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β- and γ-Actin Genes Differ in Their Mechanisms of Down-Regulation During Myogenesis

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Abstract During the differentiation of myoblasts to form myotubes, the expression patterns of the different actin isoforms change. The cytoplasmic actins, β and γ, are down-regulated and the muscle specific isoforms are up-regulated. The region responsible for the down-regulation of the β-actin gene has been located in the 3′end of the gene. Since the β- and γ-actin genes arose from a gene duplication (Erba et al. [1988] J. Cell. Biol. 8:1775–1789), it is possible that the region responsible for down-regulation of the γ-actin gene may also be in the 3′end of the gene. We have tested this by transfection of human γ-actin gene constructs into myogenic C2 cells. To our surprise, we found that the region responsible for down-regulation of the γ-actin gene during differentiation is not in the 3′ end of the gene in contrast to that for β-actin. Rather, we found that intron III is required for appropriate down-regulation of γ-actin during myogenesis. Intron III containing transcripts from the γ-actin gene were also found to accumulate during myogenesis. We, therefore, propose that excision of intron III from the primary transcript is inhibited during myogenesis resulting in degradation of the RNA. Removal of intron III from the gene allows it to escape this regulatory mechanism. J. Cell. Biochem. 84: 335–342, 2002. © 2001 Wiley-Liss, Inc.

Key words: muscle gene regulation; regulatory sequences; differentiation

At least six actin isoforms are known in mammals, each encoded by a separate gene, and they differ by less than 10% of their amino acid sequence [Vandekerckhove and Weber, 1978; Hamada et al., 1982; Gunning et al., 1984; Ng et al., 1985; Erba et al., 1988]. During differentiation of muscle tissue, the amount of mRNA coding for β- and γ-non-muscle actins is reduced, whereas the muscle specific actin mRNAs are induced [Shani et al., 1981; Schwartz and Rothblum, 1981].

C2 is a mouse myogenic cell line that can be made to differentiate from mononuclear cells, myoblasts, to multinuclear cells, myotubes. During differentiation, the expression of actin isoforms changes from non-muscle to muscle specific [Bains et al., 1984; Gunning et al., 1987, 1990]. Although both β- and γ-actin mRNAs are down-regulated during differentiation, the regulation is not coordinated; γ-actin decreases more quickly than β-actin, 7.5-fold and 2.5-fold, respectively [Erba et al., 1988].

The regulatory sequences of the β- and γ-actin genes have some similarities as well as some regions that differ. The nucleotide sequence of the human β-actin coding region compared to γ-actin has 91% homology [Erba et al., 1986]. There is significant sequence homology between the human γ-actin 5′flank and the human, rat and chicken β-actin, and the *Xenopus borealis* type 1 actin 5′flank [Erba et al., 1988]. The similarity of this region may reflect the conservation of sequences involved in the co-expression of these genes. The 3′UTR of the different

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actin isoforms are unique to each but have been shown to be highly conserved [Ponte et al., 1983]. Both the β- and γ-actin 3′UTRs are conserved between mammals and chickens, although some regions are more highly conserved than others [Yaffe et al., 1985; Erba et al., 1986]. These observations strongly suggest that regions of the 3′UTRs have been under strict evolutionary constraint and that they may have isoform-specific functions.

The region responsible for down-regulation of β-actin has been investigated, and it was concluded that the 3′end (including exon VI, 3′UTR and 3′flanking region) of the chicken β-actin gene was necessary and sufficient for this regulation [DePonte-Zilli et al., 1988; Lohse and Arnold, 1988]. DePonte-Zilli et al. [1988] found that a region sufficient for the reduction of β-actin mRNA levels during myogenesis is, at the most, 40-bp long and located in the 3′UTR of the chicken gene. This 40-bp sequence is conserved in all the vertebrate β-actin genes and is not similar to any of the 3′ processing, polyadenylation or termination sequences described. However, the 40-bp sequence was never deleted from the β-actin gene to determine if it was also necessary for down-regulation. Lohse and Arnold [1988] found the 3′flanking sequence to be necessary for down-regulation but did not test to ascertain if it was also sufficient for down-regulation. However, they found that the 3′UTR was not sufficient for down-regulation, contrasting with the findings of DePonte-Zilli et al. [1988]. Existence of multiple signals in both the 3′UTR and flank of the chicken β-actin gene might best explain the different results of DePonte-Zilli et al. [1988] and Lohse and Arnold [1988].

