selected to replace α-actin sequences in the deletion-insertion recombinant may have functionally replaced positive transcriptional control signals with surrogate elements which directed a slightly reduced level of expression in oocytes. This prospect could not be ruled out with the available data. In any case, the results indicated that the inserted plasmid DNA did not have an appreciable negative effect on the efficiency of transcription in oocytes. This observation clarified our initial conclusions based on the results of the transfection assays of the deletion-insertion recombinants. This interpretation was initially complicated by the possibility that the observed drastic reduction in activation in differentiating myoblasts may have been caused by the inability of the template DNA to permit the access of transcriptional machinery to express the adjacent gene. The results of the oocyte transcription assay indicate that this was not a valid assumption.

With the progressive removal of 5′-flanking sequences the phenotypes of deletion recombinants with graded deletion 5′ to 133 bp upstream of the α-actin mRNA cap site showed enhanced relative levels (up to 40%) of transcriptional efficiency in oocytes. A similar inhibitory phenomenon has been reported by Grosschedl and Birnstiel for the H2A gene in a *Xenopus* oocyte transcription system (23). Since the transcription assays were performed under DNA saturation conditions, the difference in expression could not have been caused by a variability in molar concentration of DNA templates (although this possibility was tested; data not shown). The apparent inhibitory influence of the upstream sequence may have resulted from nonspecific competition for transcription factors. Alternatively, the inhibition of expression in oocytes may have reflected the existence of sequences which confer host cell or tissue specificity. Preliminary experiments that tested the expression of the 5′-deletion and deletion-insertion α-actin-CAT recombinants in nonmyogeneic cells indicated that putative DNA signals that control tissue specificity appear to be distributed throughout the 200-bp *cis*-acting regulatory element, although the contributions of upstream elements could not be excluded (data not shown).

In conclusion, the results of this study established that DNA signals required for development modulation and transcription control of the chicken skeletal α-actin gene reside within 200 bp of 5′ flanking sequence, Presently we do not know how this developmental control is mediated, although we assume that *trans*-acting factors are involved. How these putative factors accomplish their regulatory roles, whether through a positive or negative mechanism, is subject to speculation, although we believe the results are more consistent with a regulatory model that includes a positive control mechanism.

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