Multiple 5'-Flanking Regions of the Human α -Skeletal Actin Gene Synergistically Modulate Muscle-Specific Expression

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Transfection into myogenic and nonmyogenic cell lines was used to investigate the transcriptional regulation of the human α -skeletal actin gene. We demonstrated that 1,300 base pairs of the 5'-flanking region directed high-level transient expression of the bacterial chloramphenicol acetyltransferase gene in differentiated mouse C2C12 and rat L8 myotubes but not in mouse nonmuscle L.TK⁻ and HuT-12 cells. Unidirectional 5' deletion analysis and heterologous promoter stimulation experiments demonstrated that at least three transcription-regulating subdomains lie in this 1,300-base-pair region. A proximal *cis*-acting transcriptional element located between positions -153 and -87 relative to the start of transcription at +1 was both sufficient and necessary for muscle-specific expression and developmental regulation during myogenesis in the two myogenic cell systems. The region 3' of position -87 interacted with factors present in both myogenic and fibroblastic cells and appeared to define, or to be a major component of, the basal promoter. In C2C12 myotubes, but not in L8 myotubes, a distal sequence domain between positions -1300 and -626 and the proximal sequence domain between positions -153 and -87 each induced transcription about 10-fold and synergistically increased CAT expression 100-fold over levels achieved by the sequences 3' of position -87. Furthermore, these *cis*-acting elements independently and synergistically modulated an enhancerless, heterologous simian virus 40 promoter in a tissue-specific manner. DNA fragments which included the proximal domain displayed classical enhancerlike properties. The central region between positions -626 and -153, although required in neither cell line, had a positive, two- to threefold, additive role in augmenting expression in L8 cells but not in C2C12 cells. This suggests that certain elements between positions -1300 and -153 appear to be differentially utilized for maximal expression in different myogenic cells and that the particular combination of domains used is dependent on the availability, in kind or amount, of *trans*-acting, transcription-modulating factors present in each cell type. Thus, multiple myogenic factors that vary qualitatively and quantitatively may be responsible for the different and complex modulatory programs of actin gene expression observed during *in vivo* muscle differentiation.

The fusion of proliferating myoblasts to form myofibers is accompanied by the developmental regulation of genes encoding a structurally diverse group of proteins that form the muscle sarcomere. These events are characterized by a sequence of isoform transitions typified by the actin multigene family. During differentiation of muscle tissue, the β - and γ -cytoskeletal actins are down-regulated. In contrast, the sarcomeric α -cardiac and α -skeletal actins, which are coexpressed in adult human heart and skeletal muscle, are synthesized in large amounts (3, 13, 45, 48) but with distinct tissue-specific and developmental patterns of expression (26, 40).

During development, α -cardiac actin appears to be the equivalent of a fetal isoform in both skeletal muscle and heart (40). In adult human skeletal muscles, the α -skeletal actin isoform predominates, but the α -cardiac mRNA represents 5% of the sarcomeric actin transcripts (26). Similarly in adult heart muscle, cardiac actin is the predominant isoform, but the proportion of skeletal actin present seems related to the size of the mammal studied (26, 38, 60). Second, primary chicken (29) and human (25a) myoblasts differentiating *in vitro* have highly similar patterns of actin isoform mRNA accumulation. Cardiac actin mRNA predominates, but skeletal actin mRNA is transiently present at moderate levels and subsequently at 10 to 15% of adult muscle levels. Finally, in the frequently studied, immortalized, rodent

myogenic cell lines C2C12, L8, and L6 the patterns of sarcomeric actin mRNA accumulation differ substantially and are different from the patterns seen in primary cells. For example, the cardiac actin transcripts that accumulate to extraordinary levels in C2C12 cells (3) are not detectable in L6 cells (31) and accumulate to 10 to 15% of adult levels in L8 cells (41). In contrast to this variation in amounts of cardiac actin mRNAs, the skeletal actin transcripts in these cell types have similar patterns of accumulation and slowly rise to 10 to 15% of adult levels (3, 29, 31).

The results of transfection experiments have demonstrated that sequences upstream of the transcription start site of the rat (39) and chicken (24) skeletal actin gene were sufficient for both stage- and tissue-specific expression. Bergsma et al. (5) delimited the 5' boundary of a *cis*-acting regulatory element in the chicken α -skeletal actin gene to be 200 base pairs (bp) upstream of the mRNA cap site. Included in this regulatory element is a *cis*-acting transcriptional control region having a 5' border 107 bp before the RNA cap site. These data depicted a noncomplex promoter, but this is not consistent with the complex pattern of expression of skeletal actin mRNA in different cell types and during differentiation. Nor would one expect a simple promoter for the α -skeletal actin gene in view of the complexity of the 5'-flanking regulatory sequences of its closely related cognate, the α -cardiac actin gene (41-43; T. Miwa, L. Boxer, and L. Kedes, *Proc. Natl. Acad. Sci. USA*, in press). Furthermore, a number of recent studies on other tissue-

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specific mammalian promoters have emphasized their general complexity by identifying *cis*-acting elements which mediate both positive (59) and negative (33, 46) modulation of transcription and that span many kilobases of 5'-flanking sequences.

To reexamine the nature of the α -skeletal actin gene promoter and to contrast it with that of the α -cardiac actin gene, we have characterized the upstream regions of the human α -skeletal actin gene, which has recently been isolated and sequenced in our laboratory (A. Taylor, H. Erba, and L. Kedes, manuscript in preparation). We created deletions to span a large region of 5'-flanking DNA with the view to evaluating whether multiple sequence elements could account for the different expression patterns observed in primary and immortalized cell populations. Our results demonstrate that the α -skeletal actin gene promoter is complex and large, spanning at least 1,300 bp. At least three segments of this region, when placed adjacent to an enhancerless simian virus 40 (SV40) promoter, act as regulatory domains which respond to muscle-specific factors for positive modulation of transcription.