In this paper, we have investigated the region of the γ-actin gene responsible for its down-regulation during myogenesis. Constructs of the human γ-actin gene, with various deletions, were transfected into myogenic C2 cells and the regulation of the exogenous gene was measured during myogenesis. Transfectant cells that did not regulate the human gene appropriately provided information about the regions of the γ-actin gene necessary for the down-regulation process. We found that the region responsible for down-regulation of the γ-actin gene during differentiation is not in the 3′UTR or 3′ flank in contrast to β-actin. Rather, the regulation of γ-actin appears to require intron III.

MATERIALS AND METHODS

γ-Actin Gene Constructs

The full length human γ-actin gene construct (γ33) was prepared as described in Lloyd and Gunning [1993]. The human γ-actin gene deletion constructs (Figs. 1 and 4) utilized were γ22, missing the entire 3′UTR and 3′flanking sequence but containing all the protein coding sequence; γ156, missing intron III; and γCD-8γ, missing most of intron I and all of the rest of the exons and introns and most of 3′UTR of the γ-actin gene replaced with the CD-8 cDNA. The preparation of γ22 and γ156 were described in Lloyd and Gunning [1993]. The γCD-8γ construct was prepared as follows. The SacI-XbaI fragment was isolated from the CD-8 gene (PG59, CD-8 cDNA cloned into the plasmid pUC-18 kindly given by Dr. J. Parnes, Zamoyska et al. [1985]). The StuI-XbaI region of the γ-actin gene (γ33) was removed and the CD-8 gene fragment was ligated into the γ-actin gene to make the hybrid construct γCD-8γ.

Cell Culture and Transfection

Mouse C2 myoblasts originally isolated in the laboratory of Dr. D. Yaffe and subcloned in the laboratory of Dr. H. Blau were grown in DMEM (Gibco labs, Glen Waverly, Australia) supplemented with 20% fetal calf serum (Commonwealth Serum Labs, Melbourne, Australia) and 0.5% chicken embryo extract (Flow Laboratories Australasia Pty. Ltd., North Ryde). To induce C2 differentiation, cells were grown to approximately 90% confluence and then cultured in DMEM containing 2% horse serum (Commonwealth Serum Labs, Australia). The cultures were then fed fusion media every day until all the cells were harvested for RNA.

DNA transfection and isolation of transfectant clones was performed as described in Schevzov et al. [1992]. The constructs used for transfection included γ33, γ22, γ156, and γCD-8γ. These constructs were all co-transfected with 1 μg of pSV2-neo.

RNA Isolation and Analysis

Total cellular RNA was isolated from the cell cultures by the method of Chomczynski and Sacchi [1987]. Several dishes were set up at the start of the time course and samples taken at various stages, myoblasts at low confluence (30–40%), and myotubes at days 2 and 4 after addition of differentiation media. RNA was

size-fractionated and transferred to nylon membranes as described [Gunning et al., 1990]. After electrophoresis, RNA was transferred to a nylon membrane as per manufacturer's instructions (either Hybond N or N^+, Amersham Australia).

