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Developmentally regulated expression of chimeric genes containing muscle actin DNA sequences in transfected myogenic cells

Danielle Melloul, Batya Aloni, Joseph Calvo[1], David Yaffe and Uri Nudel*

Department of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel and [1]Department of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA

*To whom reprint requests should be sent
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A recombinant plasmid containing 2/3 of the rat skeletal muscle actin structural gene plus 730 bp of its 5′ flanking region, spliced to the 3′ end of the human ε-globin gene, was introduced into cells of the rat myogenic line L8. Myogenic clones carrying the actin/globin chimeric gene were isolated. In many of these clones, the expression of the gene greatly increased during differentiation (up to >50-fold) and, in some clones, the amount of the chimeric gene transcripts in the differentiated cultures exceeded that of the native muscle actin gene transcripts. Furthermore, the temporal relation between differentiation of the cultures and the accumulation of the transcripts from the transferred genes was very similar to that of the native skeletal muscle actin gene, suggesting a similar mechanism of regulation. Endonuclease S1 analysis indicated a correct initiation and termination of the mRNA but suggested that a fraction of the chimeric actin/globin transcripts was not properly processed. To test whether the increased expression of the transferred gene which occurred during differentiation was determined by DNA sequences in the 5′ region of the muscle actin gene, a plasmid (pα-CAT) containing 730 bp of the 5′ flanking region of the rat skeletal muscle actin gene (plus the exon of the 5′ untranslated region, and 25 bp of the first intron), spliced to the bacterial structural gene coding for chloramphenicol acetyl transferase (CAT), was constructed and introduced into L8 cells. In the majority of the isolated clones containing this plasmid, CAT activity increased many-fold during differentiation. Very little or no increase in CAT activity during differentiation was observed when a plasmid containing the CAT gene spliced to the 5′ region of the cytoplasmic β-actin gene (pβ-CAT) was used for transformation. The results demonstrate that the increase in CAT activity during differentiation in clones containing the plasmid pα-CAT was determined by DNA sequences contained in the fragment derived from the skeletal muscle actin gene.

Key words: muscle actin genes/regulated expression/myogenic cells/transfection

Introduction

The cell type and stage specificity of expression of genes might be determined by the primary structure of the gene and its flanking regions, by the location of the gene in a specific chromosomal domain, by modifications of the DNA occurring during embryogenesis and development, or by a combination of these factors. We have investigated whether the information for stage-specific activation of a gene expressed during myogenesis is an intrinsic property of the gene, i.e., encoded in the DNA sequence of the gene and its flanking region. To this end, we tested the expression of derivatives of a skeletal muscle actin gene during cell differentiation, following stable integration into myogenic cells.

During terminal differentiation of muscle cells, mononucleated myoblasts cease dividing and fuse into multinucleated fibers. This is associated with the accumulation of large amounts of muscle-specific mRNAs and proteins. We have previously shown that genes coding for muscle-specific proteins are not preferentially sensitive to DNase I in proliferating mononucleated cells of the myogenic cell line L8, but they become so during the transition to the stage of cell fusion. These data indicate that the activation of the transcription of these genes occurs during the terminal differentiation process (Carmon *et al.*, 1982). Thus, this cell system appears suitable for following the activation during differentiation of tissue- and stage-specific genes, stably integrated into the mononucleated proliferating precursor cells. The results show that, in many clones derived from independently transfected cells, the transferred gene is expressed in an apparently developmentally regulated manner.

Results

The construction of a chimeric actin/globin gene and its introduction into myogenic cells

To distinguish the transcripts of the transferred gene from those of the endogenous skeletal muscle actin gene, we constructed a chimeric rat skeletal muscle actin/human ε-globin gene. The chimeric gene comprised ~2/3 of the 5′ region of structural gene coding for rat skeletal muscle actin (Zakut *et al.*, 1982) plus 730 bp upstream from the cap site, and ~1/3 of the 3′ region of human ε-globin gene, including the polyadenylation site, and 172 bp flanking the 3′ untranslated region (Figure 1).

Proliferating L8 mononucleated cells were co-transfected with this plasmid and $pIPB_1$, a plasmid containing a neomycin resistance gene, and selection was made in the presence of the neomycin derivative G418, as described in Materials and methods. Twenty neomycin resistant clones, each originated from a single transfected cell, were isolated. Ten of the clones were tested by Southern blot hybridization and were all found to contain the chimeric gene. The estimated copy number of the donor gene varied between 1 and 50 (one clone (#8) had >500 copies; Table I).

