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The Chicken Skeletal α-Actin Gene Promoter Region Exhibits Partial Dyad Symmetry and a Capacity To Drive Bidirectional Transcription

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The chicken skeletal α-actin gene promoter region (−202 to −12) provides myogenic transcriptional specificity. This promoter contains partial dyad symmetry about an axis at nucleotide −108 and in transfection experiments is capable of directing transcription in a bidirectional manner. At least three different transcription initiation start sites, oriented toward upstream sequences, were mapped 25 to 30 base pairs from TATA-like regions. The opposing transcriptional activity was potentiated upon the deletion of sequences proximal to the α-actin transcription start site. Thus, sequences which serve to position RNA polymerase for α-actin transcription may allow, in their absence, the selection of alternative and reverse-oriented start sites. Nuclear runoff transcription assays of embryonic muscle indicated that divergent transcription may occur in vivo but with rapid turnover of nuclear transcripts. Divergent transcriptional activity enabled us to define the 3′ regulatory boundary of the skeletal α-actin promoter which retains a high level of myogenic transcriptional activity. The 3′ regulatory border was detected when serial 3′ deletions bisected the element (−91 CCAAA TATGG −82) which reduced transcriptional activity by 80%. Previously we showed that disruption of its upstream counterpart (−127 CCAAAGAAGG −136) resulted in about a 90% decrease in activity. These element pairs, which we describe as CCAAT box-associated repeats, are conserved in all sequenced vertebrate sarcomeric actin genes and may act in a cooperative manner to facilitate transcription in myogenic cells.

The actin genes are encoded in most animal species by multigene families in which individual members are differentially expressed with a distinct pattern of tissue and developmental stage specificity (10, 17, 18, 33). Mammalian, avian, and some amphibian species express four muscle actin isotypes, which are separable into striated and smooth-muscle isoforms (50). The skeletal and cardiac α-actin genes have a common evolutionary origin, as deduced by comparing both protein sequence (51) and gene organization (7); they are often coexpressed in the same embryonic tissues (26, 36). Although these striated actin genes share some transcriptional elements within their promoter regions (23, 37), their regulation is rather complex; they are differentially regulated in primary myogenic cultures (25, 28) and during the developmental transition from fetal to adult tissues (25, 26). Nevertheless, gene transfer experiments in cultured cells (22, 34, 37), embryos (54), and transgenic animals (47) indicate that sequence elements with the capacity for selective genetic expression of the skeletal and cardiac α-actin genes reside within their 5′ promoter and immediate DNA-flanking regions.

Recently, we delimited the 5′ boundary of the *cis*-acting regulatory element of the chicken skeletal α-actin gene to a region approximately 200 base pairs (bp) upstream from the authentic mRNA cap site (5). Transcriptional analysis of several gene promoter regions has revealed that multiple elements responsible for regulated expression are often present within a few hundred base pairs of the transcription start site (44). These regions are classically viewed as having two domains, proximal and distal. The proximal domain frequently includes a TATA box sequence approximately 30 bp upstream of the mRNA cap site in higher eucaryotic cells (6). Mutagenesis of the TATA sequences has been shown to result in heterogeneous start sites and reduced transcriptional efficiency (11). Thus, the proximal domain plays a role in both the accuracy and efficiency of transcription initiation.

The distal portion of the promoter region appears to serve to modulate transcriptional activity. Conserved DNA sequence elements found in this region are highly variable between gene promoter sequences and can be organized into general and specific subclasses. General distal element sequences appear to include the hexanucleotide 5′-CCGCCC-3′, which has been shown to bind a cellular transcription factor, termed Sp1, and the pentanucleotide CCAAT box (4, 13), which binds CCAAT-binding transcription factor/nuclear factor I (30), CCAAT binding protein (21), and the Y box nuclear factor (12). One method of testing the function of distal regulatory elements is to insert the test sequences next to a surrogate promoter. This strategy has been used effectively in determining the regulatory boundaries of many transcription elements. This approach assumes that the organization of the surrogate promoter is such that it will interact with the test elements in a functional manner. However, substituting promoters may have the potential for aberrant results. Seiler-Tuyns et al. (46) found that a surrogate simian virus 40 (SV40) promoter was much less responsive to induction conferred by the vitellogenin 5′-flanking region than the herpesvirus *tk* promoter. Likewise, the activity of the human cardiac α-actin upstream sequences when linked to the SV40 promoter had a reduced effect on the potentiation of transcription in myogenic cells (37).

A notable feature of the skeletal α-actin promoter region is that the sequences are partially symmetrical about an axis at nucleotide −108. These symmetrical sequences, which include diametrically oriented CCAAT- and TATA-like sequences, led us to test whether this promoter region functioned in a bidirectional manner. Subsequent experimentation identified that reverse oriented skeletal α-actin

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promoter fragments had the capacity to drive myogenic transcription in the divergent direction. In this report, we addressed two issues concerning the transcriptional regulation of the skeletal α-actin gene. First, we identified sequences which promote unidirectional transcription. Second, we determined which sequence elements must be included at the 3′ border of the skeletal α-actin promoter to retain high levels of transcriptional activity in myogenic cells.

MATERIALS AND METHODS

Plasmid nomenclature. DNA plasmid constructions were labeled according to the general format pSx.ypCz. The S signifies that the inserted sequences are from the chicken skeletal α-actin gene. The numbers separated by a dot, represented by x.y, indicate orientation and the nucleotide distance upstream from the skeletal α-actin cap site of both ends of the inserted sequence. If the fragment includes nucleotides downstream of the cap site, this is represented by a plus sign. The p following the nucleotide indicates that a small portion of the pUC8 cassette is included at one end of the insert. The C signifies that the vector includes a *cat* structural gene immediately downstream from the inserted fragment. A lowercase t following the C indicates that the vector sequences are intact pSV0CAT. A lowercase n indicates that the vector sequences are pSV0CAT truncated at an *Nde*I site approximately 50 bp upstream of the *cat* gene.

Plasmid constructions. Two skeletal α-actin promoter constructions were made to test for bidirectional transcriptional activity. The 2-kilobase (kb) *Bam*HI fragment from p0.411α-skCAT (22), which included 0.4 kb of α-skeletal 5′-flanking sequence and 1.6 kb of vector separated by an *Hin*dIII site, was ligated into the *Bam*HI site of pUC 8. An *Hin*dIII fragment containing the skeletal α-actin promoter and flanking sequence (−424 to −12) was then removed from the pUC8 vector and ligated into the *Hin*dIII site of pSV0CAT. Recombinant DNA clones were obtained in the both the direct 5′-3′ (pS424.12pCt) and indirect 3′-5′ (pS12.424pCt) orientations.

