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Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells

(myoblast transformation/expression/β-actin regulation)

ANNE SEILER-TUYNS, JUANITA D. ELDRIDGE AND BRUCE M. PATERSON

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

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ABSTRACT **We have introduced the chicken genes for cytoplasmic β-actin, cardiac α-actin, and skeletal α-actin into C2 cells, a murine myogenic cell line, and into L cells by using the simian virus 40-derived vector PSV2-gpt. In each selection, the entire population of transformed cells was analyzed for the expression and regulation of the actin genes by nuclease S1 assay and primer extension. This was compared to the expression of the vector marker Eco-gpt. The β-actin gene is transcribed accurately and efficiently both in L-cells and in undifferentiated C2 cells. In fused C2 cells, β-actin transcripts decrease significantly in parallel with the endogenous level of mouse β-actin mRNA. Eco-gpt RNA levels remain essentially constant during myogenesis. The α-actin genes are correctly expressed at low levels in L cells but at significantly higher levels in the C2 cell background. Unlike the endogenous mouse α-actin gene, this level of expression does not change measurably with myogenesis. The skeletal α-actin gene is expressed poorly in pre- and post-fusion C2 cells, displaying no induction with differentiation. These results suggest that the tissue specificity of expression is maintained but the pattern of gene regulation for the sarcomeric actins is not. Factors in addition to the sequences flanking these genes are important for modulating gene expression during development. The decrease in the levels of β-actin RNA during C2 cell differentiation provides a model system in which to study gene repression during development.**

The differentiation process in muscle tissue culture systems has been well documented by several investigators (1–3). The dividing myoblast withdraws from the cell cycle and fuses to form a multinucleated syncitia that then begins to elaborate all the muscle-specific structural proteins, enzymes, and membrane components characteristic of skeletal muscle (for review, see ref. 4).

During this process, the pattern of actin gene expression changes. In the dividing myoblast β-actin is the predominant cytoplasmic isoform and one cannot detect either of the sarcomeric α-actins. Once fusion has taken place, synthesis of β-actin is greatly decreased and the appearance of the muscle-specific α-actins begins. This pattern is also seen at the mRNA level (5). The type of α-actin and the level of expression appears to be species related. In the chicken embryo, cardiac α-actin is the major sarcomeric isoform (unpublished data), whereas in the mouse, similar amounts of skeletal and cardiac α-actin are expressed in embryonic muscle (6). Regulation of this process is thought to occur at the transcriptional level, because there is no evidence for a change in the half-life of actin mRNA during differentiation (7, 8).

One approach to the study of cellular differentiation involves the introduction of cloned genes into cells that will differentiate in tissue culture and possibly regulate the expression of the genes of interest. To this end, we have isolated the β-actin, cardiac α-actin, and skeletal α-actin genes from the chicken (unpublished results), introduced them into mouse L cells and into mouse muscle cells, and analyzed their expression and regulation.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. The C2 murine myogenic cells were originally isolated and described by Yaffe and Saxel (9). A well differentiating subclone, C2C12, was obtained from H. Blau and was used as described (10). L cells (L929; see ref. 11) were grown in Dulbecco's modified Eagle's medium 10% fetal calf serum. Both C2 cells and L cells were transfected and selected as described (12, 13). Briefly, 48 hr after transformation by the standard calcium phosphate precipitation procedure, cells were transferred into gpt selection medium (14) and grown in that medium for 3 weeks. Only cells that have integrated the vector in their genomes are expected to survive (14). Thereafter, the selective medium was replaced by normal medium and the cells were allowed to differentiate, and actin gene expression and regulation were examined. We have also performed some assays for transient gene expression 3–4 days after transformation. Plasmid preparations and transient expression studies were carried out according to Gorman *et al.* (15). PSV2-gpt was obtained from Bruce Howard (National Cancer Institute).

