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Tissue restricted and stage specific transcription is maintained within 411 nucleotides flanking the 5' end of the chicken α-skeletal actin gene

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ABSTRACT

α-skeletal actin message levels have been shown to be tightly regulated in chicken primary myoblast cultures. To test for gene elements required for muscle cell specific expression, DNA sequences containing the 5'-flanking regions of the chicken α-skeletal actin, β-cytoplasmic actin, and the histone H2b genes were linked to the coding sequences of the chloramphenicol acetyltransferase gene and transfected into myogenic and non-myogenic cells. In contrast to β-actin CAT hybrids, the α-skeletal actin CAT constructions displayed restricted CAT expression in transfected non-myogenic cells. We showed that a 411 nucleotide fragment flanking the 5' end of of the α-skeletal actin gene was responsible for a 9-15 fold increase in CAT enzymatic activity during myoblast fusion, versus only a transient 2 fold rise for the β-actin and histone flanking sequences. These results indicate that DNA sequences within 411 bp of the 5' terminus of the α-skeletal actin gene influenced its cell type and stage specific expression.

INTRODUCTION

Actins, a major component of sarcomeres and cytoskeletons, serve as a model system for investigating tissue specific expression of gene families during development of an organism (1-8). Our laboratory (6,8) as well as others (4,7) have described a switch in the expression of actin genes during *in vitro* myogenesis. The non-muscle actins (β and γ) are the predominant actin mRNAs species in replicating prefusion myoblasts. Following the end of myoblast replication, the non-muscle actin mRNAs are reduced in content. Concomitantly, with the onset of fusion, the α-cardiac and α-skeletal actin mRNAs are both strongly induced in a sequential manner (8), and replace the non-muscle actin forms within myotubes. Whether such changes in mRNA levels are mediated by transcriptional mechanisms or by altered mRNA stability or both has not yet been resolved.

Recently, the regulation of cloned genes have been studied by introducing them into cells in culture (9-14). In several of these studies DNA sequences upstream of the TATA and CCAAT boxes were implicated in the regulation of cell specific transcription (13,14). In order to understand how the striated and

non-muscle actin genes might be regulated at the transcriptional level we decided to study their function by constructing hybrid genes with the 5' flanking and putative promoter sequences fused to the bacterial gene chloramphenicol acetyltransferase (CAT; 15,16) and transfecting these hybrid genes into myogenic and non-myogenic cells. The assay of a CAT gene product was then used to measure promoter activity.

Although, CAT assays have served to define the regulatory borders of several genes (17,18), this strategy has some limitations. The CAT methodology allows investigation only of the transcriptional promoting activity of the gene's 5' flanking sequences. Thus, sequences within the body of a gene which potentiate transcriptional or post-transcriptional control over the native transcript will not be identified. Another limitation is that CAT activities may vary between transfection experiments even though the relative levels of CAT expression do not change significantly. Finally, the CAT protein appears to be a fairly stable product, and CAT activity assays may not be helpful for examining a decrease in gene activity.

However, there are considerable advantages for using the CAT system. The assay of CAT gene product is rapid, sensitive, reproducible, and has shown to be directly proportional to the strength of the transcriptional promoter in properly constructed gene fusions (15-18). An advantage for transfecting hybrid genes into a cell differentiation system in culture is that CAT activity assayed at an early developmental stage can serve as a baseline for comparing changes in genetic activity at later stages. In this respect, primary myoblast cultures differentiate quickly in a highly specific and temporal manner and are more likely to retain natural control mechanisms in contrast to myogenic cell lines. A large number of cells can be obtained from the same pooled population of dissociated primary myoblasts. Consequently, several promoter constructions can be simultaneously tested in gene transfection experiments. Finally, transient transfections in primary cells are advantageous because transiently transfected DNA remains extrachromosomal which insures that the only cis effects are those of the construct and not due to the site of chromosomal integration (19). Results of our gene transfection experiments revealed that the DNA regions 5' to the mRNA cap site may play a major role in the regulation of the α-skeletal actin gene expression during myogenesis.

MATERIALS AND METHODS

Preparation of Plasmid DNA

A 6.2 Kb Hind III fragment (pAC6.2), and an 9.5 Kb Eco RI fragment of λAC

29 (pAC2995), which encompass the entire chicken α-skeletal and β-actin genes were subcloned into pBR322 and characterized as described by Chang et al. (1). The α-skeletal actin putative promoter fragments were derived from a Hind III-Bam HI subclone of pAC6.2 which includes 2 Kb of 5' flanking DNA and 1.6 Kb of the gene (pAC3.6). A subcloned portion of a chicken genomic histone H2b gene, pKR1α-1.3, was a generous gift of Dr. Jerry Dodgson (20). The plasmids pSV0-CAT and pSV2-CAT were gifts of Dr. Bruce Howard (15). Transformed bacteria were grown in L- broth containing ampicillin in host E. coli K-12 strain RR1, followed by selective amplification of the plasmid with chloramphenicol. Plasmid DNA was prepared by a gentle alkaline lysis procedure (21) and cesium chloride-ethidium bromide centrifugation. DNA containing greater than 50% form I DNA was used in eucaryotic cell transfection experiments.

Enzymes and Radionucleotides

Restriction endonucleases were purchased from New England Biolabs and Boehringer-Mannheim. E. coli DNA polymerase I large (Klenow) fragment was obtained from Bethesda Research Laboratories. T4 DNA ligase was a product of Collaborative Research Inc. T4 polynucleotide kinase was obtained from P-L Biochemicals, and calf alkaline phosphatase was from Worthington Diagnostics. Nuclease S_1 was purchased from Miles Laboratories, Inc. γ-^{32}P labeled ATP was purchased from New England Nuclear.