Surprisingly, several of these elements exert different degrees of regulation in different myogenic cell lines. The data show that the general organization of the human α -skeletal actin gene promoter is similar to that of the α -cardiac actin gene promoter in that both genes are modulated by physically separated *cis*-acting segments. At least one of these segments contains an element in common, the CAR_G sequence motif described by Minty and Kedes (42), which is now known to be required for muscle-specific α -cardiac actin gene expression (43; Miwa et al., in press). The differences in abilities of the α -skeletal actin regulatory domains to positively modulate expression in different cell lines suggests that the particular combination of domains used is dependent on the availability, in kind or amount, of *trans*-acting transcription-modulating factors present in each cell type. Thus, multiple myogenic factors that vary qualitatively and quantitatively may be responsible for the different and complex modulatory programs of actin gene expression observed during muscle differentiation.

MATERIALS AND METHODS

Cells and tissue culture. The origins of myogenic and fibroblastic cell lines and their culture conditions have been described previously (42).

Plasmid construction. In general the procedures suggested by Maniatis et al. (36) were followed. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and phosphorylated *Hind*III and *Xho*I linker DNA fragments were purchased from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals. A pBR322 plasmid carrying the human skeletal actin gene on a 9.5-kilobase *Hind*III fragment (Taylor et al., in preparation) was digested with the restriction endonucleases *Hind*III and *Dra*I. A 2,250-bp DNA fragment starting at a *Hind*III site 2,000 bp upstream from the site of transcription initiation (28) contains the first exon and 149 bp of the first intron and ends at the *Dra*I site. We ligated *Hind*III linkers to this DNA fragment and then digested it with *Hind*III. Then a number of subfragments were generated by cleavage with *Xba*I, *Pvu*II, *Sac*I, *Ssp*I, *Sma*I, or *Xho*I, which cleaved at position -1300, -710, -626, -432, -153, or -87 respectively, relative to the start of transcription (Fig. 1). The 2,250-bp *Hind*III-*Dra*I fragment, which contains 2,000 bp of upstream sequence, was cloned into the

*Hind*III-digested and calf intestinal alkaline phosphatase-treated pHCA0CAT vector (42) and is designated as pHSA2000CAT (Fig. 1). pHCA0CAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene and the splicing and polyadenylation signals from pSV2CAT (22) substituted for the *Hind*III-*Bam*HI fragment of pBR322. In transient expression assays after calcium phosphate-mediated DNA transfection, the plasmid pHCA0CAT yields a lower background CAT activity than pSV0CAT (22). The series of deletion fragments starting at the *Xba*I, *Pvu*II, and *Sac*I sites was cloned into a corresponding set of unique sites which had been created by insertion of an mp18 polylinker between the *Eco*RI and *Hind*III sites of the vector pHCA0CAT. The resulting plasmids were designated as pHSA1300CAT, pHSA710CAT, and pHSA626CAT, respectively. The deletion fragments starting at *Ssp*I and *Sma*I sites were cloned into the vector pHCA0CAT which had been cleaved with *Cla*I, treated with T4 DNA polymerase to create blunt ends, and then cleaved with *Hind*III. These plasmids were designated as pHSA432CAT and pHSA153CAT. The fragment starting at the *Xho*I site was cloned into the pHCA0CAT vector containing the polylinker in the following way. First the polylinker was cleaved with *Sma*I, and then *Xho*I linkers were ligated to these blunt ends and digested with *Hind*III and *Xho*I. This plasmid was designated as pHSA87CAT.

Fragments of different sizes, which were used to modulate the heterologous SV40 promoter, were generated by cleavage of the α -skeletal actin promoter at the sites previously

FIG. 1. Prototype recombinant plasmid used for the various transfections (pHSA2000CAT). A DNA fragment containing 2,000 bp of human skeletal upstream sequence plus the first exon and 147 bp of the first intron was cloned into the vector pHCA0CAT previously described by Minty and Kedes (42). A restriction map of the fragment is included as a guide to the relative positions of the 5' deletions. IVS1, Intervening sequence 1.

used to create the 5' deletions; the fragments were treated with T4 DNA polymerase or Klenow DNA polymerase I to produce blunt ends, ligated to *Bgl*II linkers, and cloned into the *Bgl*II or *Bam*HI site in pA10CAT.2N (45).

Plasmid DNAs were prepared from cleared bacterial lysates by banding on ethidium bromide-cesium chloride gradients. All preparations of plasmid DNA used for quantitative CAT assays were purified on two successive gradients, a step which ensures reproducibility of transfection efficiency (42).

Cell transfection and CAT assays. Cells were transfected by the calcium phosphate precipitation method (23) with 5 μ g of DNA. The CaPO_4 -DNA precipitate was allowed to form for 30 min at room temperature and then diluted in the cell culture medium. The C2C12, L, and HuT-12 cells were incubated for 16 h; the L8 cells were incubated for only 4 to 6 h since they did not remain viable for longer periods. The medium was then changed to normal growth medium for fibroblastic cells or growth-fusion medium in the case of myogenic cells, and the cells were left for a further 24 to 120 h depending on the cell type or myogenic stage required as described previously (41, 42). CAT activity was measured by the method of Gorman et al. (22). Samples of the cell extracts in 150 μ l with 0.1 to 0.4 μ Ci of [14 C]chloramphenicol (New England Nuclear Corp.) in the presence of 4 mM acetyl coenzyme A and 0.25 M Tris hydrochloride (pH 7.8) were incubated at 37°C. After 2 to 4 h of incubation for C2C12, L, and HuT-12 cells and 8 h for L8 cells (because of the relatively low transfectability of L8 cells), the reaction was stopped by the addition of 1 ml of ethyl acetate, used to extract the chloramphenicol and its acetylated forms. The extracted materials were analyzed on Silica gel thin-layer chromatography plates as described by Gorman et al. (22). Quantitation of CAT assays was performed by scintillation counting of the chromatograms.