Expression of the actin/globin chimeric gene during myogenesis

To investigate the expression of the actin/globin chimeric gene in the isolated clones, cells of nine clones were plated and grown under conditions which favour cell proliferation without cell fusion. RNA was extracted from cultures containing monolayers of proliferating mononucleated cells. Parallel cultures were induced to differentiate by changing the medium (as described in Materials and methods) and were

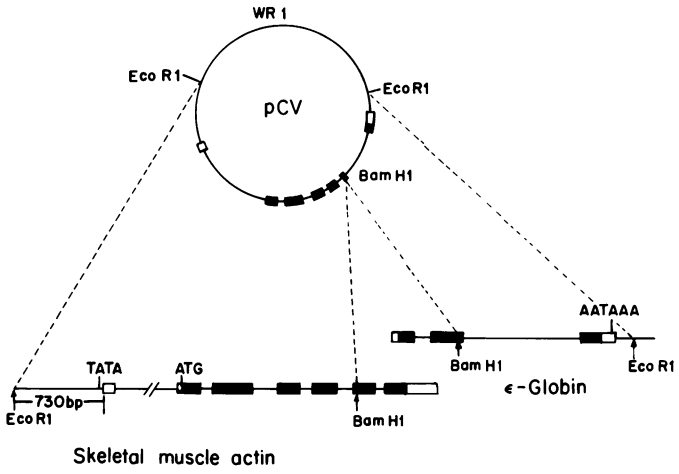

Fig. 1. The structure of the plasmid pCV containing the chimeric rat skeletal muscle actin human ϵ-globin gene. Plasmid pCV was constructed as described in Materials and methods. Empty bars represent 5′ and 3′ untranslated regions. Solid bars represent coding regions. WR1 = plasmid pWR_1.

Table I. Expression of actin/globin gene in transfected cells

Clone #	Copy number of actin/globin gene	Expression*		Induction fold
		Mononucleated	Fibers	
46	25–50	0.1 –0.3	3–10	50–80
36	10–20	0.05–0.2	2– 3	20–40
1	N.D.	0.05–0.1	>1	>20
51	2–3	0.02–0.05	0.1–0.2	5–10
8	>500	U.D.	0.02–0.05	>3
49	3–5	0.1 –0.2	0.3–0.6	2–6
47	3–5	U.D.	0.05–0.1	>3
33	1–2	U.D.	U.D.	
20	1–2	U.D.	U.D.	

*An estimation based on the intensity of bands formed on the fluorograms of the endonuclease S1 analysis of the transcription products, given in arbitrary units. 1 = The intensity of the bands formed by the probe protected by the native muscle actin mRNA in differentiated cultures of the same clone. The numbers indicate the range of values obtained in several experiments. The range of fold of induction includes values obtained also by using the ϵ-globin derived DNA probe.
U.D. = Undetectable.
N.D. = Not determined.

harvested after the formation of a dense network of multinucleated fibers (72 h after change of medium). The presence of the chimeric gene transcripts in the RNA preparation from these cultures was determined by endonuclease S1 mapping analysis, using a probe prepared from a plasmid p749 containing a rat skeletal muscle actin cDNA sequence (Katcoff *et al.*, 1980; Shani *et al.*, 1981a) as described in Figure 2. Since the endogenous actin mRNA protects the entire probe and the chimeric actin/globin mRNA protects only part of the probe, it was possible to determine the presence of transcripts from both genes in the same hybridization reaction using a single probe (Figure 2). As shown in Figure 3, and Table I, a clear increase in the amount of the actin/globin gene transcripts was found in five out of the nine clones tested. In two other clones small amounts of the actin/globin gene transcripts were detected in RNA extracted from differentiated cultures but not in RNA from mononucleated cells. In

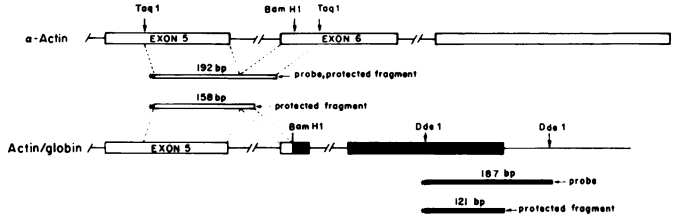

Fig. 2. The probes used for S1 analysis of the expression of the skeletal muscle actin and the actin/globin chimeric genes. The DNA probes used for S1 analysis are shown in relation to the skeletal muscle actin gene and the actin/globin chimeric genes. Thick empty bars represent exons with skeletal muscle actin DNA sequence. Thick black bars represent exons with ϵ-globin DNA sequence. The probes and the protected fragments are marked in the figure (thin bars). The probe containing actin DNA sequence was prepared from the actin cDNA clone p749 (Katcoff *et al.*, 1980; Shani *et al.*, 1981a). The probe containing human ϵ-globin DNA sequence (thin black bar) was a 187-bp long DNA fragment extending from the *Dde*I site in the 3rd exon of the globin gene to the *Dde*I site located 97 bp downstream from the polyadenylation signal. α-actin = skeletal muscle actin. Actin/globin = the junction region of the chimeric gene.