Construction of Hybrid CAT Genes

The pSV0-CAT vector, originally constructed by Gorman et al. (15), includes the entire coding sequence of the CAT gene from Tn 9 followed by two fragments from SV40. Lacking a promoter, this construct will not produce significant CAT activity as assayed in several tissue culture systems (15-18). A single Hind III site at the 5' end of the pSV0-CAT gene provides a cloning site for adding 5' flanking DNA sequences as diagramed in Figure 1. A hybrid gene was constructed to include 2.0 Kb of the 5' flanking and promoter regions of the α-skeletal actin gene. We digested pAc3.6 with Nae I which cleaves at a site just 12 nucleotides downstream from the ATAAA box and 12 nucleotides upstream from the natural transcription initiation site. Hind III linkers were then ligated and this fragment was digested with Hind III, which also cleaved at the 5' Hind III junction of pBR322. The released 2.0 Kb Hind III fragment was isolated and ligated into the Hind III site of pSV0-CAT. Recombinant clones were selected for proper orientation by restriction endonuclease mapping. In this respect we obtained clones that contained the α-actin promoter placed in the opposite orientation to the CAT gene. Fortuitously, we also obtained a 2.0 Kb fragment of the α-skeletal actin gene

in which 20 bp of DNA, including the ATAAA box, was deleted from the 3' end of the 2.0 Kb fragment. Another construct was made by a partial Nae I digestion, which provided us with a 2.3 Kb DNA fragment that contained the 5' flanking sequences, the first intron, the complete 5' noncoding mRNA leader and 115 nucleotides of the α-actin coding region fused into the pSVO-CAT gene. A shorter region of the α-actin 5' flanking sequence was made by a complete Nae I digestion of a previously subcloned Pvu II-Bam HI fragment of pAC3.6. In this construct the Pvu II site was converted to a Bam HI site with linkers. After the ligation of Hind III linkers, digestion with Hind III yielded a fragment which retains 411 nucleotides of the α-skeletal actin promoter and 346 bp of pBR322. The 346 bp region of pBR322 has been tested in both orientations in pSVO-CAT and does not induce CAT activity in our culture system.

Two other constructions were made as system controls for comparing transcriptional activity of the hybrid CAT genes. The first construct included the putative promoter region of the β-cytoplasmic gene and a portion of its first intervening sequence (22). This clone was generated by subcloning an S1 nuclease treated 2 Kb Sac II fragment of pAC2995 into the Sma site in pUC8. The Eco RI site of pUC8 was then converted to a Hind III site by filling in the sticky end with DNA polymerase I large fragment (Klenow) and nucleotide triphosphates and then ligated with linkers. This construct was then digested with Hind III and the 2.0 Kb fragment was isolated and ligated into the Hind III site of pSVO-CAT. Second, a construct was made by cloning the 5' DNA flanking sequences of the chicken H2b histone gene from pRK1α-1.3 by a Sau 3A digestion. This fragment contains about 600 bp of chicken DNA, which includes approximately 20 nucleotides of the putative non-transcribed leader sequence, and retains 314 bp of pBR322. This fragment was blunt end ligated into the filled in Hind III site in pSVO-CAT.

Nucleic Acid Sequencing

The hybrid CAT constructs used in this study were first examined by restriction endonuclease mapping, and then by nucleic acid sequencing across the ligated Hind III junctions. Hybrid DNA-CAT junctions were verified by the DNA sequencing method of Maxam and Gilbert (23).

S1 Nuclease Analysis of RNA Transcripts

Total RNA was isolated as previously described (6) from myoblast cultures 24 hours after transfection with either 4 μg or 40 μg of pO.411 α-skCAT DNA per 100 mm dish. The 458 base pair Pvu II/Stu I DNA probe was end labeled with [γ-^{32}P] and polynucleotide kinase (24) at the Pvu II site and coprecipitated with 100 μg of RNA. The pellet was resuspended in 30 μl of

hybridization buffer consisting of 15 mM PIPES [piperazine-N-N'-bis(2-ethane sulfonic acid); pH 6.4, 1 mM EDTA, and 400 mM NaCl]. The solution was heated at 95°C for 10 minutes and rapidly transferred to 68°C for 12 hours. After hybridization, 10 volumes of ice-cold nuclease S_1 buffer (300 mM NaCl, 50 mM sodium acetate pH 4.5, 2 mM zinc acetate pH 4.5 containing 2000 units of S_1 nuclease (Miles Laboratories, Inc.) was added quickly to the reaction, mixed thoroughly and incubated at 37°C for 2 hours. The reaction was stopped with the addition of 14 µl of stop buffer (0.5 M Tris-HCl pH 7.5, 0.25 M EDTA), extracted once with an equal volume of phenol-chloroform-isoamyl alcohol and precipitated with absolute ethanol. The pellet was redissolved in 0.3 M sodium acetate (pH 7.0) and ethanol precipitated. The precipitate was collected by centifugation, dried under vacuum, and dissolved in 3 µl of 99% formamide containing 0.05% xylene and bromophenol blue dyes, and the size of the S_1 protected fragment was determined by electrophoresis on polyacrylamide-urea sequencing gels with size markers produced from a DNA fragment sequenced by the technique of Maxam and Gilbert (23).