One of the following methods, (1) random priming, (2) specific priming, or (3) terminal kinasing, radioactively labeled all DNA probes. For both random and specific priming, 100 ng of probe DNA was labeled with 32P dCTP using a Bresa Oligo Labelling kit (OLK-50) for 1 h at 37°C, following the manufacturer's protocol (Bresatec, Adelaide, South Australia). Radiolabeled DNA template from either of these methods was separated from unincorporated nucleotides with a Centricon 30 microcentaur (Amicon, USA). The samples were washed twice with 0.5 ml TE and centrifuged at 2,500*g* for 15 min before collection by inversion and centrifuging at 800*g* for 5 min. The volume of the collected probe was increased to 500 μl with TE before phenol:chloroform 1:1 extraction with an equal volume. For terminal kinasing, 100 ng of template DNA was end labeled with ^{32}P γdATP using a Bresa 5′ Terminal Kinasing kit (TKK-1) for 1 h following the manufacturer's protocol (Bresatec, South Australia). Terminally labeled DNA was separated from unincorporated nucleotides on a DE52 Sepharose column of packed volume 0.2 ml. The column was equilibrated with 1 volume of TE before addition of labeled DNA. Unincorporated nucleotides were washed from the column with 5 volumes of 0.2 M NaCl in TE before the labeled probe was eluted from the column with 3 volumes of 1 M NaCl in TE.

Prehybridization of the filters for 8–16 h was carried out at 65°C in 4 × SSC, 5 × Denhardts solution (1 × Denhardts solution is 0.1 g Ficoll, 0.1 g of polyvinyl pyrrolidine, 0.1 g bovine serum albumin (fraction V), in 500 ml of water), and 50 mM NaH_2PO_4 (pH 7.0). DNA probes were hybridized to RNA blots at 10^6 dpm/ml in a solution containing 4 × SSC, 50 mM NaH_2PO_4 (pH 7.0), 5 × Denhardts solution, and 10% (w/v) dextran sulfate at 65°C for 16 h. Following hybridization, unbound and non-specifically bound probe was washed from the filters under conditions of various stringencies. Blots were washed in 0.5 × SSC, 0.1% SDS at 65°C except those hybridized with SV40 3′UTR probe which was washed in 1 × SSC, 0.1% SDS at 65°C, and the 18s probe which were washed in 4 × SSC, 0.1% SDS at 55°C. Membranes were exposed to Kodak X-OMAT AR, X-ray film (Kodak, Australia) at −80°C. The film was developed in an Ecomat 2400 automatic developer. Autoradiographs were scanned using a Molecular Dynamics Model 300 series computing densitometer (California, USA) and the analysis program Imagequant.

DNA Probes

The human γ-actin mRNA was detected using a human-specific γ-actin-specific 3′UTR probe, pHFγA-3′UT-HX [Erba et al., 1986]. Detection of transcripts containing the SV40 3′UTR was achieved using the *Eco* R1-*Hpa* 1 fragment of the plasmid pcDV1 [Okayama and Berg, 1983]. The mouse γ-actin mRNA was measured using a mouse-specific γ-actin-specific DNA fragment containing bp 199-465 of the mouse γ-actin 3′UTR (Mγ-3′UT-HA). The mouse γ-actin probe was isolated as a HpaI-AvaII DNA fragment from a mouse cDNA supplied by Dr. D. Leader and was subcloned into the SmaI site of pGEM-3. In the γCD-8γ transfectants, the mouse γ-actin mRNA was measured using a mouse-specific γ-actin-specific DNA fragment containing all of the mouse γ-actin 5′UTR. The γCD-8γ mRNA was detected using a CD-8 cDNA fragment [Zamoyska et al., 1985]. To verify that equivalent amounts of RNA were transferred, blots were hybridized to a 18s rRNA–specific oligonucleotide probe [Lloyd et al., 1992]. γ-actin intron III containing transcripts were detected using the intron III specific probe (γ-IVSIII-HD) described by Erba et al. [1988].

RESULTS

Expression of the Human γ-Actin Gene During Myogenesis in γ-Actin Gene 3′ Deletion Transfectant Lines

A 3′end deletion construct of the human γ-actin gene was engineered since it was thought likely that the region responsible for down-regulation would be found in a similar location to that in the β-actin gene. Figure 1 depicts the γ-actin gene and the region of the 3′end of the gene that was deleted. Two constructs were made: γ33, containing the entire cloned form of the human γ-actin gene; and γ22, missing the entire 3′UTR and 3′flanking sequence but containing all the protein coding sequence.