Cloning of the Chicken Actin Genes. Actin genomic clones were identified as described (16). β-actin was identified with the 590-base-pair (bp) *Hin*dIII fragment (specific for the 3′-untranslated portion of chicken β-actin) in the pBR322 plasmid pA3 from Cleveland *et al.* (17). The 800-bp *Hin*dIII/*Bgl* II fragment from the cardiac α-actin gene (see Fig. 1) specifically selected mRNA coding for α-actin (unpublished data). Subsequently, the sequence of the 5′-coding exon unambiguously identified this as the cardiac α-actin gene. Comparing our data with those of Fornwald *et al.* (18), we established the identity of our independent isolate of the skeletal α-actin. The *Eco*RI fragments containing the cardiac α-actin gene [7.0 kilobases (kb)] and the β-actin gene (8.2 kb) were cloned directly into the unique *Eco*RI site in PSV2-gpt. In the case of the skeletal α-actin gene, the 6.6-kb *Hin*dIII fragment was converted to an *Eco*RI fragment prior to insertion, using *Hin*dIII/*Eco*RI adaptors (Collaborative Research, Waltham, MA).

Nuclease S1 Analysis. Uniformly labeled single-stranded 3′-noncoding DNA probes specific for each of the chicken actin genes were prepared from actin sequences cloned in the single-stranded phages, M13-8 or M13-9 (19, 20). The following fragments were used to generate the specific probes: β-actin probe, the 590-bp *Hin*dIII fragment from plasmid pA2 [Cleveland *et al.* (16)]; cardiac α-actin probe, the 800-bp

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Abbreviations: bp, base pair(s); kb, kilobases.

*Hin*dIII/*Bgl* II fragment from the cardiac α-actin genomic clone (Fig. 1) (this fragment is either spanning an intron or there are two poly(A) addition sites; two fragments are protected from nuclease S1 digestion); skeletal α-actin probe, the 825-bp *Hin*dIII/*Bam*HI fragment from the p(α)-actin-1 cDNA plasmid [Ordahl *et al.* (21)]. Eco-gpt transcripts were measured with the 450-bp *Pvu* II/*Bgl* II fragment cut from PSV2-gpt and labeled at the 5′ end of the *Bgl* II site or at a *Hinf*I site 30 bp inside the *Bgl* II site with [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq) and polynucleotide kinase (22). Then, 5000 cpm (Cerenkov) of probe was precipitated with 10 μg of total RNA and hybridized at 45°C for 12–14 hr in 10 μl of 80% formamide/40 mM Pipes, pH 6.4/0.4 M NaCl/1 mM EDTA. After incubation, the mixture was treated with nuclease S1 as described (23) and run on 7 M urea/6% or 8% acrylamide sequencing gels (20).

Primer Extension. Extension reactions were carried out with 10 μg of total RNA as described (24). Primers for each of the actin genes were isolated from the 5′-coding exon of each gene and labeled at the appropriate 5′ terminus with [γ-^{32}P]ATP (5000 Ci/mmol) and polynucleotide kinase (22). The β-actin primer is the 252-bp *Nco* I/*Bgl* II fragment from plasmid pA1 [Cleveland *et al.* (17)]; the cardiac α-actin primer is the 138-bp *Ava* II/*Bst*NI fragment from the 5′-coding exon of the cardiac α-actin gene; the skeletal α-actin primer is the 54-bp *Fok* I/*Ava* I fragment from the 5′-coding exon of the skeletal α-actin gene. Eco-gpt transcripts were extended with the 120-bp *Hin*dIII/*Bgl* II fragment cut from PSV2-gpt and labeled at the *Bgl* II end as for the nuclease S1 probe; 5000 cpm (Cerenkov) of the various primers was hybridized with 10 μg of total RNA as described for the nuclease S1 assays. Hybrids were ethanol-precipitated and cDNA was synthesized with reverse transcriptase and unlabeled dNTP as described (24). The extension products were analyzed on gradient gels (25) or on the same gels used for the nuclease S1 assays.

RNA Purification and RNA Blot Analysis. RNA used for the nuclease S1 assays and primer extensions was prepared as described (12, 26). RNA blots were prepared according to Alwine *et al.* (27).