Primary Chicken Myoblast Cultures

Tissue from the hindlimbs of day 11 white Leghorn chick embryos was dissected free of skin and cartilage and then mechanically dissociated by the method of Fischbach (25). The cell suspension was filtered through double layered lens paper to remove aggregates, followed by preplating for 20 min. of 100-200 x 10^6 cells per 100 mm non-collagenized dish. Enriched myoblasts were then plated at a density of 2-3 x 10^6 cells per 100 mm collagenized dish at 37°C in the presence of 5% CO_2. Culture media consisted of Eagle's minimum essential medium (MEM) containing Earle's salts (Gibco) supplemented with 8.6 % horse serum (Gibco), 4.7% chick embryo extract, and 100 U/ml penicillin-streptomycin. After 24 hours, this media was replaced with a feeding medium containing 1.9% chick embryo extract. The feeding media was changed 48 hours thereafter. At the 72 hr feeding 10 µM cytosine arabinoside (Sigma) was added to kill proliferating cells and was maintained until the 120 hour time point.

Primary Chicken Brain Cell Cultures

Cerebral hemispheres from embryonic day 11 chicks were removed and mechanically dissociated as described above. Brain cells were directly cultured on non-collagenized dishes with complete media at a density of 10^7 cells per 100 mm dish. The cultured cells were washed after 24 hours, leaving about 2 x 10^6 cells attached to the plate. At 48 hours in culture the cells were transfected with 4 µg of the chimeric CAT constructs and 6 µg of pBR322 plasmid DNA as carrier. The brain cell cultures were harvested 24 hours later.

Mammalian Cell Culture

Mammalian COS 7 cells were grown according to the protocol of Gluzman (26). Cells (1 x 10^6) were subcultured onto 100 mm dishes one day before transfection. They were transfected with 10 μg of the p0.411α-SkCAT and p2.0β-Cyt-CAT plasmids and harvested 48 hours after transfection.

DNA Transfer

Tissue culture cells were transfected with DNA by the protocol of Wigler et al. (27) with minor modifications. A total of 4 μg of DNA, unless otherwise indicated, was used to transfect each 100 mm dish of tissue culture cells. Plasmid DNA was gently resuspended in sterile TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) at a concentration of 1 mg/ml. The appropriate amount of this stock solution was diluted into 0.5 ml/dish of 2x HBSS buffer (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2PO_4, pH 7.12) and an equal volume of 250 mM $CaCl_2$ was then added dropwise while vortexing the DNA solution. A fine calcium phosphate-DNA precipitate formed at room temperature within 30 minutes. Media was aspirated from the culture dish and 1 ml of the precipitate was added directly to the cells. Immediately, an additional 10 ml of media was applied to the recipient cells and incubated for 4 hours. The media was removed and 1 ml of 25% glycerol in complete Hanks was added to coat the cells. After 40 seconds the glycerol solution was removed and cells were washed with 5 ml of Hanks buffer and then fed with 10 ml of complete media. Transfections were done multiple times with different plasmid preparations and batches of primary myoblasts to control for variations in DNA quality and plating density of cells.

CAT Assay

At various times after transfection, cells were harvested and assayed for CAT activity as previously described (15). Cell pellets were lysed by repetitive freeze thaw cycles in 50 μl of 250 mM Tris-HCl pH 7.5. The production of acetylated [^{14}C] chloramphenicol (0.5 μCi per assay, 57.8 mCi/mMol, New England Nuclear) was assayed for 90 minutes at 37°C in which additional 4 mM acetyl CoA was added after 45 minutes. Acetylated chloramphenicol was then monitored by autoradiography following thin layer chromatography on silica gel plates. Separated acetylated chloramphenicol spots were quantitated by liquid scintillation counting. Since myoblast cultures undergo a transition from replication to non-replicating fusion stages, the absolute DNA content per plate is variable with time. Therefore, DNA content was determined for each set of transfected cells to insure that the number of transfected cells assayed for CAT activity was equivalent. Data

was expressed either as the percentage of converted [^{14}C] chloramphenicol per 50 ul of cell extract, or nanomoles of converted [^{14}C] chloramphenicol per ug cell DNA. Protein concentration of cell extracts was determined by the method of Bradford (28) at each time point to insure uniformity. We determined that 1.5% to 70% of the converted [^{14}C] chloramphenicol provided the maximal range for comparing CAT activity.

RESULTS

Transfection of Hybrid CAT Genes into Primary Myoblasts

Portions of the 5' flanking DNA sequences of the α-skeletal actin, β-actin and histone H2b genes were fused to the coding region of the pSVO-CAT vector, as described in the " Methods " and as diagramed in Figure 1. These hybrid CAT constructs were transiently transfected into cultured chicken myoblasts. Dissociated embryonic chicken myoblasts were plated at a density of 2-3 x 10^6 cells per 100 mm dish and allowed to attach and replicate for 24 hours. After one day in culture each hybrid construct was transfected into myoblasts at a level of 4 μg of calcium phosphate precipitated DNA per culture dish. Transfected cells where harvested at 48, 72, 96, and 120 hour time points after the initial plating. The 48 hour time point represents a pre-fusion stage, although a few small myotubes can occasionally be found beginning to form. The 72 hour time point marks midfusion, while the 96 and 120 hour time points represent postfusion and differentiated myotube stages. The addition of foreign DNA phosphate crystals to the myoblast cultures did not significantly delay the temporal appearance of myotubes.