Except where noted otherwise, the results obtained with the human α -skeletal actin gene CAT constructs were normalized to that of the pH β APr-1-CAT (25b) construct set to 100%. All CAT activity values were normalized to the protein concentration of each lysate. The pH β APr-1-CAT was a convenient benchmark, since fused β -actin-CAT genes have been shown to direct relatively high levels of CAT activity which did not change upon differentiation in chicken (24) and rodent (39) myogenic cultures; on the other hand pSV2CAT activity has been shown to decrease (24) and increase (39, 42) during myogenesis in chicken and rodent myoblasts, respectively. In addition we included in our transfection series the well-characterized promoter-CAT construct from the human α -cardiac actin gene, pHCA485CAT (42), which contains 485 bp of human α -cardiac actin upstream sequence plus the first exon and 24 bp of the first intron linked to the CAT gene. This promoter region directs muscle-specific gene expression (42) and is inducible during myogenic differentiation (41). A control construct, pHCA0CAT (42), served as the baseline indicator of CAT activity in transfected cells, since it contains the CAT gene in the exact same plasmid as all the other constructs but in the absence of any eucaryotic promoter sequences. To overcome the variability inherent in such transient transfection assays we have performed these experiments several times (six times for most plasmid constructs with a minimum of three).

RNA preparation and S1 nuclease analysis. For RNA preparations from cultured rodent cells, cells were lysed as described by Minty et al. (41) in the presence of 10 mM ribonucleoside-vanadyl complexes. Nuclei were collected

by centrifugation as described by Minty and Kedes (42), and cytoplasmic RNA was prepared by cold phenol-chloroform extraction. The 584-bp *Xho*I-*Eco*RI DNA probe was dephosphorylated with calf intestinal alkaline phosphatase before end labeling with [γ - 32 P]ATP (aqueous, >3,000 Ci/mmol) and polynucleotide kinase. Labeled DNA was separated from unincorporated nucleotides by two successive ammonium acetate precipitations. The hybridization, S1 nuclease digestion, and electrophoretic conditions were as described previously (42).

In vitro transcription. Promoter-containing eucaryotic DNA templates were transcribed in vitro in a eucaryotic transcription system (Bethesda Research Laboratories, Inc.) prepared from HeLa cells by the method of Manley et al. (37). As noted with adenovirus genes (37), accurate transcription of the human α -skeletal actin gene in these whole cell extracts was only observed at concentrations of template DNA in the range of 40 to 80 μ g/ml of lysate.

RESULTS

Muscle-specific transcription of the human α -skeletal actin gene depends on upstream flanking sequences. Previous work from this laboratory (41–43; Miwa et al., in press) on the regulation of transcription of the human α -cardiac actin gene, as well as work from several laboratories on vertebrate α -skeletal actin genes (5, 24, 39, 40), has shown that the flanking sequences 5' to the transcription initiation site are responsible for the tissue-specific expression of these genes. Thus we inferred that 5'-flanking sequences of the human α -skeletal actin gene would also prove critical for its transcription. In addition, the nucleotide sequence of the human α -skeletal actin gene is 80% similar to those of the mouse and rat for at least 200 bp of its 5'-flanking sequences (Taylor et al., in preparation). Furthermore, these upstream sequences include a TATA (7) and a CCAATATGG sequence at positions –30 to –26 and –98 to –88, respectively, relative to the start of transcription at +1 (28). The CC(A/T)GG sequence has been designated as the CAAT box (4, 17) of the rat and chicken skeletal actin genes (5, 50). However this sequence matches poorly with the consensus CAAT sequence (GGC/TCAATCT) (4), but does conform to the CArG box motif, which, as we have described (41–43; Miwa et al., in press), is critical in muscle-specific transcription of the α -cardiac actin gene.

To test the supposition that tissue-specific transcription of the human α -skeletal actin gene is regulated by upstream sequences, we examined the ability of these sequences to direct the expression of the CAT gene in muscle and nonmuscle cells. For this purpose we constructed a plasmid (pHSA2000CAT) containing 2,000 bp of human skeletal actin 5'-flanking sequence plus the first exon and 149 bp of the first intron linked at the *Dra*I site at position +239 to the CAT gene (Fig. 1). To monitor the transcription from the putative regulatory signals in this construct, we compared the CAT activity of cells transfected in parallel with two nonmyogenic promoter-CAT constructs: pSV2CAT, which contains the 21-bp repeats and the 72-bp enhancer regions of SV40 (22); and pH β APr-1-CAT, which contains a highly efficient promoter consisting of 3.0 kilobases of upstream sequences plus the first exon and the entire intervening sequence 1 of the human β -actin gene. These plasmids also acted as controls for differences in transfection efficiencies, plasmid and RNA stability, and overall transcription levels in the various cell lines tested (1). The results obtained with the human α -

TABLE 1. Levels of CAT activity in transient expression experiments

Plasmid	CAT activity (% of pH β APr-1-CAT activity $\pm$ SD) in ^a :			
	C2C12 myotubes	L8 myoblasts	L8 myotubes	L. TK ⁻ cells
pHCA0CAT	0.3 $\pm$ 0.1	4 $\pm$ 2	4 $\pm$ 2	0.6 $\pm$ 0.2
pSV2CAT	73 $\pm$ 8	15 $\pm$ 7	70 $\pm$ 20	70 $\pm$ 15
pHCA485CAT	60 $\pm$ 5	5.0 $\pm$ 1.5	20 $\pm$ 7	1.3 $\pm$ 0.7
pH β APr-1-CAT	100	100	100	100
pHSA2000CAT	94 $\pm$ 11	13 $\pm$ 5	100 $\pm$ 7	15 $\pm$ 4
pHSA1300CAT	85 $\pm$ 4	12 $\pm$ 7	80 $\pm$ 14	9.0 $\pm$ 1.5
pHSA710CAT	8.5 $\pm$ 2.5	NT	NT	NT
pHSA626CAT	6.7 $\pm$ 1.3	5.5 $\pm$ 0.7	65 $\pm$ 21	9.0 $\pm$ 1.5
pHSA432CAT	9 $\pm$ 3.6	8.5 $\pm$ 2.5	55 $\pm$ 7	7.5 $\pm$ 3.5
pHSA153CAT	6.5 $\pm$ 2.3	7.5 $\pm$ 2.5	33 $\pm$ 4	9.0 $\pm$ 1.5
pHSA87CAT	1.1 $\pm$ 0.4	7.5 $\pm$ 3.5	7.5 $\pm$ 0.8	6.5 $\pm$ 3.5

^a The values of CAT activity in extracts of cells transfected with the various deletions are expressed as percentages of the value obtained in extracts of cells transfected with pH β APr-1-CAT, the plasmid which was used as a benchmark in each experiment. NT, Not transfected.

skeletal actin gene CAT constructs were normalized to that of the pH β APr-1-CAT construct set to 100%. The pH β APr-1-CAT was a convenient benchmark, since fused β -actin-CAT genes have been shown to direct relatively high levels of CAT activity which did not change upon differentiation in chicken (24) or rodent (39) myogenic cultures. On the other hand, pSV2CAT activity, which we also measured, did not prove to be a useful benchmark, because its expression varied considerably as anticipated from the work of others who demonstrated its activity to decrease (24) or increase (39, 42) during myogenesis in chicken or rodent myoblasts, respectively. In addition we included in our transfection series the well-characterized promoter-CAT construct from the human α -cardiac actin gene, pHCA485CAT (41–43; Miwa et al., in press).