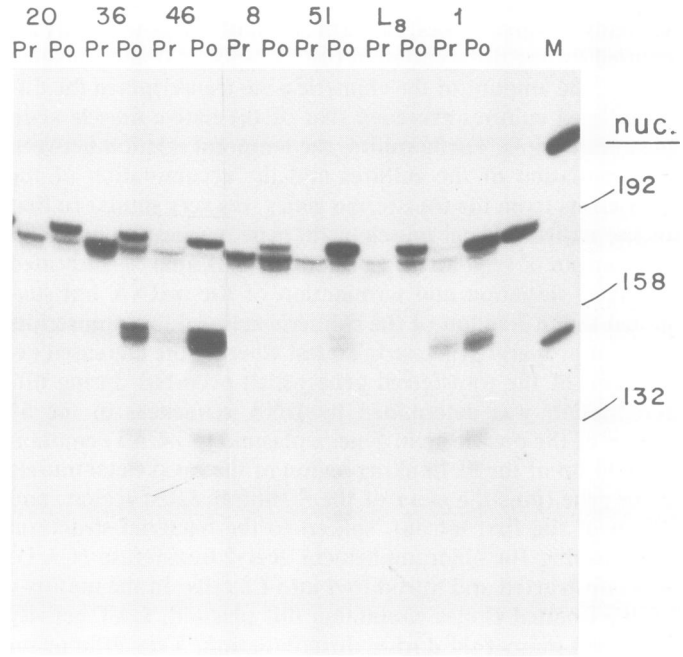

Fig. 3. S1 endonuclease analysis of RNA from clones containing the actin/globin gene. Samples containing 40 μg of total RNA isolated from undifferentiated (Pr) and differentiated (Po) cultures of the indicated clones were hybridized with ~20 ng of ^{32}P-labeled DNA from the plasmid p749 (as described in Figure 2). After hybridization for 16 h at 53°C, each sample was treated for 30 min at 37°C with 500 units endonuclease S1. The hybrids were then precipitated, electrophoresed on a polyacrylamide/urea gel, and fluorographed. The protected DNA fragment, two nucleotides shorter than the probe is the result of a certain degree of reannealing of the probe during the hybridization. Skeletal muscle actin mRNA protects the entire probe (192 nucleotides). The chimeric gene mRNA protects a fragment of 158 nucleotides.

two clones, L8-36 and L8-46, the amount of DNA sequence protected by the chimeric gene product increased >40 times during differentiation. The amount of the actin/globin gene products in differentiated cultures of these two clones was 2–5 times greater than that of the native skeletal muscle actin mRNA.

In all RNA preparations in which the chimeric gene transcripts were detected, some small DNA protected fragments

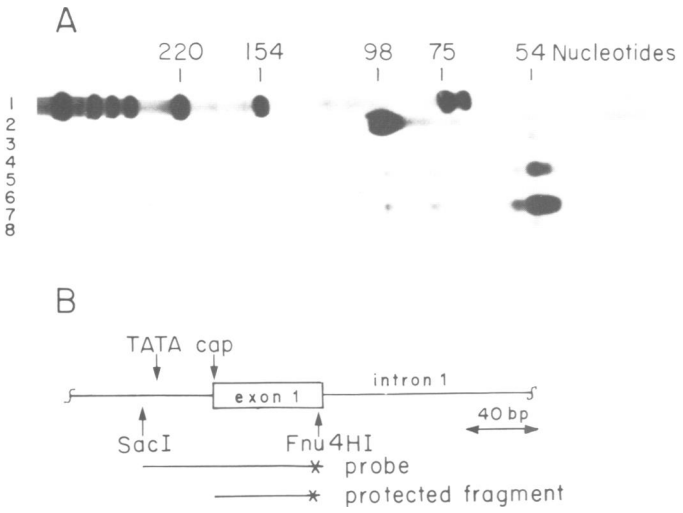

Fig. 4. Endonuclease S1 analysis of the 5′ end of the actin/globin chimeric mRNA. **(A)** The endonuclease S1 analysis was done as described in the legend to Figure 3 (hybridization at 52°C) using the end-labeled *Fnu*4HI-*Sac*I DNA fragment described in **B**, as a probe and RNA preparation from undifferentiated **(4,6,8)** and differentiated **(3,5,7)** L8, L8-36 and L8-46 cells respectively. **(B)** The 5′ end region of the rat skeletal muscle actin gene, the end-labeled probe and protected fragment.