The role of DNA sequences flanking the α-skeletal putative promoter region were evaluated by assaying CAT enzymatic activity as shown in a representative set of experiments shown in Figure 2 and Table I. Within a single experimental set of transfectants the CAT activity assays were determined within 5% to 10% of the sample's average value. The induction of α-skeletal CAT activity was reproduced in at least 5 separate experiments. The induction of CAT was quite dramatic for the 0.411 Kb α-skeletal actin construct, in which the CAT gene was fused 12 nucleotides downstream of the α-skeletal actin's ATAAA box. The p0.411α-Sk-CAT plasmid caused a 10 fold increase in CAT activity between 48 and 72 hours, and by 96 hours the activity was up to 13 fold. The induction of CAT in primary myoblasts compares well with the appearance of the α-skeletal actin mRNA. The α-skeletal actin mRNA accumulated appreciably only after fusion was complete (60-72 hours in culture). By 96 hours the α-skeletal actin mRNA had increased about 25-fold

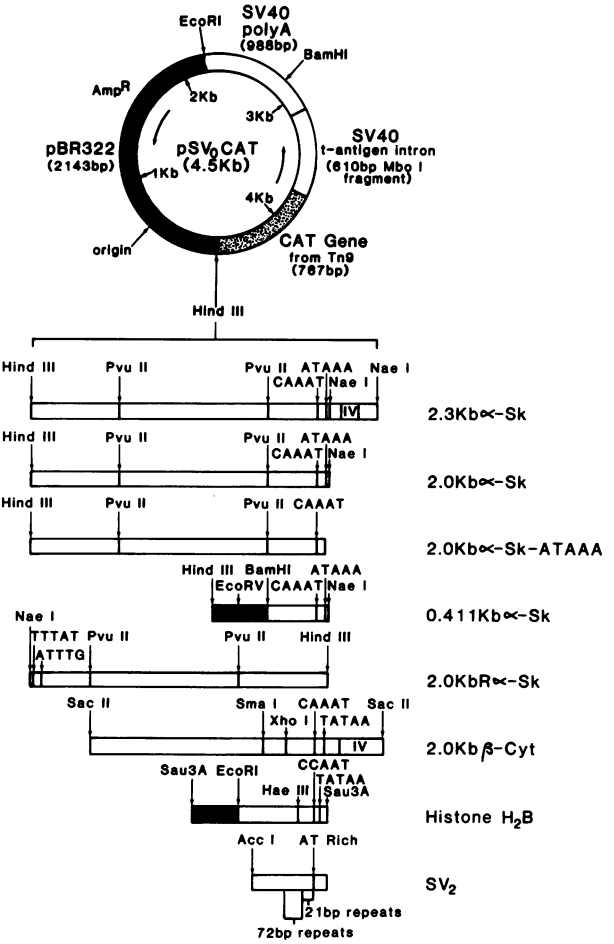

Figure 1. Construction of CAT fusion genes. The α-skeletal actin DNA fragments which encompass portions of the 5' flanking sequences described as 2.0Kb, 2.0Kb-ATAAA, 0.411Kb, and 2.0Kb reverse and a 2.0Kb fragment of β-cyt-actin and 600 bp of the 5' flanking region of the histone H2b gene were cloned into pSV0-CAT. The orientation of the SV2-CAT gene constructed by Gorman et al. (15) was illustrated. The relative positions of the TATA and CAAT boxes in the CAT constructions are indicated in the schematic diagrams. Fully blacked in regions are from pBR322. Details concerning the construction of these fused genes was described in the "Methods".

over the initial level in proliferating myoblasts (8). However, it was possible that additional DNA sequences 5' to the α-skeletal actin promoter might be responsible for even further potentiation of CAT transcription. We examined the effect of the p2.0α-Sk-CAT gene, which contains a consensus core

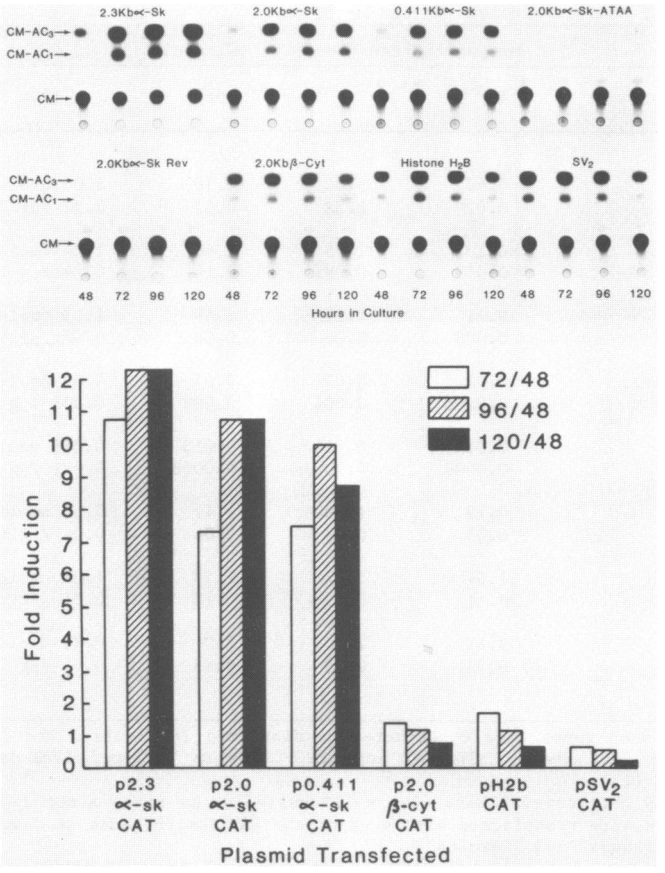

Figure 2. Analysis of CAT enzymatic activity in transfected myoblasts. Primary myoblasts from the same pooled population of dissociated cells were transfected at 24 hours after plating as described in "Methods" and "Results". Cells were harvested 48, 72, 96, 120 hours postplating and resuspended in extraction buffer and assayed for 90 minutes as described by Gorman et al. (15). The [^{14}C] chloramphenicol (CM) and acetylated products (CM-AC1 and CM-AC3) were separated by thin layer chromatography, autoradiographed on X-ray film (upper panel) and quantitated by scintillation counting as tabulated in Table I. In the lower panel, CAT activity from the 72, 96, 120 hour time points were divided by the activity expressed 48 hours ("prefusion levels") for each construct. This yields the fold increase in activity. Note that for p2.0-ATAAA and p2.0R that the CAT activity were at background levels and that induction values were not significant.