The two human γ-actin gene constructs were stably transfected into the myogenic cell line, C2. To ensure that myoblasts did not persist in

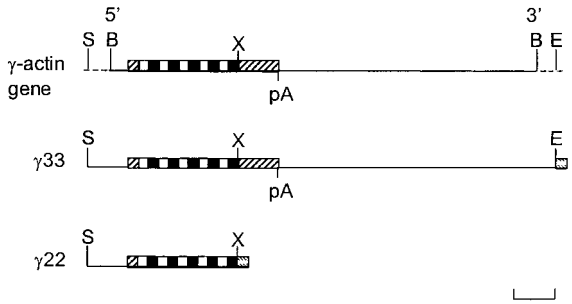

Fig. 1. 3′UTR and flanking region deletion construct of the human γ-actin gene. The human γ -actin gene is shown as a 9 kb Bam H1 fragment cloned into the plasmid pBR322. The exons are indicated by solid boxes, open boxes indicate the introns, and the 5′ and 3′UTRs are indicated by cross hatched boxes. The polyadenylation signal is depicted by pA and the SV40 3′UTR (polyadenylation and termination sequence) is depicted by a half cross hatched box. The restriction enzyme sites are shown. The lines below the gene map represent the portions of the gene cloned in the constructs; γ33 has the *Sal* 1-*Eco R*1 fragment and γ22 has the *Sal* 1-*Xba* 1 fragment. The restriction enzyme sites are abbreviated as follows: B, *Bam H*1; E, *Eco R*1; S, *Sal* 1; and X, *Xba* 1. Sizes are shown by the bar (1 kb).

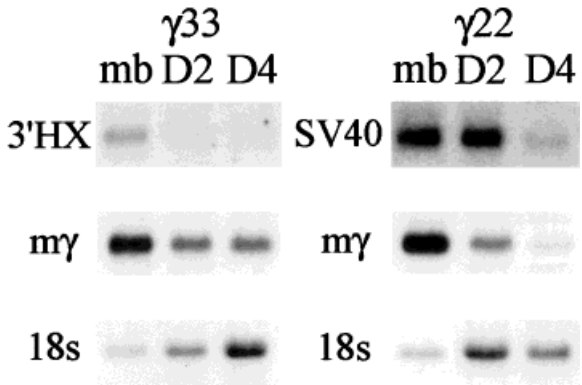

Fig. 2. Actin mRNA expression during myogenesis of pooled human γ-actin gene transfectant cultures with 3′end deletions. Total RNA was isolated from the two transfectant cultures, γ33, and γ22 during differentiation in the presence of cytosine arabinoside (AraC). The AraC was added with the fusion medium at 1×10^{-5}M final concentration to prevent proliferation of myoblasts in the differentiated cultures. The samples were taken from low confluence myoblasts (mb), 2 days (D2) and 4 (D4) days after fusion medium was added. Five micrograms of RNA was loaded in each lane. The RNA was size fractionated on a denaturing agarose gel and transferred to nylon membrane. To measure human γ-actin gene expression in the transfectant cultures, the filters were hybridized with (1) the human γ-actin specific probe, γ-3′UT-HX for the γ33 cultures and (2) the SV40 polyadenylation and termination sequence for the γ22 cultures, since γ22 did not contain the γ-actin 3′UTR. The filters were washed and autoradiographed. Subsequently, the filters were hybridized for the mouse γ-actin mRNA using the mouse specific γ-actin probe (M γ-3′UT-HA) and autoradiographed. The filters were then washed and hybridized for the 18s specific oligonucleotide in order to quantitiate the RNA levels as described by Lloyd et al. [1992].

the differentiated cultures, cytosine arabinoside (AraC) was added with the fusion media. This drug inhibits DNA synthesis, preventing cells from completing the cell cycle and, therefore, inhibiting the growth of cycling cells such as myoblasts. Cultures were harvested for total RNA from myoblasts of low confluence, 2 and 4 days after fusion media was added.