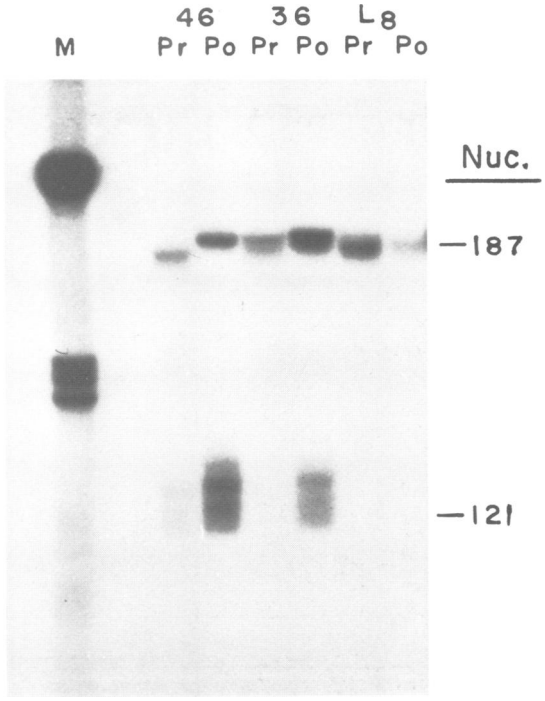

Fig. 5. S1 endonuclease analysis of the 3′ end region of the actin/globin chimeric mRNA. The end-labeled *Dde*I-*Dde*I 187-bp long DNA fragment described in Figure 2 was used as a probe. The RNA samples were from undifferentiated (Pr) and differentiated (Po) L8, L8-36 (36) and L8-46 (46) cells. Hybridization was done at 52°C and S1 analysis was done as described in legend to Figure 3.

were detected. The main one was a 132 nucleotide fragment. The size of this fragment fits the expected size of a product of S1 digestion of a hybrid formed between the probe and actin/globin gene transcripts in which either the intron 3′ to the 5th exon was not removed, or the small exon, containing the junction between actin and globin sequences, was removed during the processing of RNA (Figure 2).

The transcription of the actin/globin chimeric gene is initiated at the authentic cap site of the actin gene

The cap site of the actin/globin chimeric mRNA was determined by endonuclease S1 mapping analysis. The probe used was a 98-bp long end-labeled DNA fragment, extending from the *Sac*I site 14 bp upstream from the TATA box to the *Fnu*4HI site in the exon in the 5′ untranslated region of the actin gene. RNA isolated from differentiated parental L8 cells protected a DNA fragment 54 nucleotides long, extending from the labeled site to the cap site of the skeletal muscle actin gene (Figure 4). RNA from both undifferentiated and differentiated cells of the transfected clones L8-36 and L8-46 protected the same 54 nucleotides long fragment. The amount of 5′ probe protected by RNA from differentiated L8-36 and L8-46 was at least 10-fold greater than that protected by RNA from differentiated L8 cells. [When we used another preparation of RNA from L8 cells, a ratio of ~8 was obtained between the amounts of probe protected by RNA from differentiated L8-46 and L8 cells (not shown).] In accordance with the results shown in Figure 3, this experiment shows the presence of 5′ actin RNA sequences also in RNA from undifferentiated L8-36 and L8-46 cells but not in RNA from undifferentiated L8 cells. Therefore the transcription of the chimeric actin/globin gene in differentiated as well as undifferentiated L8-36 and L8-46 cells is initiated at the authentic cap site of the actin gene. It should be noted that the ratio between the amount of 5′ sequences in differentiated L8-46 and L8 cells is greater than that obtained using the probe derived from p749. It is possible that the discrepancy between these numbers is due to the presence in L8-36 and L8-46 cells of substantial amounts of the chimeric gene products which are initiated at the cap site but are not processed correctly (as indicated in Figures 3 and 6a).

The actin/globin chimeric mRNA uses the polyadenylation signal of the globin gene

A DNA fragment 187 bp long, extending from the *Dde*I site in the third exon of the ε-globin gene to the *Dde*I site located 97 bp downstream from the putative polyadenylation signal of the gene (Baralle *et al.*, 1980), was end-labeled by filling in with reverse transcriptase (Figure 2). The labeled probe was hybridized with RNA of undifferentiated and differentiated L8, L8-36 and L8-46 cells. After treatment with endonuclease S1 the samples were electrophoresed on a polyacrylamide/urea sequencing gel. As seen in the fluorogram of the gel (Figure 5), RNA from differentiated L8-36 and L8-46 cells protected a DNA fragment ~121 nucleotides long. A much smaller amount of the same fragment is protected by RNA from mononucleated myoblasts of the two clones. This clearly demonstrates that the chimeric actin/globin mRNA is terminated at the authentic globin poly(A)-addition site.

The correlation between the accumulation during differentiation of the native muscle actin mRNA and the transcripts of the transferred gene

We have previously described the kinetics of accumulation of actin mRNA during the process of muscle cell differentiation and showed that the main increase in the amount of the skeletal muscle actin mRNA occurs during the stage of formation of multinucleated fibers (Shani *et al.*, 1981b). To test the temporal relationship between the accumulation of the RNA transcripts of the actin/globin chimeric gene and of the endogenous actin mRNA, cultures of the transfected clone