enhancer element at -560 (GTATGGTTAGG, 29), upon CAT activity. Since, the p2.0α-Sk-CAT gene did not stimulate additional CAT activity in transfected myoblasts, the sequences 5' to -411 nucleotides in the α-skeletal actin gene

Table I:CAT Activity in Transfected Myoblasts

	Myogenic Stage (hours in culture)[b]			
DNA CAT Vector[a]	48	72h	96h	120h
p2.3αSk	0.41 0.026	5.30 0.28	6.10 0.32	6.06 nmoles[c] 0.32 /μg DNA
p2.0αSk	0.18 0.013	1.75 0.095	2.60 0.14	2.24 nmoles 0.14 /μg DNA
p411αSk	0.11 0.008	1.11 0.06	1.49 0.08	1.13 nmoles 0.07 /μg DNA
p2.0αSK-ATAAA	0.01 0.0008	0.02 0.001	0.05 0.002	0.03 nmoles 0.002 /μg DNA
p2.0RαSk	0.014 0.0009	0.014 0.0007	0.015 0.0008	0.016 nmoles 0.001 /μg DNA
pβ-Cyt	0.79 0.05	1.30 0.07	1.12 0.06	0.53 nmoles 0.04 /μg DNA
pH2b	1.61 0.11	3.57 0.19	2.23 0.13	1.05 nmoles 0.07 /μg DNA
pSV2	3.11 0.23	2.40 0.15	2.04 0.13	0.83 nmoles 0.05 /μg DNA

a) CAT fusion genes were constructed as diagramed in Figure 1 and described in the "Methods". pSV2-CAT and pSV0-CAT were obtained from Dr. Bruce Howard (15).

b) Primary chicken myoblasts (2-3 x 10^6 cells/100 mm dish) after 24 hours in culture were transfected with 4 μg of the CAT fusion genes as described in the "Methods" and "Results".

c) The amount of acetylated [^{14}C] chloramphenicol in a 90 minute assay was assayed by scintilation counting of separated spots and represent a single experiment shown in Figure 2. Radioactivity was converted to nanomoles of acetylated chloramphenicol per culture dish and per cell DNA.

were not critical for enhancing tissue specific expression in transient assay cultures.

In a control experiment the same 2Kb α-skeletal actin genomic DNA was placed in the opposite orientation to pSV0-CAT. The reversed hybrid gene was ineffective in stimulating CAT activity in transfected myoblasts and accounted for only 0.5% of the CAT activity induced by p2.0α-Sk-CAT (Table 1). In addition, very low levels of CAT was detected in myoblasts transfected with a properly oriented p2.0α-Sk-CAT gene deleted at the ATAAA box. These results are consistent with other studies, in which simple deletions at the TATA box had a primary effect on the transcription initiation complex of eucaryotic promoters (30,31).

The construction of p2.3α-Sk-CAT was used to determine a role, if any, for DNA regions immediately 3' to the promoter of the α-skeletal actin gene. Sequence analysis of the p2.3α-Sk-CAT gene allowed us to determine that the actin coding region is fused and in frame with the CAT coding sequence. Therefore, transcripts from this hybrid would most likely code for a protein fusion product with 38 amino acids of actin amino terminal sequence. In this construct both the transcript and protein product have been altered, and it may not be legitimate to compare the level of CAT activity to the other α-skeletal promoter constructs. However, comparison of the fold induction in CAT activity during differentiation is still relevant. In the experiment presented here the baseline CAT activity in 48 hour myoblasts was raised about 2-4 times higher than in myoblasts transfected with the p2.0α-Sk or p0.411α-Sk-CAT genes (Table 1). We cannot comment on the stabilities of the RNA transcript or protein product. Nevertheless, a 12 to 14 fold increase in CAT activity was detected between the 48 hour and 96 hour myogenic stages in these experiments. Since the DNA sequences immediately downstream of the α-skeletal actin promoter did not seem to have a major additive effect on potentiating transcriptional activity during myogenesis, it appears that stage specific activation was retained within 411 nucleotides of α-skeletal actin promoter sequence.

In comparison, three non-myogenic promoter CAT genes were selected for transient expression experiments (Figure 2). The p2.0β-Cyt-CAT hybrid showed a rather constant CAT activity during the first 96 hours in culture and displayed a modest decline by the late 120 hour stage. The pH2b-CAT gene increased CAT activity about 2 fold from 48 to 72 hours and then declined in later stages as well. Since the CAT activities stimulated by the p2.0β-Cyt-CAT and pH2b-CAT genes were substantially reduced following the addition of ARA C at 96 hours, the presence of proliferative cells (fibroblasts) in the primary myoblast cultures might have been responsible for inducing CAT activity. In addition, the decreased activity at these later time points in culture may be due to loss of transfected DNA. The third construct, pSV2-CAT, contains the SV40 early promoter and the 72 bp enhancer juxtaposed to the CAT gene. Transcriptional activity of pSV2-CAT appears to be expressed early in myogenesis, almost full activity at 24 hours (data not shown), and was found to decrease slowly throughout the culture period.