RESULTS

Introduction of the Chicken Actin Genes into Myogenic C2 Cells and L Cells. The PSV2-gpt constructs containing the chicken actin genes are illustrated in Fig. 1.

Although L cells can be readily transformed by the calcium phosphate procedure, it was necessary to establish that C2 cells would (*i*) continue to fuse after calcium phosphate treatment and selection, and (*ii*) regulate the β- to α-actin transition. As shown in Fig. 2 *A* and *B*, fusion continues normally after transformation, selection for nine passages, and fusion induction in C2 cells containing the cardiac α-actin PSV2-gpt construct. In addition, the β- to α-isoform shift occurs with cell fusion (Fig. 2*C*). The efficiency of transformation is approximately 100–600 transformants per 5×10^6 cells, using 10 μg of plasmid DNA. A minimum of three independent transformations for each construct was carried out and the results reported here represent the consensus.

We analyzed the entire population of transformed cells, rather than cloned isolates, in order to facilitate future studies with structural variants of the actin genes. This approach minimizes position effects, copy number problems, and artifacts due to the rearrangement of the genes during transformation and selection. Levels of transient expression were determined in some experiments as described.

Expression of the Chicken Actin Genes in C2 Cells and L Cells. The chicken actin genes introduced into the different mouse cell backgrounds were initially tested for expression and regulation using a nuclease S1 assay with 3′-specific noncoding probes to determine the level of chicken actin transcripts in the presence of homologous mouse mRNA sequences. This was compared to the gpt expression from the vector. Previous reports (4, 17, 28) have established that the region of maximal sequence divergence resides in the 3′-noncoding portion of the actin mRNA transcripts. The uniformly labeled single-stranded nuclease S1 probes used in this study contain the 3′-noncoding portion of each chicken actin gene. Hybridization of these probes with homologous RNA, followed by nuclease S1 digestion, produce discrete DNA fragments characteristic for each of the chicken actin genes (Fig. 3). No discrete fragments were observed with mRNA prepared from C2 cells or L cells that did not contain one of the chicken actin genes.

The intensity of the protected DNA fragment provides a quantitative measure of the actin mRNA transcripts expressed in the different transformed mouse cell backgrounds, because the probe is in sequence excess. As shown, all of the chicken actin genes are functional in both types of mouse cells, because specific transcripts can be detected by nuclease S1 analysis (Fig. 3). Furthermore, the level of expression is dependent on the particular mouse cell background. Both of the chicken sarcomeric α-actins are expressed poorly in L-cells, whereas cytoplasmic β-actin transcripts are measurably more abundant. In the myogenic C2 cells the cardiac α-actin and β-actin genes are efficiently