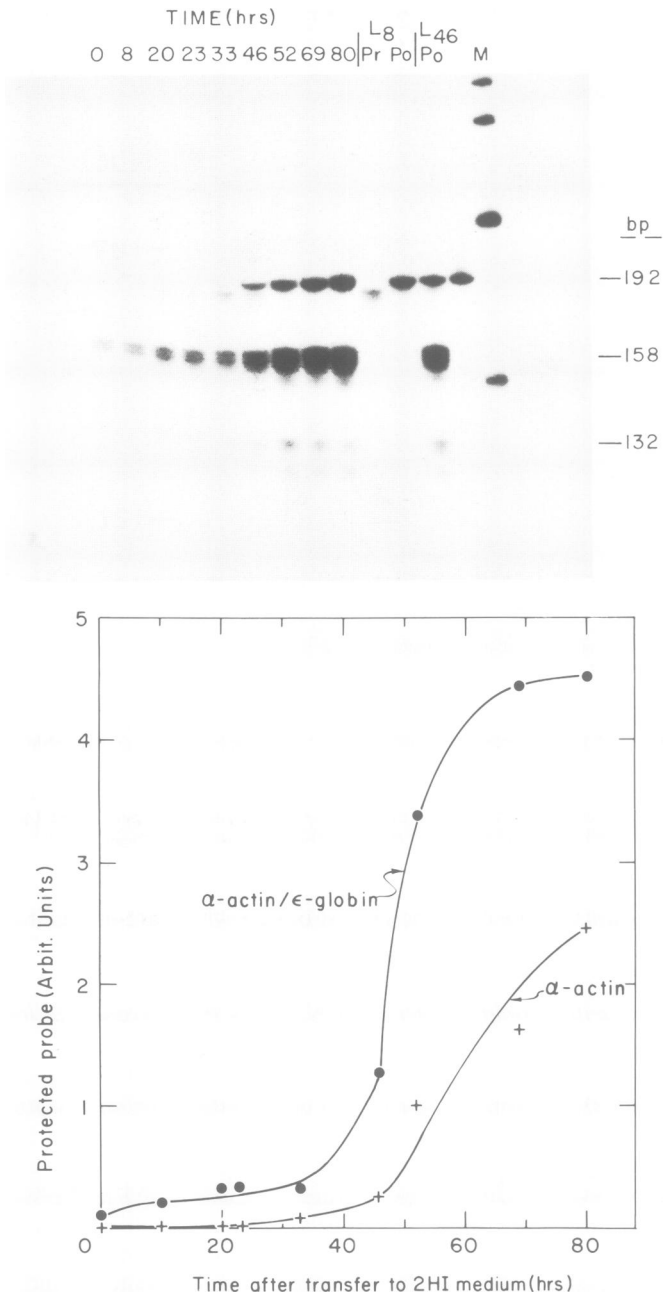

Fig. 6. The kinetics of accumulation during differentiation of actin/globin gene products and the native skeletal muscle actin mRNA in L8-46 cells. **(A)** L8-46 cultures approaching confluency were stimulated to differentiate by changing the medium to 2HI medium (0 time). At the indicated times RNA was extracted from the cultures and analyzed by the S1 endonuclease method (as described in the legend to Figure 3). Cell fusion began at ~36 h. **(B)** The autoradiograms from the S1 nuclease analysis were scanned by a Beckman DU8 spectrophotometer (using exposures which were in the linear range of the film). The relative intensities of the signals formed by the probes protected by the skeletal muscle actin mRNA (192 nuc) and by the chimeric major mRNA (158 nuc) are shown in the figures.

L8-46 were plated and induced to differentiate. Aliquots of cultures were harvested at intervals of several hours and RNA was extracted and analyzed by hybridization to the p749 probe. Following digestion with S1 nuclease, the protected DNA was fractionated on a polyacrylamide/urea sequencing gel and fluorographed. The relative amounts of protected DNA fragments were determined by scanning the fluorograms with a densitometer. As seen in Figure 6a and 6b, small amounts of transcripts of the actin/globin gene were detected in RNA extracted during the first 30 h following the change of medium. However, concomitant with the accumulation of the endogenous muscle actin mRNA transcripts, there was also a rapid increase in the amount of the actin/globin gene transcripts. The level of the actin/globin gene products at 70 h after the induction of differentiation, was more than 50-fold higher than the background level found before cell fusion, and was more than twice as high as the amount of the transcripts of the endogenous skeletal muscle actin gene. The similarity in the kinetics of accumulation of the two gene transcripts suggests a common regulatory mechanism.