DNA constructions were generated to determine the 3′ regulatory border of the skeletal α-actin promoter by *Nde*I digestion of pS12.320pCt and then by secondary digestions with *Eag*I, *Sac*II, and *Sma*I. The fragment ends were filled in with nucleotides and Klenow DNA polymerase and *Hin*dIII linked to generate pS74.320pCn, pS150.320pCn, and pS 202.320pCn, respectively. Constructs were generated by *Eag*I digestion, slow *Bal*31 exonuclease, followed by *Nde*I digestion, Klenow, and ligation with *Hin*dIII linkers to yield pS87.320pCn, pS91.320pCn, and pS99.320pCn. Further constructions were generated by *Taq*I and *Hgi*AI digestion of pS12.320pCt. The appropriate *Hin*dIII-ligated products were ligated into an *Hin*dIII-digested pCn vector (pSV0CAT truncated to the *Nde*I site). To complete this series, pS12.320pCt was recloned in both orientations into the pCn vector by *Hin*dIII digestion and yielded pS12.320pCn and pS320.12pCn.

Recombinant DNA clones were made to test the degree of divergent transcriptional activity in the presence of additional 3′ actin downstream sequences. These constructions were generated by *Stu*I digestion of p2.3kbα-skCAT and p2.0kbα-skCAT-ATAAA (22), followed by ligation with *Hin*dIII linkers. The appropriate products were ligated into pCn vector to yield pS320+312Cn and pS+312.320Cn and pS320.33Cn and pS33.320Cn, respectively. DNA constructions were generated by *Sal*I digestion of pS12.424pCt and second digestion with *Stu*I, *Sma*I, *Sac*II, or *Xma*III, nuclease S1 treatment, and ligation to yield pS12.320pCt, pS12.201pCt, S12.147pCt, and pS12.73pCt, respectively, to examine the role of 5′ deletions in divergent transcriptional activity.

The sequences at the modified junctions of the DNA hybrid constructions were verified by chemical sequencing. Transformed bacteria were grown in L-broth containing ampicillin in host *Escherichia coli* K-12 strain RR1. Plasmid DNA was prepared by gentle alkaline lysis and banded twice on cesium chloride-ethidium bromide gradient centrifugations.

Transfections and enzymatic assays. Primary myoblast cultures were established by mechanically dissociating tissue from the thighs of 11- to 12-day-old chicken embryos as described previously (22). The myoblast-enriched cell populations were seeded into collagen-coated 100-mm dishes at a density of 5×10^6 cells per plate in minimal essential medium supplemented with 10% horse serum, 5% chicken embryo extract, and gentamicin sulfate (50 μg/ml). Cultures were incubated in 5% CO_2 at 37°C. At 34 h postplating, primary cultures were transfected with a total of 10 μg (unless otherwise indicated) of the hybrid *cat* vector construct DNA plus 1.5 μg of pRSVGAL per 100-mm dish in calcium phosphate precipitates. Cells were harvested by scraping at 36 h posttransfection and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (22). To control for variations in both cell numbers and transfection efficiency, all recombinant and control clones were cotransfected with the same amount of pRSVGAL, a eucaryotic expression vector in which the *E. coli* β-galactosidase (*lacZ*) structural gene is under the transcriptional control of the Rous sarcoma virus long terminal repeat (a gift of Grant McGregor, Baylor College of Medicine, Houston, Tex.). Lysates prepared from the harvested cells were assayed for both CAT activity, which was used as a measure of promoter efficiency, and β-galactosidase activity, which was used as an internal standard to normalize the CAT activity directed by the test plasmid.

Assays of β-galactosidase activity under the conditions described by Miller (35) and Norton and Coffin (41) were performed with 10 μl of cell extract which was added to a solution containing 50 μl of 3.36 M 2-mercaptoethanol, 50 μl of 30 mM MgCl, and 650 μl of 0.1 M Na_2HPO_4 adjusted to pH 7.3 at 37°C with 0.1 M NaH_2PO_4. The reaction was started by the addition of 750 μl of 4.38 mM ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (Sigma) dissolved in the sodium phosphate buffer and incubated at 37°C for 30 to 45 minutes. The reaction was stopped by the addition of 500 μl of 1 M Na_2CO_3. The absorbance at 410 nm was then recorded. Units were determined by the method of Miller (35) per microliter of cell extract as described by the manufacturer. Transfection experiments were performed in duplicate with two independently isolated sets of chimeras and control vectors, and the results were averaged. The range of individual sample values averaged 10% of the normalized value. Data are expressed as nanomoles of acetylated [^{14}C]chloramphenicol per hour per microgram of *cat* vector DNA transfected per unit of galactosidase activity.

S1 nuclease mapping. The initiation sites of divergently directed transcripts were located by S1 nuclease mapping. A DNA probe was constructed by double digestion of plasmid pS12.424pCt with *Xma*III (site at −77) and *Pvu*II, which cuts 150 bp into the *cat* gene. This 550-bp fragment was *Xho*I linked and ligated into the *Sal*I site of M13mp10. Single-stranded uniformly ^{32}P-labeled DNA probes were prepared

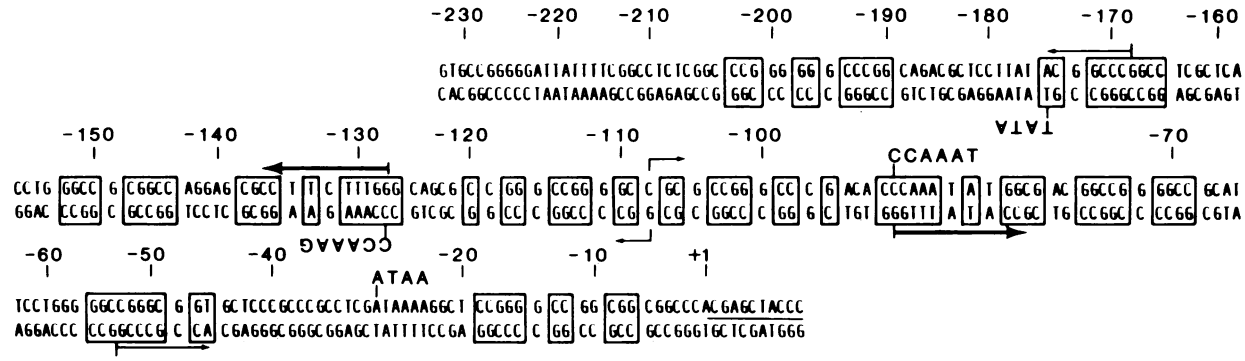

FIG. 1. Chicken skeletal α-actin promoter region exhibits partial dyad symmetry. The native transcription initiation site is labeled +1. CCAAAT and TATA sequences are indicated on the appropriate strand. The thick arrows indicate the direction and location of the conserved CBAR elements, and the fine arrows indicate the CGGGC(C/G)GT sequence. The central axis of symmetry is represented by bent arrows at −108. Nucleotide regions which are present in an inverted orientation in a similar location on the other side of the axis are boxed.

from the M13 DNA recombinant with Sequenase enzyme (United States Biochemicals) followed by *Eco*RI digestion as previously described (28). RNA was isolated from myoblasts transfected with 40 μg of plasmid DNA per ml as described previously (22). Additionally, RNA isolated from myoblasts and embryonic day 15 brain, cardiac, and breast tissue was tested for endogenous divergent transcripts. Uniformly ^{32}P-labeled probe (4×10^5 cpm) was coprecipitated with 50 μg of RNA in the presence of 0.3 M sodium acetate and 2 volumes of ethanol, and the pellet was suspended in 20 μl of hybridization buffer (50% formamide, 400 mM NaCl, 15 mM PIPES [piperazine-*N*,*N*′-bis(2-ethane sulfonic acid), pH 6.4], and 1 mM EDTA]. The solution was heated at 95°C for 10 min, and hybrids were allowed to form first at 70°C for 6 h and then at 55°C for 10 h. Following this reaction, 330 μl of ice-cold nuclease S1 buffer (300 mM NaCl, 50 mM sodium acetate [pH 4.5], 2 mM zinc acetate, including 725 Units of S1 nuclease [Pharmacia]) was added to the hybrids and digested at 30°C for 70 min. The digestions were terminated by phenol-chloroform extractions, and protected probes were coprecipitated with 5 μg of carrier tRNA. The digestion products were suspended in 5 μl of 99% formamide containing 0.05% xylene cyanole FF and bromophenol blue dyes and 2.5 μl of this mixture and loaded on a 6% polyacrylamide–urea sequencing gel adjacent to sequencing ladders.