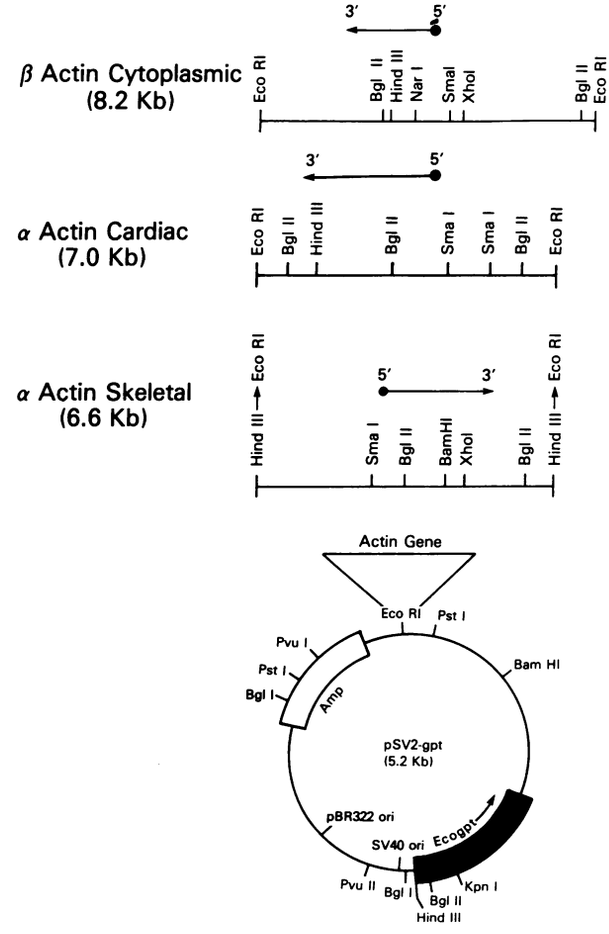

FIG. 1. PSV2-gpt constructs containing the chicken actin genes. The cytoplasmic β-actin, cardiac α-actin, and skeletal α-actin genes were inserted into the unique *Eco*RI site in the vector in the orientation indicated by the large arrow. The 5′ end of the arrow marks the position of the initiator methionine in the sequence and the extent of the arrow indicates the span of the coding exons. The direction of transcription for gpt is shown by the small arrow. The *Xho* I and *Sma* I sites in β-actin are reversed.

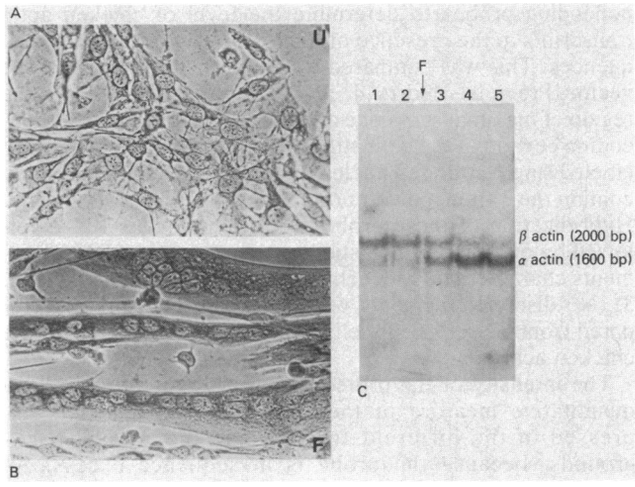

FIG. 2. The effect of transformation and selection on fusion and the β- to α-actin transition in C2-cells transformed with the PSV2-gpt cardiac α-actin construction. (*A*) Stable transformants prior to fusion induction. (*B*) Sixty hours after fusion induction. (*C*) Effect of cell fusion on the expression of the endogenous mouse α-actin and β-actin genes. Ten-microgram portions of total RNA harvested at 24-hr intervals after fusion induction of C2 cells were fractionated on a 1.5% agarose/glyoxal gel, blotted onto DBM-paper and probed with a nick-translated equimolar mixture of the three chicken actin genes. The positions of the endogenous mouse α-actin (1600 bp) and β-actin (2000 bp) mRNA transcripts are shown. Lanes: 1, zero time; 2, 24 hr; 3, 48 hr; 4, 72 hr; 5, 96 hr after fusion induction. F marks the onset of fusion.

transcribed in the undifferentiated cell cultures, whereas the skeletal α-actin gene is transcribed at a much lower level. After cell fusion in the C2 cultures, the levels of RNA from the sarcomeric actin genes remains essentially unchanged; however, there is a substantial decrease in the level of β-actin transcripts. This decrease is not accompanied by a similar decrease in the vector Eco-gpt transcript level: densitometric scans reveal β-actin transcripts decrease by a factor of 7 to 8, whereas gpt varies no more than 2-fold (Fig. 4). Thus, by nuclease S1 analysis, all of the chicken actin genes are functional in a mouse cell background. Only the β-actin transcript level decreases in parallel with the endogenous gene (Fig. 2*C*) during differentiation of the C2 cells. An induction of either of the sarcomeric α-actin transcripts is not detectable even though the endogenous mouse α-actin mRNA transcripts increase with differentiation (Fig. 2*C*).