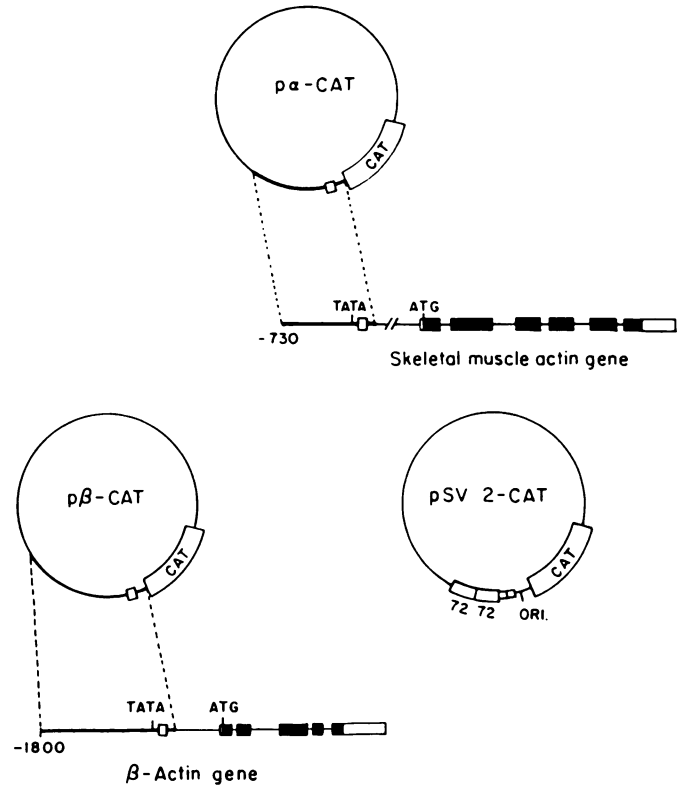

Fig. 7. The structure of plasmids containing the chimeric CAT genes. pSV2-CAT, pα-CAT and pβ-CAT are plasmids in which the SV40 early promoter, rat skeletal muscle actin promoter region and rat β-actin promoter region, respectively, were inserted 5′ to bacterial CAT structural gene. The schemes show the region of the actin genes that were used. Open and black bars represent untranslated and translated regions of the exons in the actin genes respectively. The construction of the plasmids is described in Materials and methods.

The expression of the bacterial CAT gene spliced to the promoter region of the skeletal muscle actin gene is developmentally regulated in myogenic cells

To test whether the increased expression following differentiation of the transferred gene was a specific, developmentally regulated process determined by a DNA sequence in the promoter region of the muscle gene, we compared the expression in myogenic cells of the bacterial CAT gene spliced to the 5′ region of the skeletal muscle actin gene (plasmid pα-CAT) with the expression of the same structural gene spliced to the 5′ region of the non-muscle cytoplasmic β-actin gene (pβ-CAT). It has been shown earlier that cytoplasmic actin mRNAs are present in mononucleated L8 cells in substantial amounts. The amount of this mRNA decreases after cell fu-