Dose Dependent Response

In stably transfected cell lines it is difficult to correlate DNA copy number with transcriptional gene activity, because chromosomal integration

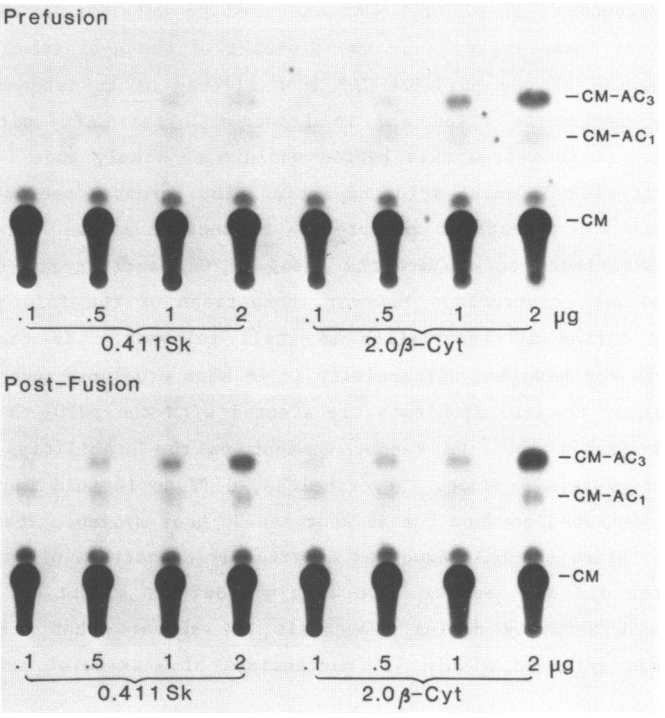

Figure 3. CAT activity in chicken myoblasts is dependent upon the amount of transfected DNA. Myoblasts were transfected 24 hours postplating with increasing quantities (0.1 µg to 2.0 µg) of pO.411α-Sk-CAT and p2.0β-Cyt-CAT DNA. Cells were harvested at 48 hours (prefusion) and 84 hours (post-fusion) postplating and assayed for CAT activity.

sites may influence gene expression (32). We explored the possibility of further defining the myoblast transient transfection system by manipulating the copy number of DNA which remains extrachromosomal (Figure 3). Myoblasts were transfected with increasing amounts of hybrid DNA (0.1 ug to 2 ug of DNA) of the pO.411α-Sk-CAT and the p2.0β-Cyt-CAT genes in the presence of a constant amount of plasmid DNA (supplemented to 10 ug with pBR322). Cell extracts were made after the 42nd hour and the 74th hour in culture. In prefusion myoblasts, CAT activity appeared to increase, although weakly, in a dose dependent manner for both the β-cytoplasmic and, the α-skeletal actin promoter constructions. Little change in CAT activity (1 to 2 fold change) was detected between the prefusion and postfusion developmental time points for myoblasts transfected with the β-actin hybrid gene. However, the α-skeletal actin CAT construct caused a 5 to 10 fold enhancement in CAT activity between the two time points, and was dependent upon the amount of the hybrid

clone DNA. Thus, we show that stage specific activation can in fact be shown to increase in a dose dependant manner.

Transfection of Actin Hybrid Genes into Non-Muscle Cells

We sought to evaluate the tissue specificity of the 411 nucleotide α-skeletal actin fragment in non-myogenic tissues. Direct comparison between different cell types of a single construct is difficult due to different transfection efficiencies, DNA, RNA, and CAT protein stabilities that might exist in various cell types. However, comparison of different promoter activities in the same cell type is valid, since transfected DNA should be stabilized to the same extent and CAT RNAs should have similar half lives (19). The pO.411α-Sk-CAT, and p2.0β-Cyt-CAT (cytoplasmic) constructs were separately transfected into primary cultures of chick embryonic day 11 brain cells and mammalian COS 7 cells. These cells were chosen because they are non-myogenic cell types. Transient activity assays revealed that COS 7 cells and the heterogeneous primary brain cell cultures transfected with the β-CAT gene converted over 15 % and over 20% of the substrate to the acetylated forms (Figure 4). Thus, the β-actin promoter displayed strong transcriptional activity consistent with other actively transcribed genes such as pSV2-CAT. In comparison, less than 1% of the radioactive chloramphenicol was converted to the acetylated forms in the presence of the α-skeletal actin promoter in either of these non-muscle cell types. These levels are similar to the transcriptional activity of the promoterless pSV0-CAT (data not shown). It is important to note that the same α-Sk-CAT calcium phosphate precipitate was found to have full activity when transfected into fusing chick myoblasts. These results suggest that transcription from the α-actin promoter may be tightly restricted in the presence of non-muscle cells.