The expression of the human γ-actin gene was examined in these samples during the differentiation process. This was achieved using the human γ-actin specific probe, γA-3′UT-HX, and for the γ22 transfectants, the SV40 polyadenylation and termination sequence was used as a probe since γ22 did not contain the γ-actin 3′UTR. Figure 2 shows the expression of human γ-actin and the mouse γ-actin mRNAs during myogenesis in the presence of AraC. These results were analyzed by densitometry, and the expression levels were normalized for loading (18s levels) and are depicted in graphic form in Figure 3. The pattern of expression of the exogenous and endogenous actin mRNAs in the γ-actin gene deletion transfectants was the same as that in the γ33 cells, i.e., a ten-fold reduction in expression. This indicated that the region necessary for down-regulation during myogenesis was not in the 3′end of the γ-actin gene, in contrast to the β-actin gene, demonstrating that the two non-muscle actin genes are differently regulated. These results were also found in pooled transfectant lines without the addition of AraC (data not shown).

Region in the Introns and/or Exons of the Human γ-Actin Gene is Important for Correct Myogenic Regulation

We next investigated whether the region necessary for the down-regulation of the γ-actin gene during differentiation lies in the gene body, i.e., exons or introns. In order to achieve this, another human γ-actin gene construct was engineered, γCD-8γ. This construct, depicted in Figure 4, contained the γ-actin promoter, 5′flanking sequence, 5′UTR, a portion of the 3′UTR, and the entire 3′flanking sequence but had the CD-8 cDNA in place of the γ-actin coding region. CD8 is a murine differentiation antigen expressed on most immature T lymphocytes as well as the cytotoxic suppressor T cell subset and functions as a cell adhesion molecule involved in the binding of the major histocom-

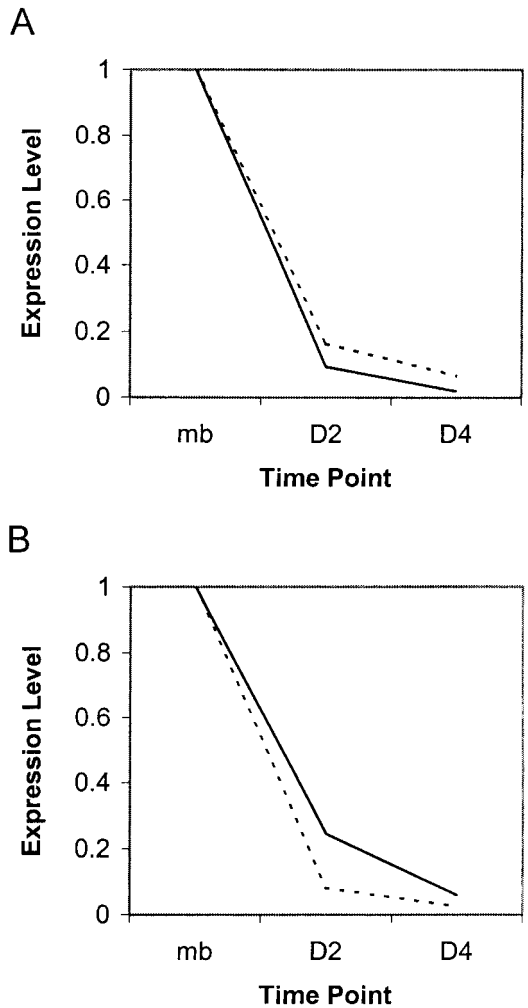

Fig. 3. Actin mRNA levels during myogenesis of pooled human γ-actin gene transfectant cultures with 3′end deletions. Human γ-actin (—) and mouse γ-actin (- - -) mRNA levels were measured in the pooled cultures of (A) γ33 and (B) γ22 during differentiation by scanning densitometry of the autoradiographs in Figure 2. These levels were normalized for loading discrepancies using the measured 18s values [Lloyd et al., 1992]. The expression levels were shown as a factor of those found in the myoblasts that were set at 1.

patibility complex class I [Nakauchi et al., 1987; Norment et al., 1988]. This construct was stably transfected into C2 cells. The cultures were differentiated, and RNA was harvested from myoblasts at low confluence, 2 and 4 days after fusion medium was added. AraC was also added with the fusion media to prevent myoblast proliferation. The expression of the human γ-actin-CD-8 hybrid gene and the mouse γ-actin genes was examined in these cultures, and the results are shown in Figure 5. The expression levels, after normalization for loading levels, are shown graphically in Figure 6A. The