The Actin Transcripts Originate from the Appropriate Chicken Actin Gene Promoter Regions. To determine whether the various actin gene transcripts were initiated correctly from their respective promoters, a primer-extension assay was used. An end-labeled restriction fragment from the 5′-coding exon of each actin gene was used as a primer for cDNA synthesis on L cell and C2 cell RNA templates. Even though there is potential homology within the chicken and mouse 5′ exons, the length of the primer extended fragment is likely to be unique for each gene. This cross homology is only apparent in the case of the β-actin genes and, when observed, serves as an additional control for the regulated expression of the mouse β-actin gene during C2 cell differentiation. As shown in Fig. 4, the RNA transcribed from the cardiac β- and α-actin genes, when used as template in the primer extension assay, produces extended fragments identical in length to those synthesized on control RNA templates. Even the polymerase pause regions seen with the control β-RNA are noted for the transcripts produced from the transfected chicken β-actin gene. The low levels of expression from the skeletal α-actin gene in C2 cells precluded routine primer extension analysis. However, results with long term exposures show the correct 5′ end for these transcripts (data not shown).

The intensity of the extended primer band is proportional to the transcript level for each of the actin genes, because the primer is in sequence excess. The 5′ analysis reveals a pattern similar to the one shown in the 3′ nuclease S1 studies. Low levels of the cardiac α-actin transcripts are detectable in the L cells; however, the β-actin gene RNA is severalfold more abundant. In the C2 cells, both β- and α-actin cardiac RNAs are well expressed in the dividing myoblasts. After cell fusion, the level of β-actin RNA again is seen to decrease (by a factor of 7 to 8, as shown by densitometric