Nuclear runoff transcription analysis. The relative activity of the divergent promoter was assessed by nuclear runoff analysis. Selected restriction fragments from the chicken skeletal α-actin gene (10) were subcloned in both orientations into the phage vectors M13mp18 and -19. Single-stranded phage DNA was isolated and verified by dideoxy sequencing, and 1-μg portions were dot-blotted onto Zeta-Probe blotting membranes (Bio-Rad Laboratories, Rockville Centre, N.Y.). Nuclei were isolated from primary chicken myoblast cultures at prefusion (36 h) and postfusion (92 h) time points by a standard procedure (24). Embryonic day 19 breast muscle was first dispersed into reticulocyte standard buffer (24) with a polytron homogenizer before being subjected to the same procedure. Nuclear runoff transcripts were prepared by the method of Groudine et al. (24) as modified by Linial et al. (32). After prehybridization, the dot-blots were hybridized with ^{32}P-labeled nuclear transcripts at a concentration of 10^7 cpm/ml at 50°C for 48 h in 50% formamide–1.5× SSPE (0.27 M NaCl, 0.015 M $NaPO_4$ [pH 7.0], 0.015 M disodium EDTA)–1% sodium dodecyl sulfate (SDS)–0.5% Blotto–0.2 mg of *E. coli* tRNA per ml. After hybridization, the membranes were washed at increasing stringencies, culminating with a 30-min wash in 0.1× SSC–1% SDS at 60°C. The dot-blots were rinsed at room temperature in 0.1× SSC, blotted dry, and autoradiographed for 5 days at −70°C with an intensifying screen.

RESULTS

Capacity of the symmetrical skeletal α-actin promoter region to drive bidirectional transcription. The DNA sequences of the 5′-flanking region of the skeletal α-actin gene harbor a *cis*-acting regulatory promoter element, a region extending from nucleotides −202 to −12, that influences cell type and developmental stage expression (5). Examination of the sequences in this core promoter region revealed an imperfect dyad symmetry about a central axis at −108 (Fig. 1). Although part of this pattern is a natural consequence of the G+C-rich structure and allowances for insertion or deletion of nucleotides within this region, sequence analysis revealed symmetrical and homologous sequences in the promoter regions of the avian and rodent skeletal α-actin genes (23). These sequences included the repeated element CGGGC(C/G)GT, found to be approximately 60 bp from the central axis (represented by the lighter arrows in Fig. 1) and a second symmetrical element, CCCAAA(T/G)A(T/A)GGCG, which we have termed the CCAAT box-associated repeat (CBAR), found approximately 20 bp from the central axis (represented by the darker arrows in Fig. 1). In addition, TATA-like sequences were spaced symmetrically to the ATAAAA sequences (−29 to −24) of the native oriented promoter fragment (Fig. 1; −175 TATAA −179).

The symmetrical nature of the skeletal α-actin promoter region was examined for its capacity to drive transcription in a bidirectional manner. To achieve this, recombinant clones were made by inserting a 413-nucleotide fragment, which encompassed the core promoter, in both orientations upstream of the *Hin*dIII site of pSV0CAT. These *cat* gene fusions, pS424.12pCt and pS12.424pCt, were transfected into chicken primary myoblasts. For comparison, parallel transfections were performed with plasmid pSV2CAT, a vector in which the bacterial *cat* gene is under the control of the SV40 enhancer and early promoter region or plasmid pSV0CAT, which lacks any defined eucaryotic promoter (20).

The native oriented fragment of the 5′-flanking region and skeletal α-actin promoter, pS424.12pCt, was effective in

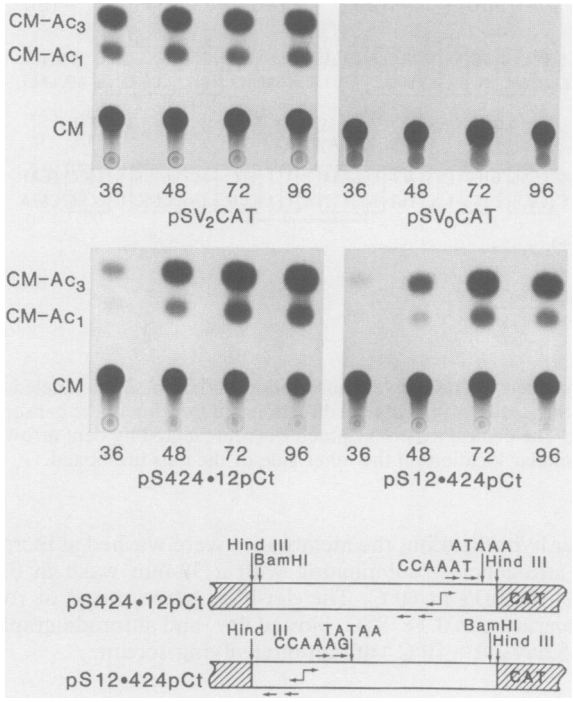

FIG. 2. Myogenic bidirectional transcriptional activity of the skeletal α-actin promoter. Primary myoblasts from the same pooled population of dissociated cells were transfected 24 h after plating with 4 μg of pSV2CAT, pSV0CAT, pS424.12pCt, or pS12.424pCt DNA. Cells were harvested at 36, 48, 72, and 96 h postplating and assayed for CAT activity. The upper panel shows the autoradiographic results of CAT assay analysis. CM, CM-Ac_1, and CM-Ac_3, Chloramphenicol and its acetylated forms. The lower panel diagrammatically represents the relative structures of pS424.12pCt and pS12.424pCt including the location of the symmetrical region from the *cat* gene and the respective CCAAT and TATA sequences.

driving increased levels of CAT activity, as the myoblasts biochemically differentiated into myotubes, as shown in Fig. 2. The same fragment oriented in the opposite direction, pS12.424pCt, was also capable of driving appreciable CAT activity and exhibited an activation profile during myogenesis similar to that of the naturally oriented promoter (Fig. 2). In marked contrast to the skeletal α-actin promoter constructs, pSV2CAT produced relatively high levels of CAT activity in the early 36-h myoblasts, which remained relatively stable throughout myogenesis. Divergent CAT activity was approximately 10 to 30% of that directed by the native orientation. Thus, this skeletal α-actin promoter fragment drives transcription in either orientation, and transcriptional activity is directed preferentially in the native orientation.