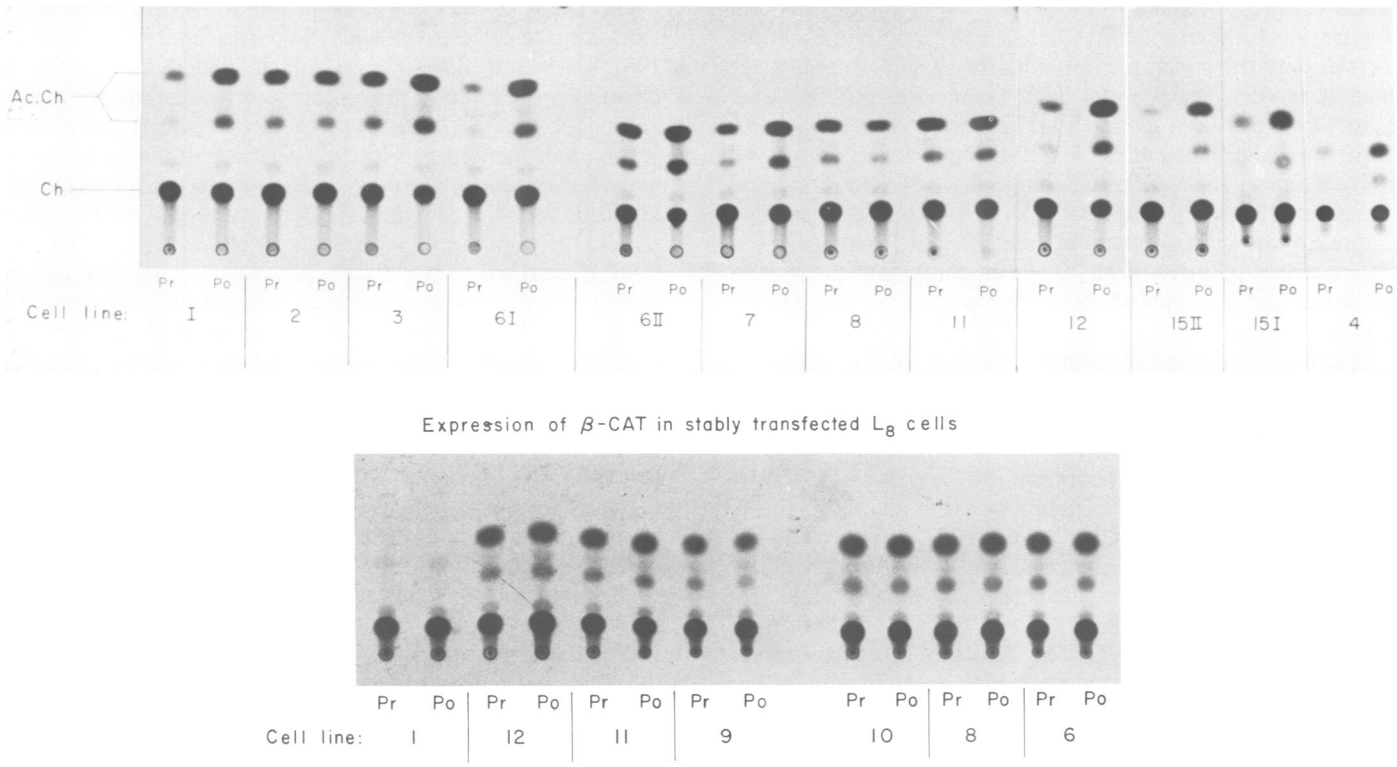

Fig. 8. (A) CAT activity in extracts of undifferentiated and differentiated cultures of the clones containing pα-CAT. CAT activity was assayed as described in Materials and methods. For each clone the same amount of extract from undifferentiated (Pr) and differentiated (Po) cell cultures was used. (Clone #8 did not undergo cell fusion; 'Po' extract was prepared from a culture grown for 72 h in 2HI medium). The [^{14}C]chloramphenicol (Ch) was separated from its acetylated forms (Ac.Ch.) by thin-layer chromatography and subsequently autoradiographed. **(B)** CAT activity in extracts of undifferentiated and differentiated cultures of representative clones containing pβ-CAT. The CAT assay was done as described in Materials and methods and in legend to Figure 8A. (The clone numbers in Figure 8A bear no relation to the clone numbers in Figure 8B.)

sion (Katcoff *et al.*, 1980; Shani *et al.*, 1981b). The inserted DNA in pα-CAT was an 810-bp long fragment extending from the *Eco*RI site 730 bp upstream from the cap site to the *Bst*EII site near the 5′ border of the intron in the 5′-untranslated region of the skeletal muscle actin gene (Figure 7). The inserted rat DNA in pβ-CAT was a 1.8-kb long fragment, extending from the *Xba*I site 1.6 kb upstream from the cap site to the *Hind*III site near the 5′ border of the first intron in the 5′ untranslated region of the rat β-actin gene (Figure 7). A third chimeric plasmid we used, pSV2-CAT, was obtained from C.Gorman and B.Howard (Gorman *et al.*, 1982); it contains the SV40 early promoter region 5′ to the CAT gene (Figure 7).

Myogenic L8 cells were co-transfected with each of the recombinant plasmids containing chimeric CAT genes and the plasmid $pIPB_1$ containing the neomycin resistance marker. Clones resistant to the neomycin derivative G418 were isolated. About 15 clones containing each of the chimeric plasmids were isolated, and most of them could be induced to differentiate. Southern blot analysis of DNA from these clones showed a great variability in gene copy number which ranged from a few to several hundred copies per haploid genome (not shown).

Extracts were prepared from undifferentiated and differentiated cultures of the clones containing the plasmids, and CAT activity was assayed. For each clone, the same amount of extract from differentiated and undifferentiated cells was assayed. The conversion of chloramphenicol to acetylated chloramphenicol by extracts from the clones containing the plasmids pα-CAT and from representative clones containing

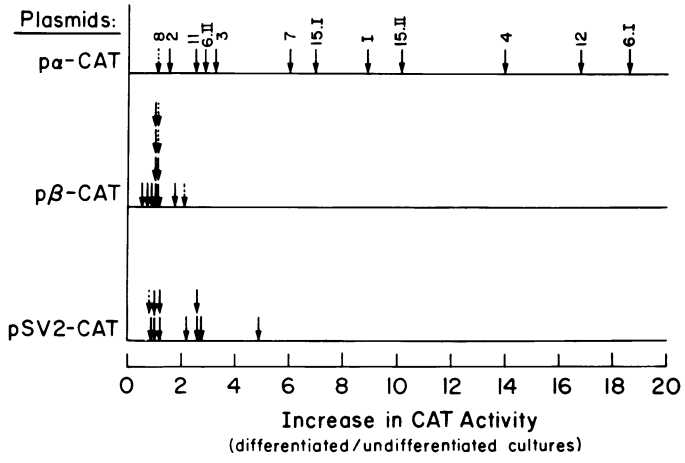

Fig. 9. The increase in CAT activity following differentiation of the clones containing pα-CAT, pβ-CAT or pSV2-CAT. Quantitation of CAT activity was done as described in Materials and methods. The numbers on the abscissa indicate the ratio between CAT activity in extracts from differentiated versus undifferentiated cultures of the same clone. Each arrow represents the ratio of activities from one clone. Broken arrows represent clones in which no cell fusion occurred after growth for 72 h in the differentiation stimulating 2HI medium.

the plasmid pβ-CAT, is shown in Figures 8a and 8b, respectively. The quantitation of the increase in CAT activity during differentiation is shown in Figure 9.