Transfection of pHSA2000CAT into myoblasts of the mouse C2C12 and the rat L8 myogenic cell lines, using the calcium phosphate precipitation method, led to high levels of transient CAT activity when these cells were allowed to differentiate into myotubes (Table 1) with respect to pHCA485CAT and pH β APr-1-CAT. In contrast, transfection of pHSA2000CAT into the mouse fibroblastic L.TK⁻ cell line led only to a basal level of CAT activity (Table 1) with respect to the two nonmyogenic promoter-CAT constructs, pSV2CAT and pH β APr-1-CAT. The level of CAT activity from pHSA2000CAT in the myogenic cell lines was 10-fold higher than that in the L-cell line. Furthermore, a recently established human fibroblastic cell line, HuT-12, does not express pHSA2000CAT, i.e., <1% of pH β APr-1-CAT (data not shown). These results indicate that the upstream region of the human α -skeletal actin gene contains sequences which are sufficient to confer high-level transcription and muscle-specific expression.

Multiple regions are important in the regulation of transcription. Alternative explanations for the tissue-specific transcription of the α -skeletal actin gene promoter construct would be the interaction of the upstream region with *trans*-acting positive-regulatory factors in muscle cells or with negative-regulatory factors in nonmuscle cells. To distinguish between these possibilities and to identify functional domains within the 5'-flanking sequences, we constructed a series of deletions starting at position -2000 and extending toward the site of transcription initiation (position +1) and

assayed their activity in the transient CAT expression system. The relative levels of CAT enzyme activity of these constructs in C2C12 myotubes are displayed in Fig. 2, and the data are summarized in Table 1.

The results of these deletion experiments in C2C12 cells reveal that the muscle-specific human α -skeletal actin gene promoter is complex and consists of at least three distinguishable domains that interact with *trans*-acting positive-regulatory factors. We have designated these regions as the distal (positions -1300 to -626), proximal (positions -153 to -87), and basal (3' end to position -87) domains. Deletion of the 5'-most 700 nucleotides (pHSA1300CAT) had a minimal effect on the level of CAT gene expression with respect to pHSA2000CAT (Fig. 2, lanes e and f). However, deletion of the distal segment (positions -1300 to -626) reduced CAT activity to levels 8- to 12-fold lower than those of pHSA2000CAT (Fig. 2, compare lanes g and e; Table 1). Further deletion to position -432 or -153 had no additional effect on CAT expression. However, deletion of the proximal segment to within 87 nucleotides upstream of the RNA initiation site led to another major loss of CAT activity (5- to 10-fold; Fig. 2, compare lane j with lanes g, h, and i). These results in C2C12 myotubes are plotted in Fig. 3 relative to the deletion breakpoints.

Such data suggest that at least two distinct domains, the distal and proximal, are important in the high-level transcription of the human α -skeletal actin gene in C2C12 cells. The distal region is required for maximal muscle specific transcription, whereas the proximal region supports significant but lower levels of expression, 8 to 10% of that supported by the β -actin gene promoter. Within the proximal region, deletion of nucleotides -153 to -87 removes the sequence CCAATATGG (-98 to -88), which is a typical CArG box motif known to be important in regulating tissue-specific transcription of the human cardiac actin gene (52–55; Miwa et al., in press). Deletion of segments which include this CArG sequence profoundly effect transcription (Fig. 3).

We determined that the nonmyogenic (i.e., basal) promoter sequences are primarily located within 87 bp of the transcription initiation site. The deletion series of constructs were transfected into the mouse fibroblastic L cells, and the

FIG. 2. Transcriptional activity of the upstream regions of the human skeletal actin genes in C2C12 myotubes. Plasmid DNA (5 μ g) was transfected into proliferating C2C12 myoblast cells by calcium phosphate precipitation. The C2C12 cells were allowed to fuse into multinucleated myotubes. Transient levels of CAT expression were determined for cell extracts after normalization of the extracts for protein as described in Materials and Methods. The 1- and 3-monoacetylated chloramphenicol (aaCM) were separated from unacetylated chloramphenicol by thin-layer chromatography and autoradiographed. Lanes: a, pHCA0CAT; b, pSV2CAT; c, pHCA485CAT; d, pH β APr-1-CAT; e, pHSA2000CAT; f, pHSA1300CAT; g, pHSA626CAT; h, pHSA432CAT; i, pHSA153CAT; j, pHSA87CAT. All lanes are from the same transfection experiment, enzyme assay, and autoradiograph, which was spliced to eliminate irrelevant data.

FIG. 3. Schematic representation of the transcriptional activity of upstream regions of the human skeletal actin gene in C2C12 and L8 myotubes. CAT activities were normalized to the activity of pHSA2000CAT in each cell line, which was taken as 100%. IVS, Intervening sequence.

results of CAT assays are shown in Table 1. The level of transcriptional activity from all the constructs was essentially similar and 7- to 12-fold lower than expression from β -CAT. We conclude that sequences from positions -2000 to -87 are not required for the activity of the human α -skeletal actin gene promoter in fibroblastic cells and that sequences downstream of position -87 appear to be sufficient to interact with factors in L cells, giving rise to modest levels of transcriptional activity.