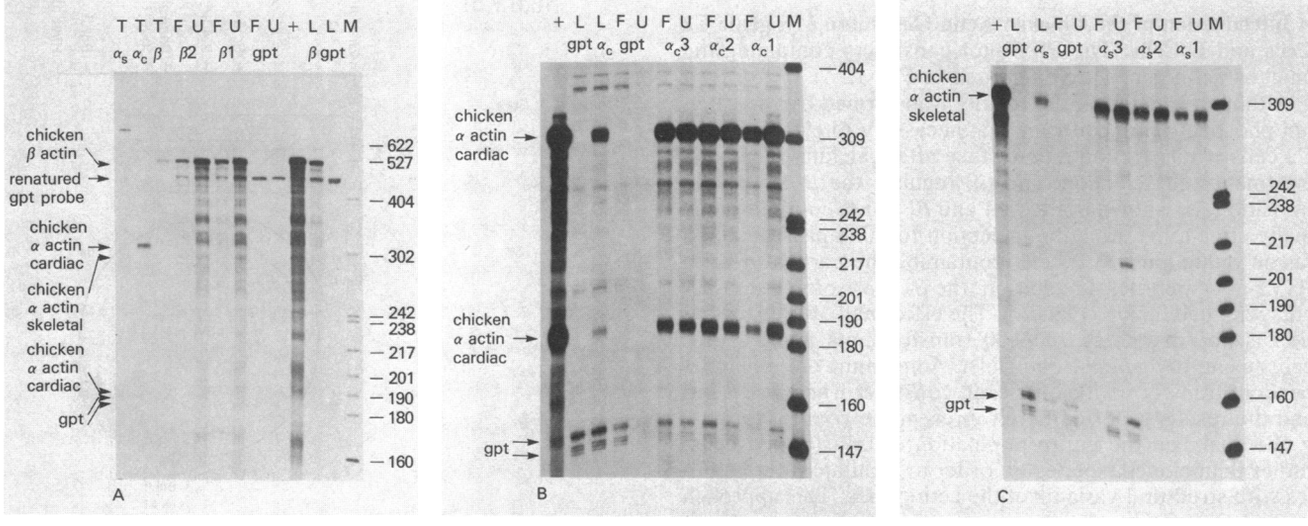

FIG. 3. 3′ nuclease S1 analysis of the RNA from transformed L cells and C2 cells containing the PSV2-gpt actin constructs. (*A*) Transient assays of α- and β-actin genes and the β-actin transformants. The lanes marked T are the transient expressions of the indicated actin gene hybridized with its corresponding probe using RNA extracted 60 hr after fusion induction. (*B*) The cardiac α-actin transformants. (*C*) The skeletal α-actin transformants. M, *Hpa* II digest of PBR 322; L gpt, L cells transformed with PSV2-gpt; (Lβ, Lαc, Lαs) L cells transformed with the β-actin, cardiac α-actin, and skeletal PSV2-gpt α-actin constructs, respectively; +, the positive control with chicken muscle RNA; U, unfused C2-cell RNA; F, fused C2-cell RNA. The numerical subscripts indicate independent transformation experiments. *A* was exposed 12 hr, and *B* and *C* were exposed 36 hr. Longer exposures of *A* show the gpt, but the β-actin transcripts are then overexposed. The same RNA is used in the primer extensions in Fig. 4, where the gpt can be seen. In Fαc1 a portion of the RNA was lost, which accounts for the decreased signal.

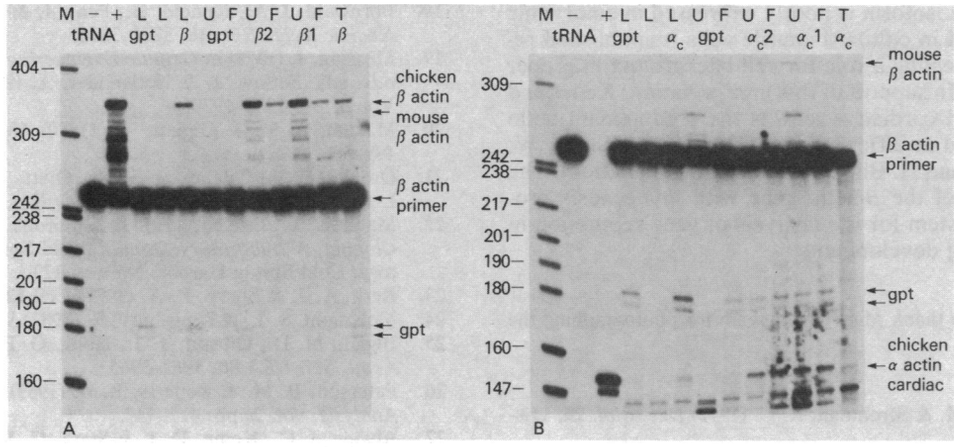

FIG. 4. Primer-extension analysis of the 5′ ends of the chicken actin transcripts expressed in L cells and C2 cells. (*A*) The β-actin transformants. (*B*) The cardiac α-actin transformants. − tRNA, extension with 10 μg of tRNA; +, positive control with chicken muscle RNA; L gpt, L-cells transformed with PSV2-gpt; L, L cells alone; Lβ, $L\alpha_c$, L cells transformed with the β-actin and cardiac PSV2-gpt α-actin constructs, respectively; U, unfused C2-cell RNA; F, fused C2-cell RNA; T transient expression of the indicated actin gene in C2-cells. The numerical subscripts indicate independent transformation experiments. β-actin primer was included as a control for the endogenous mouse β-actin shift and is clearly noted on longer exposures. All assays were set up with 10 μg of total RNA. Exposure was for 15 hr.

scan), whereas the cardiac α-actin transcripts remain unchanged. With long exposures, the correct skeletal α-actin transcripts can be seen weakly in the RNA from fused cultures (data not shown). The level of gpt transcripts, as expected, does not change substantially (2-fold variability by densitometric scan), during differentiation of the C2 cells. This implies that the decrease in the concentration of β-actin transcripts with cell fusion is not a property of all the genes on the β-PSV2-gpt plasmid, but rather is intrinsic to the β-actin gene.