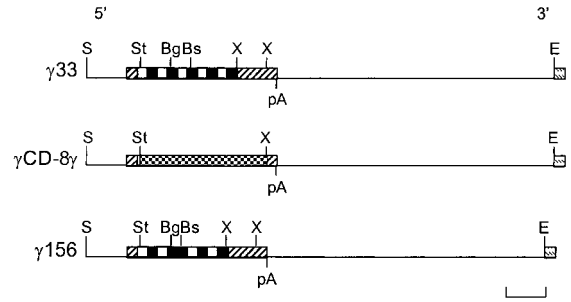

Fig. 4. Internal deletion constructs of the human γ-actin gene. The γCD-8γ and γ156 constructs are shown below that of γ33 to indicate the regions of the γ-actin gene that are missing in these constructs. The γ-actin exons are indicated by solid boxes, open boxes indicate the introns, and the 5′ and 3′UTRs are indicated by cross hatched boxes. The γ-actin polyadenylation signal is depicted by pA and the SV40 3′UTR (polyadenylation and termination sequence) is depicted by a half cross hatched box. A checked box depicts the CD-8 cDNA. The restriction enzyme sites are abbreviated as follows: Bg, *BglII*; Bs, *BstEII*; E, *Eco R*1; S, *Sal* 1; St, *Stu* 1; and X, *Xba* 1. Sizes are shown by the bar (1 kb).

transfected gene was not down-regulated appropriately during differentiation. There was only a small degree of down-regulation. It is possible that CD-8 itself may have impacted on

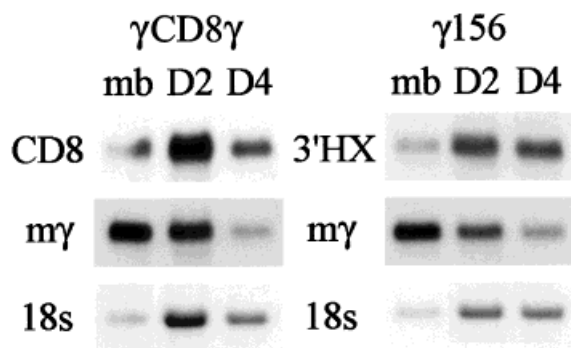

Fig. 5. Actin mRNA expression during myogenesis of pooled human γ-actin gene transfectant cultures with internal deletions. Total RNA was isolated from the two transfectant cultures, γCD-8γ, and γ156 during differentiation in the presence of cytosine arabinoside (AraC). The AraC was added with the fusion medium at 1×10^{-5}M final concentration to prevent proliferation of myoblasts in the differentiated cultures. The samples were taken from low confluence myoblasts (mb), 2 days (D2) and 4 days (D4) after fusion medium was added. Five micrograms of RNA was loaded in each lane. The RNA was size fractionated on a denaturing agarose gel and transferred to nylon membrane. The probe used to detect the transfected gene in the γCD-8γ RNA was a CD-8 cDNA fragment [Zamoyska et al., 1985], and the probe used to detect the mouse γ-actin mRNA was a 5′UTR fragment. The probes used to detect human γ-actin and mouse γ-actin gene expression in the γ156 cultures were as described in Figure 2 for the γ33 cultures. The filters were then washed and hybridized for the 18s specific oligonucleotide in order to quantitate the RNA levels as described by Lloyd et al. [1992].