Transcription initiation of the pO.411α-skCAT construct

Since the native cap site had been removed in the pO.411α-SkCAT plasmid, by inserting a Hind III linker in the Nae I site eleven base pairs downstream from the ATAAA box, we wanted to locate the initiation site of the surrogate CAT RNA transcripts. Total RNA from chick myoblasts transfected with pO.411α-SkCAT was hybridized with a ^{32}P end labeled 458 nucleotide Stu I- Pvu II fragment which overlapped the junction between the α-skeletal promoter and the CAT gene. An S_1 nuclease protected fragment of 146 nucleotides was detected by autoradiography following electrophoresis on a 6% polyacrylamide sequencing gel (Figure 5). The size of the protected DNA fragment revealed that the major CAT RNA product is initiated 18 nucleotides downstream from the ATAAA box. The predominate start position of the transcribed RNA begins at the

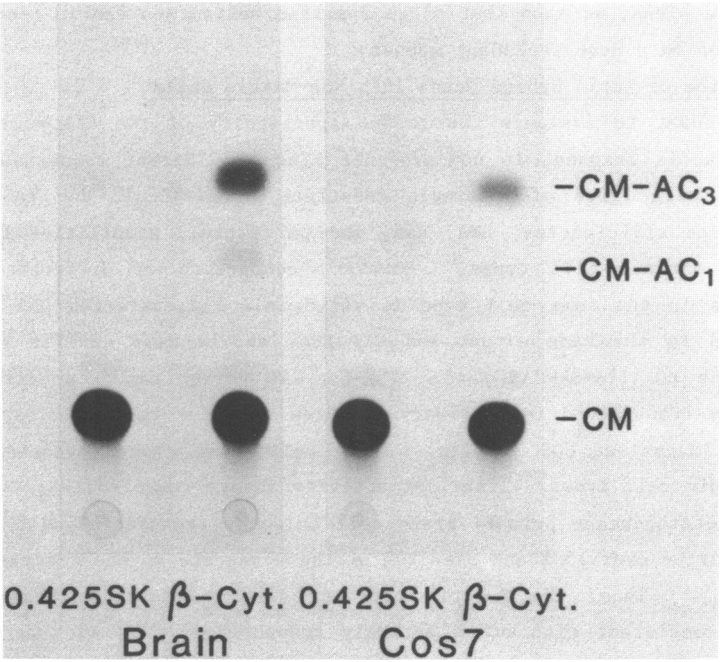

Figure 4. Restricted expression of the α-skeletal actin CAT fusion gene in non-myogenic cells. Cultures of 11 day embryonic chicken brain cells were transfected with 4 ug of pO.411α-Sk-CAT and p2.0β-Cyt-CAT plasmids plus 6 ug pBR322 as carrier. These cells were harvested 24 hours later and assayed for CAT activity. Cultures of the COS 7 were transfected with 10 ug of pO.411α-Sk-CAT and p2.0β-Cyt-CAT plasmids. These cells were harvested 48 hours later and assayed for CAT activity.

first thymidine residue in the Hind III site (AAGCTT) directly preceding the bacterial CAT gene. The predominate chimeric mRNA CAT product has approximately a 32 base pair leader before the AUG translation start codon for the CAT protein. Secondary RNA start sites fall 1-2 nucleotides immediately flanking this site. These may be genuine alternative start positions or the result of S_1 nibbling (33). In comparison, the natural α-skeletal actin mRNA initiates 24 nucleotides downstream from the ATAAA box.

DISCUSSION

It is well established that tissue culture cells transfected with recombinant DNA can express cloned genes if the regulatory sequences are present (9-14). We demonstrated that DNA flanking sequences which contain 411 nucleotides of the α-skeletal actin gene appear to be responsible for its tissue and

developmentally specific transcription in the chicken primary myoblast system. This observation is based upon the restricted expression of the actin CAT fused genes in muscle cells versus non-muscle cells, and the 9-15 fold induction of CAT activity which coincides with the normal appearance of the α-skeletal actin mRNA during myogenesis in culture. Additional DNA regions immediately flanking these 411 nucleotides did not substantially affect the induction of CAT during the myoblast fusion. The simplest interpretation of these results is the appearance of trans-acting factors in myotubes which stimulate the transcription of the α-skeletal actin gene. Currently, we do not know whether other sequences associated with or situated within the α-skeletal actin gene contribute to its myoblast specific functions.

In comparison to studies of the α-skeletal actin gene, constructions made with the promoter regions of the β-cytoplasmic actin gene and the histone H2b gene in pSV0-CAT did not demonstrate an induction of CAT activity during latter stage of myogenesis. Although the steady state levels of β-actin mRNA is reduced following the end of myoblast replication and the onset of fusion (6,8), we did not observe a reduction in assayable CAT activity. These results might be interpreted to suggest that DNA regulatory sequences, which are involved in the down regulation of the β-actin and histone genes are absent in these constructions. However, since the stability of CAT is thought to be exceedingly high in transfected cells in contrast to its mRNA product, it is difficult to evaluate events associated with the down regulation of gene transcription utilizing the CAT assay.

Our finding that a short 5' flanking sequence in the α-skeletal actin gene is sufficient to provide tissue specific expression is in agreement with transient expression studies in several other primary and tissue culture systems. Sequences situated between nucleotide positions -247 and -302 of the rat insulin gene and between positions -192 and -274 of the rat chymotrypsin gene were sufficient to promote CAT activity in a tissue specific manner when inserted upstream of the CAT gene and transfected into cultured pancreatic cells (17). In the chicken α-A crystallin gene 362 bp of 5' flanking sequences can activate the CAT gene in transfected embryonic chicken lens epithelia (18). Our data allowed us to localize the α-skeletal actin promoter region to within 411 bp of the gene. Experiments are currently in progress to further delineate the extent of the regulatory elements. We expect that the sequence ATAAAA (-24 to -30) is an important element of the promoter, because of its similarity to the cannonical TATA box. Point mutations within this sequence greatly reduce transcription of the ovalbumin gene in vitro (30). In