Regulation during myogenic differentiation: identification of an essential *cis*-acting region. We next determined that the same sequence domains responsible for high-level, muscle cell-specific transcription were also required for increased expression of the α -skeletal actin gene during myogenic differentiation. This conclusion was reached by examining the ability of myogenic cells to modulate the expression of the various transfected human α -skeletal actin gene CAT constructs during differentiation from myoblasts to myotubes. The patterns of expression of the full-length α -skeletal actin gene CAT constructs proved to be similar to those we previously reported (41, 42) for the human α -cardiac actin CAT constructs. However, the activities of the deletion series were strikingly different in these two cell types, and

this disparate expression pattern had not been seen in our previous work.

In C2C12 cells, the plasmid pHSA2000CAT displayed high levels of transcriptional activity in dividing myoblasts comparable to that seen in differentiated myotubes in stably and transiently transfected cells (data not shown). We conclude that transcriptional factors sufficient for high-level skeletal actin gene expression are already present in C2C12 myoblasts before differentiation. However, such factors do not accumulate in L8 cells until myoblasts differentiate into myotubes. The data in Fig. 4 and Table 1 contrast the level of CAT enzyme activity of the deletion series in extracts of transiently transfected L8 cells in the myoblast and myotube stages. pHSA2000CAT up-regulated during differentiation approximately 10-fold with respect to β -CAT (compare lanes d and e in Fig. 4A and B). This construct is also developmentally regulated in stably transfected L8 cells (data not shown). This result paralleled that found by others for the rodent and chicken α -skeletal actin promoter-CAT constructs in transfected L8 (39) and chicken (24) primary myogenic cells. Our results and those discussed above parallel the reports by Seiler-Tuyns et al. (55) and Nudel et al. (47), in which the chicken skeletal actin gene, stably

FIG. 4. (A) Transcriptional activity of the upstream regions of the human skeletal actin genes in L8 myoblasts. Plasmid DNA (5 μ g) was transfected into proliferating L8 myoblast cells by calcium phosphate precipitation. The L8 myoblasts were harvested 24 h after transfection. Transient levels of CAT expression were determined by autoradiography as described in the legend to Fig. 2. Lanes: a, pHCA0CAT; b, pSV2CAT; c, pHCA485CAT; d, pH β APr-1-CAT; e, pHSA2000CAT; f, pHSA1300CAT; g, pHSA626CAT; h, pHSA432CAT; i, pHSA153CAT; j, pHSA87CAT. (B) Transcriptional activity of the upstream regions of the human skeletal actin genes in L8 myotubes. Plasmid DNA (5 μ g) was transfected into proliferating L8 myoblast cells by calcium phosphate precipitation. The L8 cells were induced to fuse into multinucleated myotubes. Transient levels of CAT expression were determined as described in the text. Lanes: a, pHCA0CAT; b, pSV2CAT; c, pHCA485CAT; d, pH β APr-1-CAT; e, pHSA2000CAT; f, pHSA1300CAT; g, pHSA626CAT; h, pHSA432CAT; i, pHSA153CAT; j, pHSA87CAT. All lanes are from the same transfection experiment, enzyme assay, and autoradiograph, which was spliced to eliminate irrelevant data.

integrated in C2C12 and L8 myogenic cells, respectively, was induced only during L8 cell (42) but not C2C12 cell (55) differentiation. These results reinforce the model of Minty et al. (41), which proposes that muscle-specific factors have already accumulated in C2C12 myoblasts before cell differentiation, in contrast to L8 cells in which the levels of such factor are low in myoblasts and increase during differentiation.

Deletion of both distal and proximal upstream sequences of the α -skeletal actin-CAT constructs had no apparent effect on their transcriptional activity in L8 myoblasts, i.e., all of the constructs express approximately 10% of the CAT activity driven by the intact β -actin promoter (Fig. 4A, compare lanes e through j with lane d; Table 1). In L8 myotubes, however, the transcription of all the α -skeletal actin gene promoter constructs—with the exception pHSA87CAT (Fig. 4B, lane j)—is upregulated with respect to that of β -CAT. Although the relative levels of transcription decrease as the deletions extend closer toward the site of RNA initiation (Fig. 4B, compare lanes e through j, with lane d; Table 1), even pHSA153CAT expresses about 35% of the activity of β -CAT.

The data derived from the deletion series highlight the importance of the proximal region between positions -153 and -87 in the upregulation of the human α -skeletal actin gene during muscle cell differentiation.

There are several distinct differences in the results obtained with the deletion series of C2C12 and L8 cells. First, the distal region between positions -1300 and -710 appears to contain an important *cis*-acting sequence element that interacts in C2C12 cells with constitutively expressed, muscle-specific transcription factors because it elevates activity 10-fold. In contrast this distal region only increases expression in L8 myotubes approximately 1- to 2.5-fold over the downstream deletions. However, in L8 myotubes, but not in C2C12 myotubes, the downstream regions between positions -626 and -153 alone appear to be nearly sufficient to respond maximally to muscle-specific regulatory factors. These data suggest that some myogenic factors which interacted with the human α -skeletal actin gene-regulatory sequences in C2C12 and L8 cells varied qualitatively and quantitatively and that some or all of these factors were regulated during the differentiation of L8 cells, but not during the differentiation of C2C12 cells. This result with the α -skeletal actin gene promoter was distinct from our previous result with the α -cardiac actin promoter (41, 42), whose analogous proximal and distal domains had similar effects in both myogenic cell lines.

Finally, when compared with the expression activity of β -CAT, the pHSA87CAT construct had a fivefold higher level of transcriptional activity in L8 myoblasts and myotubes than it did in C2C12 myotubes (Table 1). This discrepancy appears to result from the impact of reduced transcriptional activity directed by the benchmark β -actin promoter in L8 cells relative to the activity of the baseline control plasmid, pHCA0CAT (Table 1). In fact, the activities of pHSA87CAT relative to pHCA0CAT are similar in C2C12 and L8 cells.

The developmental modulation of SV40-CAT constructs during rodent myoblast differentiation, such as we observed with the L8 cells, has been noted previously by Melloul et al. (39) and Minty et al. (41). This observation does not diminish the importance of the developmentally modulated increase in the expression of pHSA2000CAT, since the SV40 promoter has been shown to contain multiple segments, each with different cell type specificity (53). For example, one

such segment has been shown to interact with a factor which is developmentally regulated during B-cell maturation and critical to the expression of the SV40 and the kappa-immunoglobulin enhancers (2).