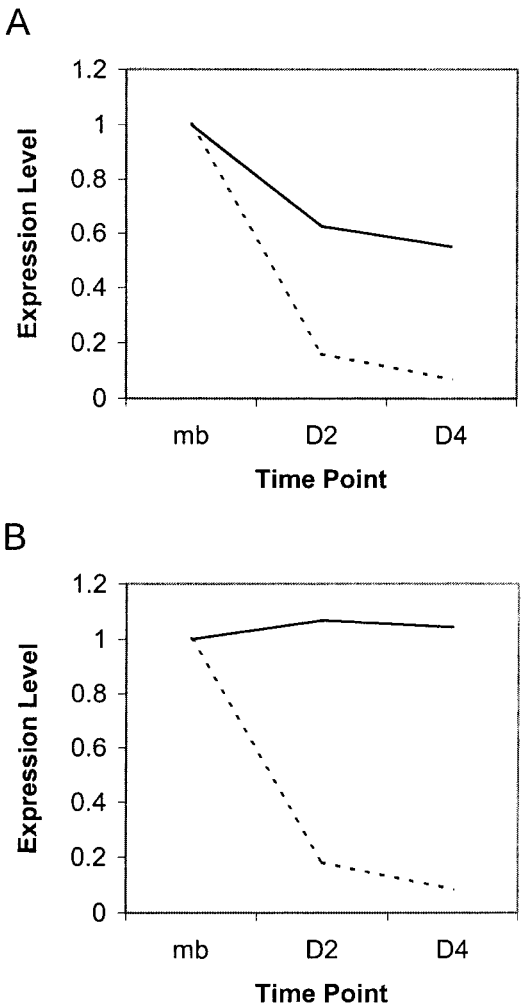

Fig. 6. Actin mRNA levels during myogenesis of pooled human γ-actin gene transfectant cultures with internal deletions. Human γ-actin (—), exogenous CD-8 (—), and mouse γ-actin (- - -) levels were measured in the pooled cultures of (A) γCD-8γ and (B) γ156 during differentiation by scanning densitometry of the autoradiographs in Figure 5. These levels were normalized for loading discrepancies using the measured 18s values [Lloyd et al., 1992]. The expression levels were shown as a factor of those found in the myoblasts that were set at 1.

this regulation. In contrast, however, the mouse γ-actin mRNAs were regulated normally (tenfold reduction) indicating that the cultures did indeed differentiate but that the transfected gene was not capable of normal regulation. This result demonstrates that the 5′ plus 3′ends of the γ-actin gene together could not mimic the regulation of the intact gene, therefore, indicating that γ-actin intronic or exonic sequences are required for appropriate regulation during myogenesis.

We chose to investigate the involvement of intron III in γ-actin regulation during myogenesis since intron III has been highly conserved during evolution [Erba et al., 1988], and a regulatory role for it was previously suggested by Lloyd and Gunning [1993]. The γ156 gene construct, missing intron III, was engineered as described in Lloyd and Gunning [1993]. It is shown in Figure 4. This construct was stably transfected into C2 cells, and the cultures were differentiated (AraC was added with the fusion media), and RNA was harvested from myoblasts at low confluence, 2 and 4 days after fusion medium was added. The expression of the human and the mouse γ-actin genes was examined in these cultures, and the results are shown in Figures 5 and 6B. The transfected gene was not down-regulated at all during myogenesis. In contrast, however, the mouse γ-actin mRNAs were regulated normally indicating that the cultures did indeed differentiate but that the transfected gene was not capable of normal regulation. Therefore, intron III is necessary for down-regulation of the γ-actin gene during myogenesis.

γ-Actin Intron III Containing Transcript is Induced During Myogenesis

During myogenesis of γ-actin gene transfectants, an additional transcript to that of the human γ-actin mRNA was observed after long exposures were taken. A larger transcript was induced during myogenesis in the γ33B5 culture and was found to contain intron III as determined by hybridization with an intron III specific probe (Fig. 7). This induction was not due to a case of poor differentiation since it can be seen that the human γ-actin mRNA levels decreased with myogenesis as expected. Normalization for loading levels (using the 18s rRNA hybridization results) showed an increase in the intron III containing transcript of up to 160% of that in the myoblast while the γ-actin mRNA decreased to 10% of that in the myoblast. Additionally, the induction of this transcript was observed in other γ-actin gene transfectant cultures (γ33-B1, γ58-B2, and γ58-A2; data not shown). We, therefore, propose that removal of intron III may be a limiting step in γ-actin mRNA production. Inhibition of its removal may promote degradation of the transcript with a consequent decrease in mRNA levels.