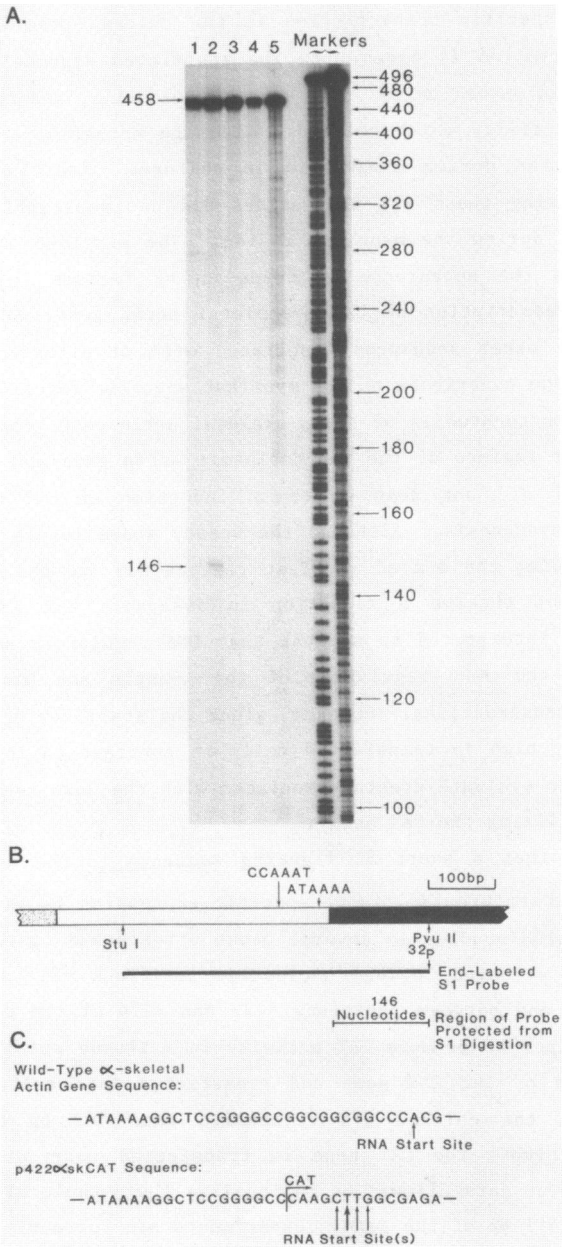

Figure 5. Nuclease S1 assay of p0.411α-Sk-CAT transcription initiation start site. Total RNA from chick myoblasts transfected with p0.411α-Sk-CAT was hybridized with a ^{32}P end labeled 456 nucleotide Stu I-Pvu II fragment which overlapped the fusion between the α-skeletal promoter and the CAT gene. RNA

isolated from myoblasts transfected with 4ug DNA/plate (Slot 1), RNA isolated from myoblasts transfected with 40 μg DNA/plate (Slot 2), RNA isolated from non-transfected myoblasts (Slot 3), control tRNA (Slot 4), and probe alone (Slot 5), were all hybridized with probe, digested with S_1 nuclease, and the products were run on a 6% polyacrylamide sequencing gel.

studies within the rabbit β-globin gene (31,32), HSV TK gene (35,36), and conalbumin gene (37) the TATA sequence was necessary for efficient transcription in vivo. In our study, the 3' deletion of 20 nucleotides which included the ATAAAA region from 2 Kb DNA flanking α-skeletal actin gene eliminated the induction of CAT activity in myoblasts. One explanation for these results is that the deleted ATAAA box could have directly inhibited transcriptional activity. Alternatively, the loss of the ATAAA region might have significantly altered the spacing of regulatory elements 5' to the CAT gene. In this way defective transcripts with insufficient leader sequences would not allow translation of a CAT enzyme product. However in either case, this observation is consistent with transcription being initiated at a single locus in the hybrid α-skeletal actin CAT gene as shown in this study.

The results of our transient transfection experiments differ from the report of Seiler-Tuyns et al. (38). In that study, the chicken α-skeletal and β-cytoplasmic actin genes were stably integrated into C_2C_{12} cells, a murine myogenic cell line. Chicken skeletal actin mRNA did not appear to be induced during C_2C_{12} myogenesis, but was considerably elevated over levels detected in transfected L cells. One explanation for these findings is that the chicken α-skeletal actin gene was actually induced earlier than its murine counterpart in response to regulatory factors perhaps already present in the prefusion C_2C_{12} myoblasts. Several other possibilities have also been discussed by Nudel et al. (40). In contrast the chicken β-actin gene was appropriately down regulated during C_2C_{12} myogenesis, while in the present study the β-actin promoter CAT hybrid gene did not appear to be strongly repressed. Currently, for reasons we have already discussed, it is premature to eliminate the promoter region from a role in the down regulation of the β-actin gene.

The results of our transient transfection experiments in chicken primary myoblasts are consistent with the results of Melloul et al. (39). In their study 730bp of the rat α-skeletal actin gene promoter was found to give a many fold increase of CAT activity when the myogenic L8 cell line made the transition from replication to fusion. In the same study the β-cytoplasmic promoter was shown to induce only a minor increase in activity. However, their results in these stably transformed cells could not be correlated with the amount of

integrated DNA and CAT activities were variable between the clonal isolates under study (39). Nevertheless, comparisons of the chicken and rat α-skeletal actin gene promoters within these 411 nucleotides have shown several regions of nucleic acid sequence homology (29,40). In addition, the chicken α-skeletal actin gene has recently been shown to be developmentally regulated in rat L8 cells (40). Although we can not rule out that other sequences present in and around the chicken α-skeletal actin gene in its native chromosomal location have regulatory functions, we suspect that the conserved regions within this 411 base pair fragment are the best locations for detecting DNA sequences involved with transcriptional control of a muscle regulated gene.

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