5′-Flanking sequences potentiate divergent transcription activity. A series of deletions made in the 5′-flanking region located between the *cat* gene and the core promoter were generated to determine the minimal sequences required for potentiating transcription in the divergent direction. Five chimeric clones, which contained progressively deleted sequences from −424 to −73, were constructed and transfected into primary myoblasts. The reverse-oriented inserts including −12 to −73 (pS12.73pCt) or −147 (pS12.147pCt) were ineffective in driving *cat* transcriptional activity above background levels (Fig. 3). However, the retention of sequences to −201 allowed a low level of divergently directed CAT activity. These results indicate that 5′-flanking sequences, including −201, which contain the core promoter region, must be retained in order to drive divergent transcription. The addition of 5′-flanking sequences up to −320 (pS12.320pCt) or −424 (pS12.424pCt) resulted in threefold and sixfold increases in divergent transcriptional activity over that of the −201 clone, respectively. The potentiation of divergent activity by the maintenance of 5′-flanking sequences could result from a positive interaction with *trans*-acting factors, increased translation efficiency of the transcripts, or additional sites for transcription initiation.

Location of opposing transcription initiation sites. The role of the sequences between −201 and −424 was examined for divergent transcription initiation sites. A single-stranded DNA probe was constructed to detect and map transcript start positions. RNA isolated from myoblasts transfected with the native oriented promoter-*cat* construct (pS 424.12pCt) was first analyzed by nuclease S1 digestion. As shown in Fig. 4, a 145-bp protected fragment mapped to the *Hin*dIII junction of the *cat* vector and was the expected size for a protected transcript initiating in the native orientation. From the RNA isolated from myogenic cells transfected with the reverse-oriented vector pS12.424pCt, divergent transcripts mapped to a broad population of start sites at which the probe was protected at 360, 390, 410, 430, and 565 bp. The protected 360-, 390-, and 430-nucleotide DNA fragments, which mapped to divergent transcription initiation start sites at −210, −230, and −275 bp, respectively, were located approximately 30 bp from TATA box-like elements. Potentially these A+T-rich blocks may serve to position RNA polymerase for transcription initiation. The band at 410 bp represents another potential transcription start site region, although due to nuclease S1 nibbling, its position adjacent to an 8-bp A+T stretch of DNA may represent an ambiguous map position. Furthermore, the protected band at 565 bp may represent additional transcripts initiated upstream of the probe.

Previously we demonstrated that divergent transcripts were not detectable from the endogenous skeletal α-actin gene by RNA dot-blot assay (23). Nuclease S1 analysis of steady-state RNA isolated from 15-day embryonic cardiac, breast, and brain tissue and muscle culture tissue failed to protect any region of the probe (data not shown). Thus, if the skeletal α-actin promoter region serves to drive divergent transcripts endogenously, these transcripts must be highly unstable. We therefore used nuclear runoff transcription analysis (24, 32) to assay for ^{32}P-labeled, nascent RNA purified from isolated nuclei. Figure 5 demonstrates that a faint signal corresponding to divergent transcription, hybridized with strand-specific probe a from −424 to −202, was obtained from primary cultures of 92-h myotubes. A stronger signal was observed for divergent transcripts from 19-day embryonic breast muscle. The divergent signal from embryonic muscle was reproduced in three separate experiments and was always significantly higher than the background signal obtained from M13 vector containing no insert. In comparison to transcripts identified in the forward direction which hybridized to probe c (+1600 to +2702), divergent transcripts accounted for about 1% of the transcriptional activity emanating in both directions from the skeletal α-actin promoter region (−202 to −12).

Sequences flanking the native start site inhibit divergent transcriptional activity. The native TATA box and proximal surrounding regions were examined for a role in determining the direction of transcription from the symmetrical skeletal actin promoter. For this experiment, three skeletal α-actin

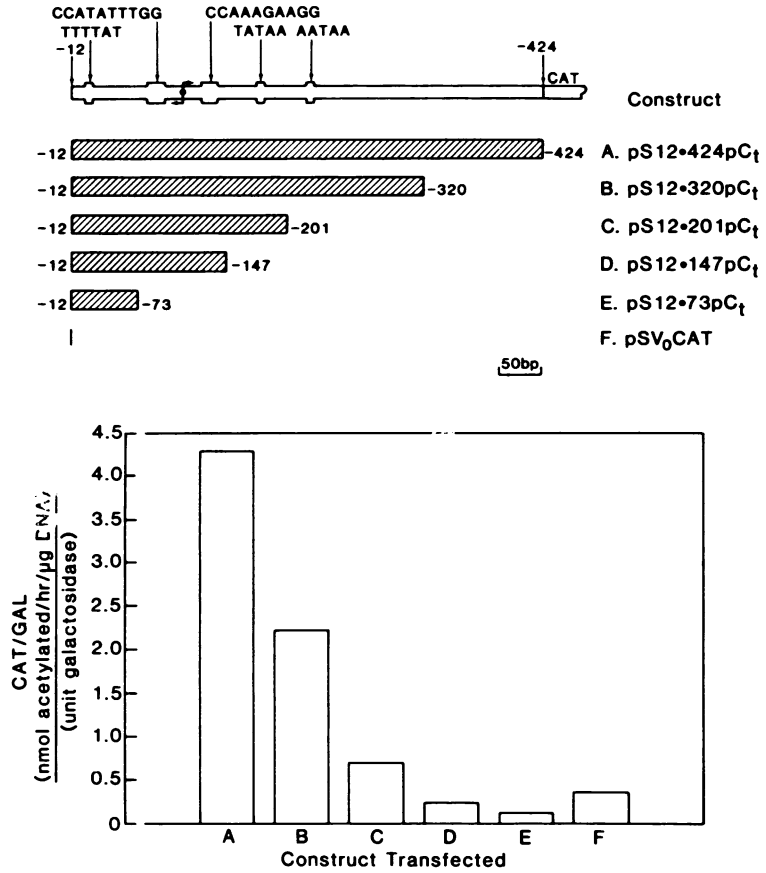

FIG. 3. Divergent activity is potentiated by 5'-flanking sequences. The schematic in the upper panel depicts the structure of the skeletal α-actin promoter region reverse oriented upstream of the *cat* gene. The CBAR and TATA-like sequences are indicated by their direct sequence. The striped horizontal bars below the schematic represent the location and size of the skeletal α-actin promoter fragments inserted upstream of the *cat* gene. The histogram in the lower panel is a representation of the resultant average CAT activity from the transfection of two independent sets of reverse-oriented constructs (a) pS12.424pCt, (b) pS12.320pCt, (c) pS12.201pCt, (d) p12.147pCt, (e) pS12.73pCt, and control (f) pSV0CAT into differentiating primary myoblasts.

gene fragments were selected and inserted in both orientations upstream of pSV0CAT as shown in Fig. 6. The first pair of clones (pS320.33Cn and pS33.320Cn), contained inserts from −320 to −33, in which the native TATA box was deleted. The second pair (pS320.12pCn and pS12.320pCn) had inserts from −320 to −12, which include the native TATA box. The third pair of clones (pS320+312Cn and pS+312.320Cn), with an insert from −320 to +312, contained the native cap site, first intervening sequence, and the actin-coding region of the first 38 amino acids. In the native orientation these sequences, which included the native cap site (pS320+312Cn), gave rise to high levels of CAT activity (Fig. 6). Sequences truncated just upstream of the cap site but retaining the TATA box (pS320.12Cn) yielded moderate levels of CAT activity. Removal of the TATA box region in the native orientation (pS320.33) reduced CAT activity to background levels.