Preliminary studies on the transient expression of the PSV2-gpt actin constructs in C2 cells (Figs. 3 and 4) demonstrate that the three actin genes have 5′ and 3′ ends indistinguishable from the authentic transcripts (primer-extension analysis of the skeletal α-actin transient expression is not shown). The level of transient expression 60 hr after transformation and fusion for each of the chicken actin genes, when compared to gpt, reflects the relative levels of expression seen in the stably transformed C2 cells; i.e., β-actin transcripts are well expressed, α-actin cardiac transcripts are less abundant, and α-actin skeletal transcripts are on the threshold of detection.

DISCUSSION

Even though a variety of cloned eukaryotic genes are expressed in heterologous cell backgrounds after DNA-mediated gene transfer (29–32), and a few of these appear to be transcriptionally activated given the proper induction signal (33, 34), cell background plays an important role in the regulation of tissue-specific gene expression (33, 35–37). The myogenic culture system provides a convenient example of tissue formation *in vitro*, either from primary cell cultures or from established cell lines. The introduction of the chicken actin genes into mouse muscle cells, therefore, provides the opportunity to define the regulatory mechanisms governing the expression of the various actin isoforms during myogenesis.

The preliminary results reported here clearly demonstrate that the chicken actin genes are transcriptionally active in two types of mouse cells and produce RNA templates with the correct 5′ and 3′ termini. Whether these templates are translationally active remains unknown.

The level of expression from the different chicken actin genes in mouse cells is a reflection of the cell background and mirrors, to a degree, the situation *in vivo*. Both the sarcomeric actins are expressed in L cells (fibroblasts) at significantly lower levels than the β-actin gene, which is active in most dividing cells of different germ layer origin. By comparison, all the chicken sarcomeric actin gene transcripts are more abundantly expressed in C2 cells, the normal muscle cell background for these genes. This again suggests that the quantitative differences in the levels of expression are tissue related.

As shown in other systems (38, 39), the orientation of the insert is probably not a major factor in the regulation of expression for the chicken actin genes, because the genes are being transcribed from their respective promotors, as judged by primer extensions, and are flanked on their 5′ and 3′ sides by a similar length of sequence. The level of expression may, however, depend on orientation, and this is under study.

The striking result is the decrease in the level of the β-actin transcripts in parallel with the endogenous mouse β-actin mRNA during C2 cell differentiation. All of the transformations to date have given the same result, with a decrease by a factor of 7 to 8 in β-actin transcripts relative to a 2-fold variation in GPT expression. The results of Singer and Kessler-Kekson (8, 9) suggest that the quantitative differences in β-actin gene expression during myogenesis are not explained by differential mRNA stability since the half-life of actin mRNA changes very little during cell fusion ($t_{1/2}$ = 20–25 hr). Nuclear transcription assays will be used to confirm this result (33).

Transcription from both of the sarcomeric genes is unchanged with C2 cell differentiation, even though the endogenous mouse α-actin transcripts increase (Fig. 2*C*). A variety of explanations for this difference in the exogenous and endogenous genes can be considered. Species differences may play a role as mouse factors may not recognize the chicken sequences in the α-actin genes. However, our results obtained with the chicken β-actin gene, heterokaryon studies (11), and numerous examples of regulation in heterologous systems (29–32, 34, 38) do not support this interpretation. Likewise, the presence of the simian virus 40 enhancer sequence in the PSV2 gpt vector may influence the expression of the inserted genes (40). However, chromosomal location and/or structure (41, 42), the extent of flanking DNA sequence (24), and methylation (43) are likely to play more substantial roles in the regulation of tissue-specific gene expression with differentiation. The skeletal α-actin is expressed at rather low levels in C2 cells and L cells, and it is not regulated in differentiating C2 cells. This may reflect the

fact that the adult isoform is poorly expressed in embryonic muscle tissue and in cultured muscle cells (unpublished results), again suggesting a role for cell background in proper gene expression. In support of this interpretation, Kedes and co-workers report cardiac α-actin is the predominant actin isoform expressed in differentiated C2 cells (L. Kedes, W. Bains, P. Ponte, and H. Blau, personal communication). The down-regulation of the β-actin gene with myogenesis provides a model system for the analysis of gene repression in eukaryotes during development.

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