Transcription initiation of the pHSA-CAT constructs. The initiation point for transcription of human α -skeletal actin mRNA was previously determined by Hanauer et al. (28) by using primer extension analysis. Their analysis indicated that a minor cap site existed two to four nucleotides upstream of the major cap site. We found similar heterogeneity in human adult skeletal muscle RNA by using S1 mapping with probes that overlapped the junction between the promoter and the 5' untranslated region (data not shown). To determine whether the α -skeletal-CAT hybrid gene was transcribed from the expected promoter, we analyzed RNA from C2C12 cells transfected with pHSA2000CAT. S1 and primer extension analyses with a variety of probes were unsuccessful in mapping the transcriptional start site in the α -skeletal-CAT hybrid gene (although they were successful with RNA from adult human skeletal muscle), possibly due to the instability of CAT transcripts in a eucaryotic cell environment. This has been observed with a variety of tissue-specific genes with S1 analysis and primer extension, including transfected cardiac myosin light-chain 2A (63) and microinjected α -crystallin (49) hybrid genes. To accurately map the start of transcription in this hybrid gene, we tested the ability of the α -skeletal actin promoter to accurately initiate transcription in a HeLa whole cell extract (37). Such whole cell extracts have been shown to accurately initiate transcription from a variety of tissue-specific genes (chicken conalbumin and ovalbumin [32, 51, 58, 61], human, rabbit, and mouse β -globin [25, 35], α -globin [57], fibroin [56], immunoglobulin kappa light chain [44]), which are normally expressed only in differentiated tissues. The 5' ends of the *in vitro* products have been identical to the *in vivo* 5' ends in all cases. Plasmid pHSA626 was digested with *Hind*III, which cleaved 239 bases downstream of the transcription start site at the junction between the truncated IVS1 and the CAT gene. Figure 5 shows the result of such an *in vitro* transcription assay with the truncated pHSA626 CAT plasmid as a template. A runoff transcript of 239 nucleotides was generated, which indicated accurate initiation from the human α -skeletal actin promoter identical to the *in vivo* start site in skeletal muscle established by Hanauer et al. (28). Lanes 2, 3, and 4 contained pHSA626 CAT DNA at concentrations of 20, 40, and 80 μ g/ml, respectively. Lanes 5 and 6 contained the vector DNA and no DNA, respectively. Thus the 239-nucleotide runoff product was dependent on the presence of the α -skeletal actin gene promoter and was DNA concentration dependent in the range of 40 to 80 μ g/ml. There remains the unlikely possibility that in transfected muscle cells the transcripts from our constructs start elsewhere.

Skeletal actin upstream domains modulate transcription from a heterologous promoter. The complex nature of the α -skeletal actin gene promoter revealed by the deletion experiments suggested that at least two domains, the distal and proximal, interact synergistically and in a tissue-specific manner to positively modulate the expression of the gene. To investigate this further, we examined the intrinsic modulating capacity of the α -skeletal actin *cis*-acting domains by subcloning them into the test plasmid, pA10.CAT.2N (34). This plasmid contains an enhancerless SV40 early gene promoter linked to the CAT gene (Fig. 6A). When this plasmid is used in transient transfection experiments with C2C12 myotubes it engenders less than 1% of the CAT activity induced by either pSV2CAT or pHSA2000CAT (Fig.

FIG. 5. Transcription of the α -skeletal actin gene in HeLa whole cell extracts. The *Hind*III-digested plasmid pHA626CAT was transcribed in 50- μ l reaction mixtures containing 1, 2, and 4 μ g of DNA in lanes 2, 3, and 4, respectively. Lanes 5 and 6 contained vector DNA (pHCA0CAT) and no DNA, respectively. Lane 1 contained pBR322 digested with *Hpa*II as markers. The arrow indicates the position of an accurately initiated 239-nucleotide runoff transcript. All lanes are from the same polyacrylamide-urea sequencing gel and autoradiograph, which was spliced to eliminate irrelevant data.

6B). Thus, this test plasmid can be used to monitor the ability of the α -skeletal actin promoter domains, inserted either 5' or 3' of the pA10.CAT.2N promoter and in either orientation, to regulate the expression of a heterologous promoter. Twenty-one such constructs were tested.

The α -skeletal actin *cis*-acting sequences that we tested are depicted in Fig. 6B. Initially each was cloned in the sense orientation, 5' of the CAT gene in the *Bgl*II site of pA10CAT.2N (34). These constructs were transiently expressed

in C2C12 myotubes, and CAT activity was measured. Each result was expressed as a fold increase in activity relative to pA10CAT.2N arbitrarily set to 1. Fragments which were tested included (i) positions -2000 to -710; (ii) positions -1300 to -710 (distal domain); (iii) positions -710 to -87; (iv) positions -710 to -36 (v); positions -153 to -87 (proximal domain); and (vi) positions -153 to -36. Fragments containing only the proximal or distal domains each led to a 3.5-fold increase in CAT signal in C2C12 myotubes, indicating that these domains can transcriptionally modulate the heterologous SV40 promoter. Flanking sequences added to either side of the proximal domain or to the 5' side of the distal domain did not have a major effect on the ability of the elongated DNA segments to modulate expression.

We next tested the ability of these two domains to interact by inserting them together in front of the pA10CAT.2N gene. Two DNA fragments, from positions -2000 to -87 or -2000 to -36, which contain the entire 5' flanking region either with or without the basal promoter segment, respectively, increased CAT expression 35- to 45-fold (i.e., at levels 30 to 40% of pSV2CAT), showing that the distal and proximal domains can synergistically interact to positively modulate transcription from a heterologous promoter. These experiments do not speak directly to the role, if any, of the central domain from positions -153 to -710 in this synergism or to the spatial requirements of the interaction.

We next examined the tissue-specific nature of these modulating events by testing each of these constructs in transient transfection assays in the human diploid fibroblast cell line HuT-12. Each of these constructs expressed CAT at levels only 0.2 to 0.4% of those of pSV2CAT (data not shown), and we conclude that the ability of the proximal and distal domains of the α -skeletal actin gene promoter to enhance gene expression and to operate synergistically is tissue specific.