In contrast, when these three fragments were inserted in the reverse orientation, the removal of native downstream sequences, the leader, and TATA box region to −33 yielded the highest levels of divergent CAT activity (approximately 40 pmol/h per μl). Maintenance of the native TATA box (pS12.320pCn) in the DNA insert truncated at −12 gave lower levels of activity (approximately 6 pmol/h per μl). The retention of sequences downstream of the native TATA box inhibited divergent CAT transcriptional activity. These results suggest that proximal sequences, including the TATA box and immediate 3'-flanking downstream sequences, function to orient transcription from the symmetrical skeletal α-actin promoter region. In the presence of the appropriate *trans*-acting factors, these proximal sequences may control the levels of divergent transcriptional activity in transfected myogenic cells.

Delimitation of the 3' regulatory border. The capacity of the skeletal α-actin promoter to drive transcription in the reverse orientation was used to delimit the boundary of the 3' *cis*-acting transcriptional regulatory elements without the introduction of foreign promoter sequences. A total of nine 3' deletion constructs, progressively removing sequences from −12 to −202 and linked to *cat* at −320, were generated and inserted into a pSV0CAT (pCn) vector truncated at the *Nde*I site (Fig. 7).

The 3' regulatory boundary of the downstream region of the α-actin gene required for the induction of CAT expression during late myoblast differentiation was indicated from the experimental data summarized in Fig. 7. Deletion of sequences to −30 removed the native TATA box and stimulated divergent transcriptional activity by twofold. Elimination of nucleotides to −47, which deleted one of two tentative Sp1 sites (at −36 to −41 and −47 to −52), further potentiated CAT transcriptional activity to a level fourfold over that of the chimeric DNA retaining the native TATA

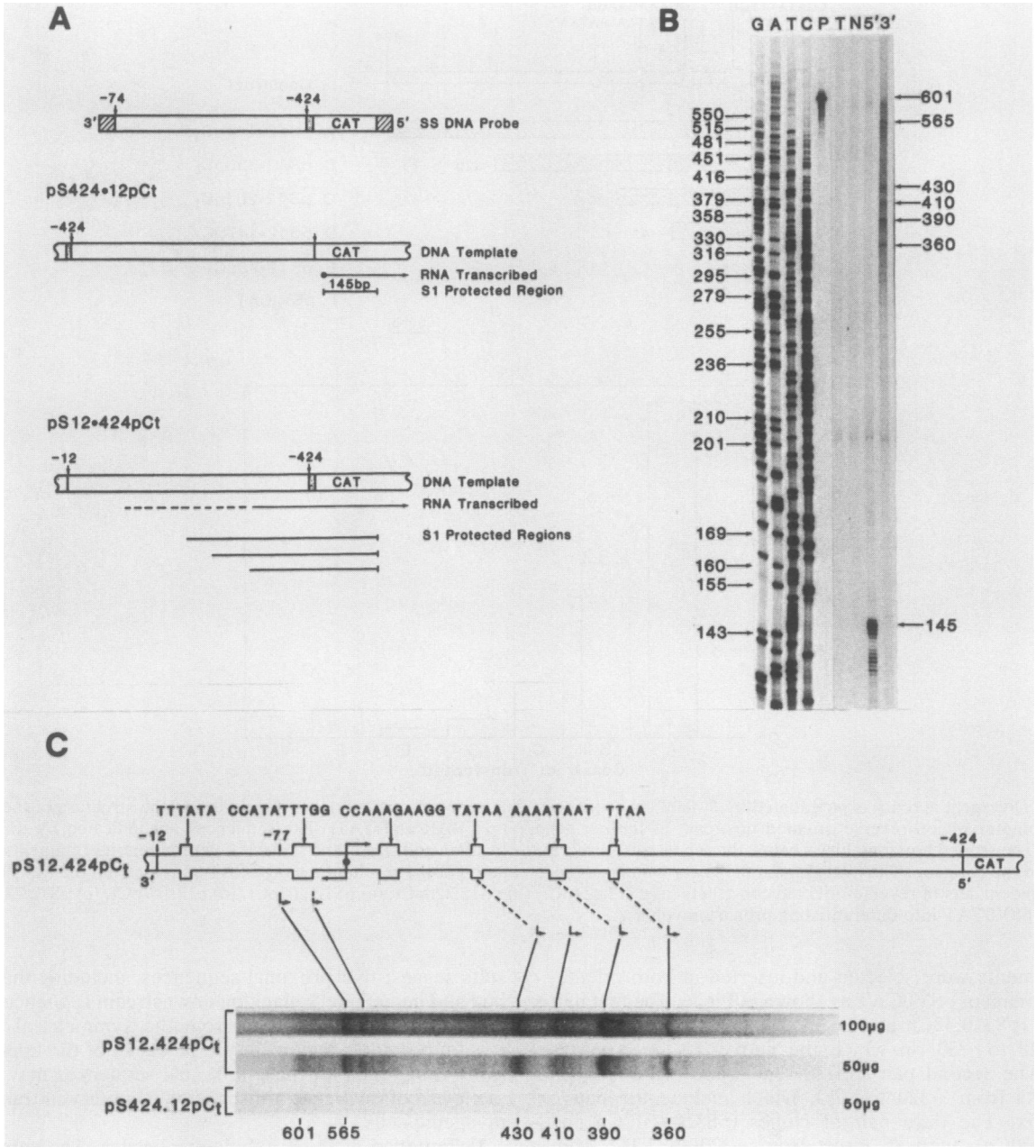

FIG. 4. Localization of transcript start sites by nuclease S1 analysis. (A) Schematic representation of the uniformly labeled single-stranded probe, the constructs pS424.12pCt and pS12.424pCT, with their respective RNA transcripts and regions of probe protection. The striped regions at either side of the 601-bp probe represent 41 and 36 bp of nonhomologous sequence at its 5′ and 3′ ends, respectively. (B) Total RNA isolated from transfected cells was hybridized, nuclease S1 digested, and electrophoresed on a 6% polyacrylamide gel as described in the text. The first four lanes, labeled G, A, T, and C, include DNA fragment length markers generated by sequencing of the XXP3.10 probe. Lane P is the undigested probe, followed by nuclease S1 digestion of probe hybridized with tRNA (T) and RNA from nontransfected myotubes (N). Myotubes transfected with pS424.12pCt in the native orientation are labeled 5′, and myotubes transfected with pS12.424pCt in the reverse orientation are labeled 3′. The sizes of the DNA fragments (in nucleotides) are indicated to either side of the gel. (C) Schematic diagram of the reverse-oriented pS12.424pCt construct. This diagram shows the sequences of the CBAR elements and additional A+T-rich regions. The upper portion of the sequencing gel was expanded, and the nuclease S1-protected bands were mapped. Their locations are indicated by the bent arrows. Three of these bands mapped approximately 30 bp downstream from A+T-rich regions (represented by the dashed lines).

box (pS12.320pCn). These results demonstrate that sequences including the native TATA box as well as the surrounding regions bordered from −47 to at least the cap site may play an important role in restricting divergent transcriptional activity.