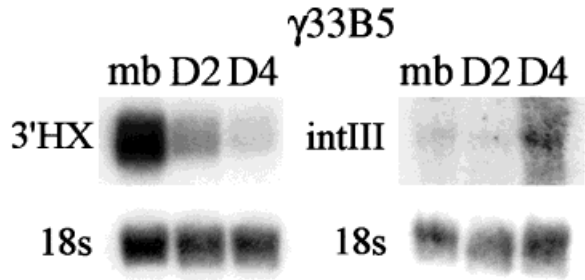

Fig. 7. A γ-actin intron III containing transcript accumulates during myogenesis. Total RNA was isolated from the clonal transfectant line, γ33-B5, during differentiation [Lloyd et al., 1992]. The samples were taken from low confluence myoblasts (mb), 2 days (D2), and 4 days (D4) after fusion medium was added. The RNA was size fractionated on a denaturing agarose gel and transferred to nylon membrane. The filters were hybridized with probes for human γ-actin (γ-3′UT-HX) and γ-actin intron III, and autoradiographed. The filters were then washed and hybridized for the 18s specific oligonucleotide in order to quantitate the RNA levels as described by Lloyd et al., 1992. Five micrograms of RNA was loaded in each lane.

DISCUSSION

Region of the γ-Actin Gene Necessary for Down-Regulation During Myogenesis is not in the 3′End

The 3′UTR and 3′flanking sequence of the γ-actin gene is not necessary for down-regulation during myogenesis, whereas the 3′end of β-actin is necessary for down-regulation [Lohse and Arnold, 1988]. Analysis of expression during myogenesis of cultures of the transfected human γ-actin gene constructs found that normal γ-actin regulation was observed with the 3′end deletion construct of the γ-actin gene. Therefore, the region necessary for down-regulation during myogenesis of the human γ-actin gene does not lie in the 3′UTR or 3′flanking region.

Additionally, the 3′UTR and 3′flanking sequence of the γ-actin gene is not sufficient for down-regulation during myogenesis, whereas the 3′end of β-actin is sufficient for down-regulation [DePonte-Zilli et al., 1988]. The expression of a hybrid gene construct, containing the human α-skeletal actin gene minus its 3′UTR and 3′flanking region attached to the human γ-actin 3′UTR and 3′flanking region, was found to be constitutive during myogenesis [Hardeman, Brennan, and Gunning, personal communication] as expected from the α-skeletal actin promoter studies. Therefore, the human γ-actin 3′end is not sufficient to cause down-regulation.

γ-Actin Intron III Sequence is Necessary for Down-Regulation During Myogenesis

The human γ-actin mRNA was not down-regulated during myogenesis in the transfectant culture γ156, indicating that intron III was necessary for down-regulation. Additionally, intron III containing transcripts accumulated in myogenic cultures of full length γ-actin transfectants. The removal of intron III may be an important step in the control of γ-actin levels. For example, the presence of intron III in the partially processed mRNA may mediate a regulatory step involving a decision to either complete processing to form the γ-actin mRNA or to degrade the transcript. The different regulatory mechanisms of the two non-muscle actin isoforms β and γ would allow the cell alternative pathways to regulate non-muscle actin levels. Since the actin isoforms originated from a gene duplication event, the apparent differences in their modes of regulation may reflect evolutionary pressure to independently regulate these genes.

Although there was a small degree of down-regulation of the transfected gene in the γCD-8γ cultures, the key conclusion was to demonstrate that the 5′ plus 3′ends of the γ-actin gene together could not mimic the regulation of the intact gene, therefore, indicating that γ-actin intronic or exonic sequences are required for appropriate regulation during myogenesis. The deletion of intron III, then, allows us to conclude that this internal sequence is absolutely required for correct regulation.

It will be interesting in future studies to determine the mechanism of action of intron III for γ-actin gene regulation. Answers to questions such as will the regulatory sequence need to be in an intronic context to function or do other introns play a role will add a new facet to the research of gene regulatory mechanisms.

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