The proximal domain is an enhancer. The ability of the upstream domains to modulate transcription from a heterologous promoter suggested the possibility that they contained tissue-specific, enhancerlike activity. To determine whether they could act in an orientation- and position-independent manner, these skeletal actin *cis*-acting DNA sequences were

FIG. 6. Quantitation of the transcriptional activity of upstream sequences of the human skeletal actin in C2C12 myotubes and cloning into pA10CAT.2N. (A) Schematic representation of pA10CAT.2N showing the *Bgl*II and *Bam*HI cloning sites 5' and 3' of the CAT gene. Ori, Origin of replication. (B) Different regions of the 5'-flanking sequences represented by the bars were subcloned into pA10CAT.2N. The activity of these plasmids in C2C12 myotubes is expressed relative to pA10CAT.2N arbitrarily set to 1. The square and the triangle show the relative position of the CarG and TATA boxes. N.D., Not done; S, sense; AS, antisense.

cloned into the *Bam*HI site of pA10.CAT.2N, located 3' to the polyadenylation signal of the CAT gene, in both the sense and antisense orientations. The resulting constructs were transiently expressed in C2C12 myotubes (Fig. 6B). The fragments, which include the proximal domain (i.e., positions -710 to -87 and -710 to -36), were functional in both orientations at this position, increasing the activity of the SV40 promoter 10- to 14-fold (Fig. 6B). Thus the DNA segment between positions -710 and -87, which includes the proximal domain, appears to be a tissue-specific enhancer since it functions in either orientation and at a great distance relative to the start of transcription.

The DNA segment between positions -2000 and -710 had little or no ability to modulate the expression of the heterologous promoter (less than twofold activity) when placed in the antisense orientation at the 5' or 3' end of the gene. Thus the distal domain does not seem to have the position- and orientation-independent characteristics of an enhancer element. However, the synergistic activity between the proximal and distal domains does appear to be orientation and position independent: when fragments from positions -2000 to -87 and -2000 to -36 were tested in the antisense orientation at the 3' location they augmented the activity of the SV40 promoter 25- to 45-fold. Peculiarly, this striking synergistic effect was not apparent in the antisense orientation at the 5' end of the gene (i.e., CAT expression increased only 5-fold) or in the sense orientation at the 3' end of the gene (i.e., CAT expression increased only 11-fold). One way of restating this unusual result is to consider that the tissue-specific synergistic effect of the 5'-flanking sequences functions at either end of the gene but only when the proximal region is nearer to the gene than is the distal domain.

DISCUSSION

The promoter region of the human α -skeletal actin gene is complex. Tissue culture cells transcribe transfected genes containing regulatory sequences (5, 9, 10, 14, 19, 21, 24, 30). We have demonstrated by transient expression studies in two myogenic (C2C12 and L8) and two fibroblastic (L.TK⁻ and HuT-12) cell lines that the upstream 2,000 bp of the human skeletal actin gene (plus the first exon of the 5' untranslated region and 149 bp of intervening sequence 1) mediates both tissue-specific expression and regulation during *in vitro* myogenesis.

Deletion of increasing amounts of upstream sequences of the human skeletal actin gene diminished transcription in muscle cells but not in fibroblastic cells, suggesting that sequences in this region interact with muscle-specific, positively acting transcription factors. Upstream regions have likewise been implicated in the expression of a number of genes, including those encoding insulin (16), lysozyme (59), immunoglobulin (21), retinol-binding proteins (14), α -2(I) collagen (54), and α -cardiac actin (42).

At least two regions upstream of the human skeletal actin gene were important in the positive modulation of its transcription, and a third region acted as a moderately strong basal promoter in both muscle and nonmuscle cells. We designate these regions as the distal (positions -1300 to -626), proximal (positions -153 to -87), and basal (3' end to position -87) promoter domains. There was a 10-fold loss in transcriptional activity in C2C12 cells accompanying the deletion of the distal domain and a further 10-fold loss of activity from deletion of the proximal domain. The 80- to 100-fold loss of CAT activity (with respect to pHSA2000 CAT) when the deletions extend to within 87 nucleotides

upstream of the RNA initiation site suggests that the distal and proximal domains interact synergistically. This hypothesis is strengthened by our experiments, which demonstrate that DNA segments carrying the proximal and distal domains increase transcriptional activity of the heterologous SV40 early promoter 35- to 45-fold, but these *cis*-acting domains induce activity only 3.5- to 6-fold when tested independently. The distal region may extend slightly further 5' of position -1300, since there was a stimulatory effect on the expression of pA10CAT.2N (34) when we inserted the DNA fragment encompassing positions -2000 to -1300 upstream of the promoter (data not shown). The pA10 CAT.2N experiments also established that sequences that include the proximal domain display all of the properties of classical enhancers.

These data suggest that the human α -skeletal promoter activity is effected by at least two synergistically interacting—but physically separated—*cis*-acting sequence domains and that these interact with muscle-specific *trans*-acting factors. There is evidence in prokaryotes for physical contact between proteins bound to regulatory DNA sequences that are separated by such large distances. These proteins presumably interact by flexing of the intervening nucleic acids (15). The failure of synergy between the proximal and distal domains to function when the distal domain was juxtaposed to the SV40-CAT sequences suggests that there is a third interaction important in the transcriptional modulation of the promoter, which is between the flanking sequences and the basal promoter or gene sequences, and that this interaction may be constrained by distance. Tests of this hypothesis must await a more detailed deletion and replacement analysis of the function of these domains.

Regulation during myogenesis: identification of essential domains. The proximal region (positions -153 to -87) of the skeletal α -actin gene promoter is essential for modulating the increased transcription of the gene during myogenesis in L8 cells, whereas the distal region does not appear to be required. Sequences more proximal than position -87 do not support muscle-specific expression or regulation. The relative importance of the proximal region and the reduced modulatory capacity of the distal region in L8 cells contrasts with the major effect of the distal sequences in our experiments with C2C12 cells. However, our results with L8 cells are similar to those of Grichnick et al. (24) and Bergsma et al. (5), who examined the promoter of the chicken α -skeletal actin gene promoter. These authors demonstrated that in chicken primary myoblasts about 200 bp of 5'-flanking DNA was sufficient for tissue-specific transcription and induction during myogenesis and that further deletion to position -76 almost abolished transcription.