Further deletions into the core promoter from the 3′ border reduced divergent transcriptional activity. Removal of sequences to −74, in clone pS74.320pCn, resulted in a 35% decrease of activity in comparison with the maximal activity induced by pS47.320pCn. Removal of an additional 13 nucleotides to −87 (pS87.320pCn) bisected the native downstream CBAR and resulted in a marked 4.5-fold reduction in *cat* transcriptional activity. Transient activity levels remained low, although small fluctuations were observed in

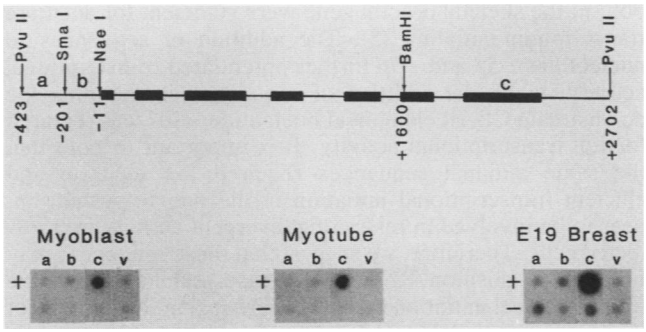

FIG. 5. Nuclear runoff transcription analysis of divergent activity. A restriction map indicating the subcloned fragments (a to c) and their locations relative to the seven exons of the chicken skeletal α-actin gene is illustrated at top. Autoradiographs of dot-blots are shown below, where a to c indicate the subcloned fragments and v indicates the M13mp18 vector with no insert. The strandedness of the nuclear transcripts responsible for the signal relative to native skeletal α-actin transcription is indicated at left with + or − signs. Radiolabeled nuclear transcripts were prepared by the method of Linial et al. (32) with nuclei isolated from chicken primary cell cultures at 36 h (myoblast) and 92 h (myotube) postplating as well as from chicken embryonic day 19 breast muscle (E19 breast).

sequences deleted to −87, -99, and −150. Deletions to the end of the core promoter, at −202, gave levels essentially equivalent to background CAT activity.

These experiments demonstrated that disruption of sequences including the downstream CBAR motif resulted in a marked decrease in transcriptional activity. These findings mirror those of the 5′ deletion series, in which removal of the upstream CBAR element resulted in a five- to sevenfold reduction in transcriptional activity (5) (Fig. 8). As presented above, these CBAR sequences form part of the symmetrical elements in the skeletal α-actin promoter and appear to play a critical role in potentiating transcription of the sarcomeric actin genes.

DISCUSSION

Bidirectional transcriptional activity has been associated with gene promoter regions in procaryotes (31, 43), mitochondria (14), *Saccharomyces cerevisiae* (48), viruses (19), and cellular genes (11, 27, 53). These bidirectional promoter regions support transcripts which are expressed constitutively (11, 48) as well as in a tissue-specific (27) and constitutive manner (19, 43, 48). Stimulation of divergent transcriptional activity from a gene promoter region may also be a consequence of mutagenesis of regulatory promoter elements. In the case of the adenovirus *IVa2* gene, which is separated from the major late promoter (MLP) cap site by

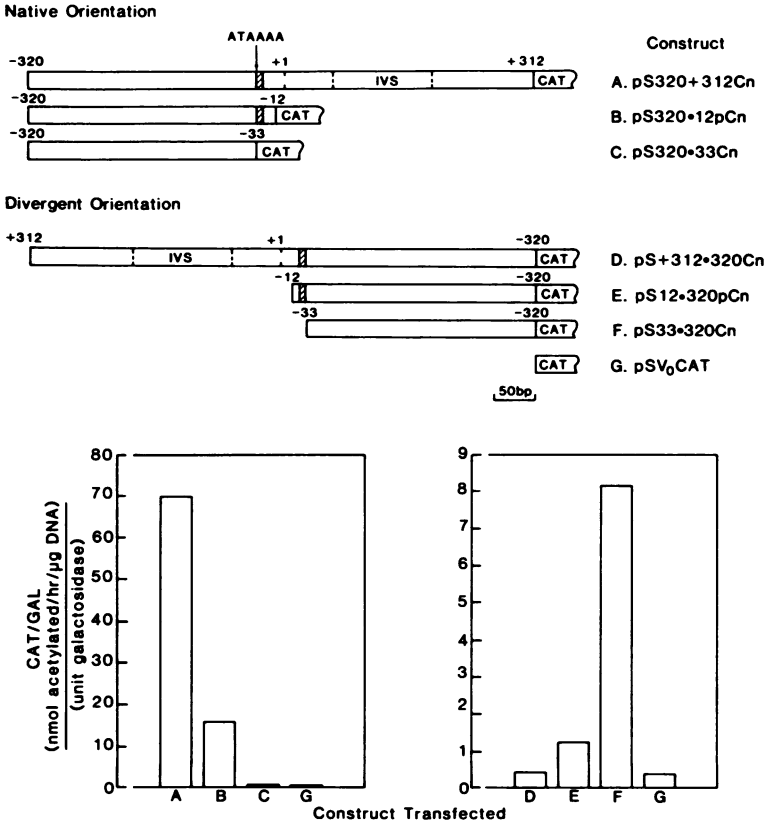

FIG. 6. Intact proximal sequences inhibit the detection of divergent transcriptional activity. The orientation, size, and location of the skeletal α-actin fragments upstream of *cat* are illustrated. The upper three constructs were positioned in the native orientation 5′ to 3′ upstream of the *cat* gene. The second set of constructs were oriented 3′ to 5′ upstream of the *cat* gene. The position of the native TATA box is indicated by the striped region. The first intervening sequence (IVS) is also indicated in the pS320+312Cn and pS+312.320Cn constructs. The histogram below the construct schematics shows the average resultant CAT activity from the transfection of 5 μg of two independent sets of plasmid constructs.

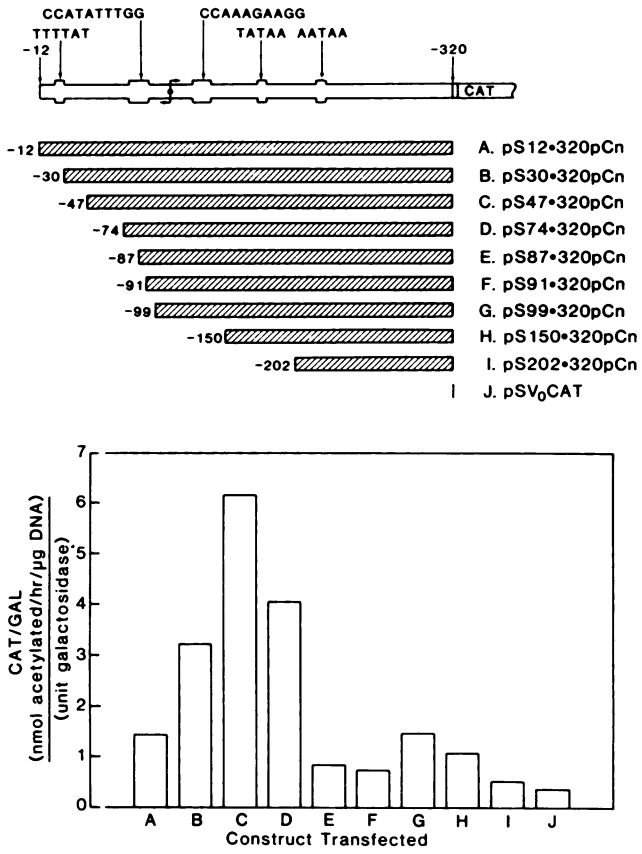

FIG. 7. Sequences retaining the most-proximal CBAR element potentiate divergent transcriptional activity. The reverse-oriented skeletal α-actin promoter fragment upstream of the *cat* gene is illustrated schematically at the top of the figure. The sequences of the CBAR and TATA-like elements are shown. The size and location of the 3′ deletions are represented by the striped horizontal bars. A histogram in the lower panel depicts the average resultant CAT activity from the transfection of two sets of the constructs (a) pS12.320pCn, (b) pS30.320pCn, (c) pS47.320pCn, (d) pS87.320pCn, (e) pS91.320pCn, (f) pS91.320pCn, (g) pS99.320pCn, (h) pS 150.320pCn, (i) pS202.320pCn, and (j) pSV0CAT.

210 bp and transcribed from the opposite strand, suppression of *IVa2* gene transcription in vitro is relieved when the region containing the MLP cap site is deleted (40). Similarly, in the SV40 promoter the removal of the 21-bp repeats from the early promoter and enhancer regions increased the levels of expression from the late promoter (42). As shown here, the detection of divergent activity from the skeletal α-actin core promoter region was also dependent on the removal of sequences in the vicinity of the native transcription start site.

Stimulation of divergent activity from the skeletal α-actin promoter region coincided with the deletion of downstream sequences. The sequences which were primarily involved in inhibiting divergent activity in the skeletal α-actin promoter region bordered 3′ of nucleotide −47 (Fig. 8). They included the transcription start site, the TATA box, and a potential Sp1-binding site spanning nucleotides −41 to −36. Immediately 5′ of this region an additional potential Sp1-binding site spanning nucleotides −52 to −47 and a skeletal α-actin conserved sequence CATTCCT. . .GGGC, can be identified. Transcription activity assays in the constitutive *Xenopus* oocyte system revealed that retention of sequences downstream of nucleotide −31, which included the TATA box, in the skeletal α-actin gene were sufficient for accurate transcription initiation (5). The addition of sequences to nucleotides −55 and −76 further potentiated transcriptional activity, while the addition of sequences surrounding the downstream CBAR element at nucleotide −107 was required for full transcriptional activity. It is important to note that the same minimal sequences required for accurate and efficient transcriptional initiation in the oocyte system appear to be involved in restricting divergent activity in transfected cells. Therefore, we suggest that these sequences may function to position RNA polymerase and help form the transcriptional initiation complex. Perhaps, in the absence of these sequences, RNA polymerase sequestered by orientation-independent transcriptional factors might search for alternative start sites, such as those shown in Fig. 4, which have similarities with TATA sequences.

Hybridization measurements have indicated the apparent lack of divergent transcripts in steady-state RNA isolated from embryonic chicken tissues. However, nuclear runoff transcription assays allowed us to detect DNA strand-specific divergent transcripts in nuclei isolated from myogenic cell cultures and embryonic muscle. These divergent transcripts represent only a minor portion of the total transcriptional activity of the skeletal α-actin gene. This observation is consistent with the reduced level of divergent transcriptional activity in cells transfected with chimeric constructions which retained the native cap site. Thus, divergent transcription might actually be inhibited in vivo due to the presence of *cis*-acting restricting sequences which surround the native initiation start site. We conclude that divergent transcription may occur in vivo, but with rapid turnover of nuclear transcripts.

The capacity of the skeletal α-actin promoter to drive transcription in the divergent orientation allowed us to map the 3′ regulatory border without the use of an alternative promoter. As shown in Fig. 7, the largest decrease in divergent transcriptional activity correlated with the disruption of the −95 bp downstream CBAR sequence. The critical nature of these sequences was first identified by gene transfection experiments with mutant skeletal α-actin promoters serially deleted at the 5′ border (5) (Fig. 8). In these experiments a major decrease in native-oriented transcriptional activity was detected following the deletion of the upstream −123 CBAR element. While other sequences in this promoter region were shown to be required for full regulatory activity, it appears that transcription in either orientation is highly dependent on sequences harboring both CBAR sequences. The CBARs are a highly conserved subset of 16-bp elements (Fig. 9) which are also included in a more general consensus sequence described as CArG boxes (37). Sequence analysis of vertebrate striated α-actin gene promoter sequences revealed that the CBAR element presented in Fig. 9 has maintained a high degree of sequence conservation through at least 300 million years of evolution. These sequences appear in similar locations in the sarcomeric α-actin promoters; one CBAR sequence is located approximately 80 bp from the initiation of transcription, and the second CBAR is in the reverse orientation and located approximately 150 bp upstream for skeletal and 200 bp (best homology) for cardiac α-actin. In addition, mutagenesis of the human cardiac α-actin gene promoter region (37, 38) has shown the important nature of these CBAR elements in potentiating transcription.

The CBAR motif bears a striking resemblance to the canonical CCAAT box pentanucleotide sequence located approximately 80 bp upstream of the mRNA cap site in many