Similarly, the work by Melloul et al. (39) and Nudel et al. (48) suggested that far distal sequences of the rat skeletal α -actin gene promoter, beyond -780 bp, were not needed in L8 cells for maximal tissue-specific expression and upregulation during differentiation. Furthermore, the rat skeletal α -actin gene assumes a DNase I-sensitive conformation in differentiating L8 cells, indicative of a transcriptionally active state, but the limit of DNase I sensitivity of the gene in myotubes ends between 70 and 700 bp 5' to the structural gene (8). Thus more distal regions of the rat skeletal α -actin gene do not appear to undergo a major alteration of chromatin conformation during myogenesis of L8 cells.

One possible explanation for the failure of distal sequences to influence promoter activity in L8 cells may stem from the effect of the central domain, those sequences between the distal and proximal domains (positions -626 to

–153). Although these sequences are not essential in C2C12 cells, they mediated positive modulation of transcription from the human α -skeletal actin gene promoter in L8 cells. The ability of this central domain to maximally augment the activity of the proximal region of the α -skeletal actin promoter during L8 myogenesis may account for the failure of the far-distal sequences to have a further modulating effect in this cell line. Furthermore, the impact of the central domain may have obscured the importance of the distal promoter region in the work of others.

The number and functional diversity of regulatory domains and the distance they span raise the issue of their role in the distinctive patterns of α -skeletal actin mRNA accumulation during myogenesis in rodent cell lines (3, 31, 41) and primary myoblasts (25a, 29). Certainly it is possible that the immortalized aneuploid cell lines may differ in the kind and amount of *trans*-acting factors they produce. Although this might explain why we detect differences in the role of various *cis*-acting sequences in L8 and C2C12 cells, the observations only serve to emphasize the complexity of the promoter region and the variety of interactions that regulate its function. The promoter subdomains may respond differentially and subtly to variations in the type and quantity of myogenic factors and are used in a hierarchical manner with respect to maximal expression in different cell lines. Such promoter complexity could also account for different modulatory programs during embryonic, fetal, and adult muscle development. Davidson et al. (11) proposed a similar model to explain the activity of the SV40 enhancer in different cell types. These authors suggested that multiple *cis*-acting sequence elements can interact with different *trans*-acting factors in a cell-specific manner: overlapping motifs and surrounding sequences may be differentially involved in different cell types depending on the various ratios of available interacting factors (12). Recently, Goodbourn et al. (20), Ryals et al. (52), and Fugita et al. (18) identified repeated elements at the 5' ends of the alpha and beta interferon genes. Their data suggested that the number of repeated elements required for maximal induction varied and may have been a reflection of the cell type in which the gene was expressed. Similarly, the nonadditive activity of the three enhancer regions in the α -fetoprotein gene (*AFP*) in the HepG2 cell line has been attributed to a putative role in the diverse patterns of expression of the *AFP* gene in endoderm, fetal liver, and fetal gut (19, 27).

The CCAAAT sequence is part of a CARG motif and is not a CAAT box. The nucleotide sequences in the proximal promoter region are strongly conserved during evolution in chickens, mice, rats, and humans (A. Taylor, H. Erba, G. Muscat, and L. Kedes, unpublished data). This proximal region also contains a CARG box motif identified and implicated by Minty and Kedes (42)—and demonstrated by mutation analysis (43)—as a required sequence for muscle-specific transcription and induction during myogenic differentiation of the α -cardiac actin gene. The transcription studies of Bergsma et al. (5) indicated that the DNA segment between positions –107 and –76 nucleotides upstream of the mRNA cap site of the chicken α -skeletal actin promoter and encompassing a sequence, CCAAAT, was required for maximal expression in *Xenopus laevis* oocytes. They have referred to this sequence as a variant of the classical CAAT box. It seems more likely that the chicken CCAAAT sequence is not part of the basal promoter, which in the human α -skeletal actin gene we have located 3' of position –87 and downstream of the CARG motif. The chicken CCAAAT sequence is, in fact, a part of a typical CARG motif. The

contrast between the importance of sequences bearing the CARG motif for maximal expression in the *Xenopus* oocyte test system used by Bergsma et al. and its insignificance for the basal activity of the human promoter in rodent myogenic cell lines may reflect different *trans*-acting factors in each test system. The significance of this proximal CARG-bearing domain is further supported by the experiments of Nudel et al. (48), who suggested that the regions between positions +4 and –140 are sufficient to confer developmentally regulated expression on the rodent α -skeletal actin and myosin light-chain 2 genes. The CARG motif is present in the rodent α -skeletal actin and myosin light-chain 2 genes at positions –92 and –122, respectively, as pointed out previously (42).

Conclusions. The experiments reported here reveal the complexity of the human α -skeletal actin gene promoter. The proximal domain (positions –153 to –87) is critical for tissue-specific expression and regulation in two distinct myogenic cell systems. The *cis*-acting sequences 3' of position –87 interact with factors present in both myogenic and fibroblastic cells and appear to define—or act as a major component of—the basal promoter domain. The 5'-distal domain from positions –1300 to –626 and the proximal domain from positions –153 to –87 respond to muscle-specific factors and positively modulate transcription in a synergistic fashion in C2C12 cells but not in L8 cells. The central domain between positions –626 and –153, although not required in either cell line, has a positive, additive role in augmenting expression in L8 cells (two- to threefold) but not in C2C12 cells. Thus these elements appear to be differentially utilized for maximal expression in different myogenic cells. These observations are compatible with the possibility that the particular combination of domains used in vivo depends on the availability or relative levels of *trans*-acting factors in each cell type and the accessibility of the different domains to those factors.

Based on data discussed by Yamamoto (62) and Bienz and Pelham (6), we can interpret our results in terms of a model in which one or more upstream regions of the human α -skeletal actin gene are differentially used during muscle differentiation or development to positively modulate transcription (depending on factor availability) via an interaction with an essential promoter domain (positions –153 to –87). Such structural changes could allow an improved interaction with the basic transcriptional machinery, resulting in increased rates of RNA polymerase initiation.

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