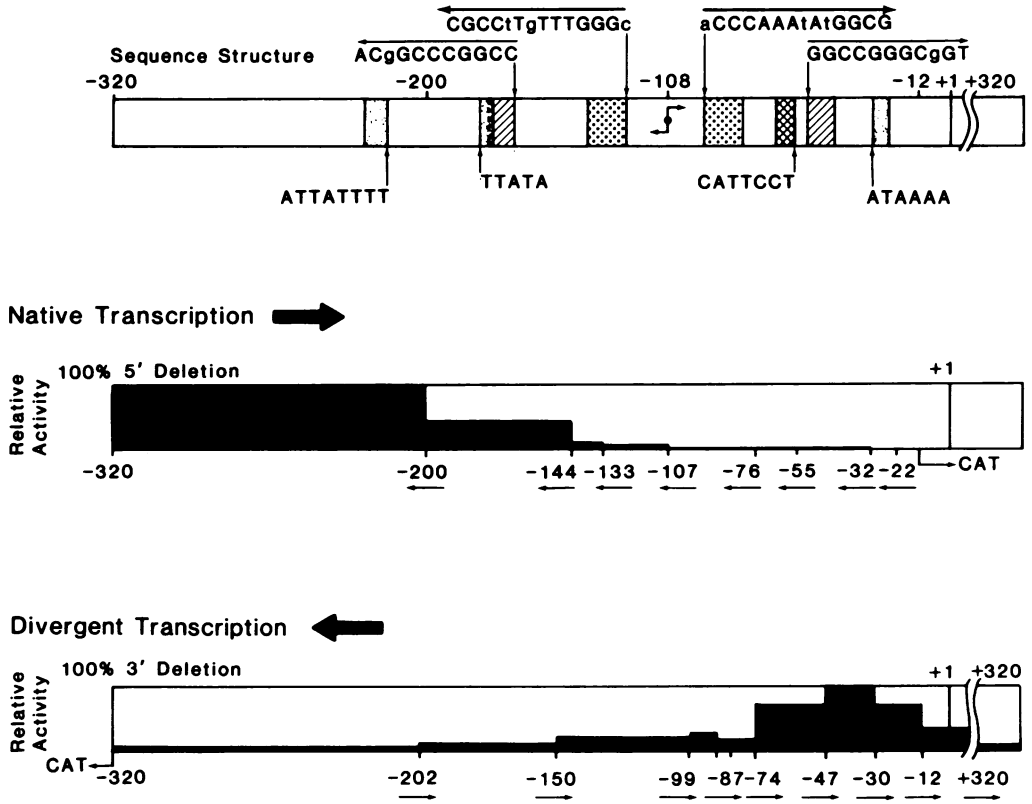

FIG. 8. Analysis of transcriptional promoter sequences. The sequence structure depicted in the top bar includes the central axis of symmetry at −108, the location of the CBAR elements (thick arrows), the location of the conserved CGGGC(C/G)GT sequence, tentative TATA sequences, and a conserved CATTCCT sequence. The second part of this figure summarizes the results of the deletion analysis. The middle bar depicts the effect of 5′ deletions on native transcription (5). The activity of the −202 clone was designated 100% (full) activity and is indicated by the height of the darkened region to the left of the deletions. The lower bar depicts the effect of the 3′ deletions on divergent transcription. The activity of the −47 clone (pS47.320pCn) was designated 100% activity and is indicated by the height of the darkened region to the right of the deletions. Activity for the native orientation is illustrated to the left of the construct number, while activity for the divergent orientation is illustrated to the right of the deletion nucleotide.

RNA polymerase II-dependent promoters. Sequences including this CCAAT box element have been shown to influence the efficiency of mRNA synthesis in several eucaryotic genes. Recently, at least three different proteins have been identified which specifically interact with the CCAAT box motif (12, 21, 30). Additionally, potential negative CCAAT-binding factors have been identified in extracts from nonexpressing cells for both histone H2b-1 (2) and rat albumin (8). The heterogeneous nature of this class of binding proteins has led us to suspect that an additional CCAAT box-binding factor(s) might exist which interacts preferentially with the myogenic CBAR sequence. DNase protection (P. Fong, unpublished data) and methylation interference footprints (52; P. Fong, unpublished data) have shown that the CBAR elements in the skeletal α-actin promoter are bound by a factor. Walsh and Schimmel (52) have shown that at least two different factors may bind to the downstream CBAR. It will be important to determine whether CBAR-binding factors are enriched in myogenic tissues and whether other CCAAT box-binding factors are present and have a regulatory role in determining the transcriptional activity and specificity of the skeletal α-actin gene promoter.

Deletion-transfection analysis indicated that skeletal α-actin promoter sequences including both CBAR sequences must be retained to potentiate transcriptional activity. Additional studies on the human cardiac α-actin gene have also

SKELETAL α- ACTIN					
CHICKEN	+	(- 95)	ACac	CCAAATATGG	CG (- 80)
	-	(-123)	CTGc	CCAAAgAAGG	CG (-138)
RAT	+	(- 96)	ACac	CCAAATATGG	Ct (- 81)
	-	(-155)	CTGA	CCAAAgAAGG	AG (-170)
MOUSE	+	(-100)	ACac	CCAAATATGG	Ct (- 85)
	-	(-163)	CTGA	CCAAAgAAGG	AG (-178)
FROG	+	(- 97)	CTGt	CCAAATATGG	AG (- 82)
	-	(-150)	GTGA	CCAAATATaG	tG (-165)
CARDIAC α-ACTIN					
CHICKEN	+	(- 94)	CCGG	CCAAATAAGG	AG (- 79)
	-	(-197)	ATGG	CCAAATAgGG	AG (-212)
HUMAN	+	(-113)	GgGA	CCAAATAAGG	Ca (- 98)
	-	(-224)	ATGG	CCAAATAgGG	AG (-239)
MOUSE	+	(-115)	GCGA	CCAAATAAGG	Ca (-100)
	-	(-227)	ATGG	CCAtATAgGG	AG (-242)
FROG	+	(- 94)	GCtA	CCAAATAAGG	gc (- 79)
	-	(-214)	ATGG	CCAAATAgGG	At (-229)
CONSENSUS			UYGU	CCAAATA(A/T)GG	(A/C)G

FIG. 9. Sequence analysis of the conserved CBAR element. The orientation, location, and sequence structure of the conserved CBAR elements of the chicken (5, 15), rat (55), mouse (29), and frog (49) skeletal α-actin and chicken (9), human (37), mouse (37), and frog (49) cardiac α-actin gene promoters are illustrated. The + or − before the nucleotide sequence indicates orientation. The central region of 10 nucleotides which are the most highly conserved is boxed. Below the sequences is the consensus sequence derived from the 16 sequence motifs, where U denotes purine nucleotides and Y denotes pyrimidine nucleotides. The nucleotides which do not fit the consensus sequence are indicated as lowercase letters.

suggested mutually dependent roles (38). The requirement for two or more copies of a transcriptional element appears to be a common feature in many genes. This synergistic effect of two or more regulatory elements is clearly demonstrated in transcriptional studies in which synthetic nucleotides containing regulatory sequences are reintroduced into promoter sequences (16, 39, 45). Two or more regulatory elements may enhance specificity though cooperative interaction to facilitate a protein DNA structure or conformation which might enhance polymerase entry into the promoter. Thus, cooperative interactions may be generated by pairs of CBAR elements and may constitute an orientation-independent regulatory core of the skeletal α-actin gene promoter. Core regulatory factors may potentially elicit an orientation-independent localization by binding the repeating heptapeptide C-terminal "tail" of the large subunit of RNA polymerase (1, 3). Since the inhibition of divergent activity in the skeletal α-actin promoter region required sequences which included the cap and TATA sites, these proximal sequences may serve to drive an orientation-dependent transcriptional event. Therefore, the entry of RNA polymerase and formation of the initation complex in the skeletal α-actin gene may involve these two levels of control; the first is the attraction of RNA polymerase by *trans*-acting factors bound to orientation-independent core regulatory sequences, and the second level of control functions to position RNA polymerase to a transcription start site.

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