In spite of the considerable variability in the pattern of CAT activity in the transfected myogenic clone, it can be seen that the pattern of expression of CAT during differentiation

of the cultures is related to the plasmid used for transfection (Figure 9). In seven out of the 11 myogenic clones transformed with the plasmid containing the skeletal muscle actin gene promoter region, the activity in the differentiated cultures was between six and 19 times higher than the activity in the mononucleated cells. The kinetics of increase in CAT activity during differentiation (clone 6I) was very similar to the accumulation of the actin/globin transcripts shown in Figure 6b (data not shown). In contrast, CAT activity in the differentiated cultures of all 10 clones containing the β-actin promoter did not exceed twice the activity in the mononucleated cells.

The activity in the differentiated cultures of five out of 10 clones containing the plasmid pSV2-CAT gene was not higher than that of the mononucleated cells; in four clones the increase was ~2–3 times and in one clone 4.9 times (Figure 9).

The significance of the wider range of increase of CAT activity following differentiation observed in pSV2-CAT-containing clones compared to pβ-CAT clones is not known. It may be due to DNA sequences in the SV40 early promoter which are more active in differentiated muscle cells. Sequences responding to the induction of differentiation of erythroleukemic cells have been detected in the promoter region of the *Herpes* thymidine kinase gene (Wright *et al.*, 1983). Alternatively, the slight increase in activity may be due to unspecific mechanisms of stabilization of mRNAs or proteins after differentiation. If the latter is the case then the lack of increase in activity observed in the pβ-CAT-containing clones may be the net result of a down regulation of the β-actin gene promoter in differentiating muscle cells and stabilization of the CAT mRNA or of the enzyme in these cells.

Discussion

We have shown that a chimeric rat skeletal muscle actin/human ε-globin gene, introduced into myogenic cells and stably integrated into the genome, is expressed in an apparently developmentally regulated manner. Likewise, the expression of a chimeric gene composed of the promoter region of the rat skeletal muscle actin gene spliced to the bacterial CAT structural gene, stably integrated into the genome of myogenic cells, is induced during differentiation. On the other hand, the expression of CAT gene fused to the promoter region of a non-muscle actin is not changed or only slightly increased during differentiation of myogenic cells. The kinetics of accumulation in L8-46 cells of the chimeric actin/globin gene products during differentiaton is very similar to the kinetics of accumulation of endogenous skeletal muscle actin mRNA in the same cells. It seems, therefore, that the two genes respond to the same developmental signal. We have previously shown that the skeletal muscle actin gene and other muscle-specific genes are not preferentially sensitive to DNase I digestion in proliferating myoblasts, but become DNase I sensitive in differentiated cultures expressing these genes (Carmon *et al.*, 1982). Preferential sensitivity of genes to DNase I digestion has been correlated with transcriptional activity of the genes (Weintraub and Groudine, 1976; Garel and Axel, 1976). It is therefore likely that both the skeletal muscle actin gene and the transferred actin/globin chimeric gene are activated during differentiation at the transcriptional level.

Transfected DNA usually integrates at random, most often into a single or very few chromosomal sites; the sites of integration are different in each clone (de Saint Vincent *et al.*, 1981; Lacy *et al.*, 1983). Thus, it is unlikely that the regulated expression observed in the majority of the clones carrying the actin/globin or CAT genes was due to the integration of these genes in all the clones into chromosomal domains which are activated during the terminal differentiation of muscle cells. Had this been the case the expected results would be a similar pattern of donor gene expression in clones transfected with CAT gene fused to the β-actin promoter region. Rather, the results indicate that the region extending from −730 bp to the beginning of the intron in the 5′ untranslated region of the muscle actin gene contains information for the regulated expression of the gene during terminal differentiation.

This, however, does not exclude the possibility of the existence of additional control mechanisms acting at different levels. The expression of the transfecting genes varied greatly between the clones. Such variations were observed also in the expression of globin genes introduced into erythroleukemic cells (Chao *et al.*, 1983; Wright *et al.*, 1983). As in the investigations with the globin genes, no clear relation was found between the number of gene copies integrated into the genome and the expression of the donor gene. The variation in expression might reflect differences in the nature of the chromatin domain in the chromosomal site of integration, including long distance acting enhancer-like sequences, situated in the neighboring host DNA. It is quite possible that the inserted tissue-specific genes are expressed and responsive to the developmentally regulated activating signals only in those clones in which integration occurs in 'permissive' chromatin domains. No selection was made for clones expressing the actin chimeric genes. Nevertheless, the genes were expressed in a large proportion of the isolated clones. Since it seems that all or most of the donor DNA integrates in tandem into a single chromosomal site, it is most probable that by selection for neomycin resistant clones, one selects for those clones in which the donor DNA happened to integrate into a chromatin domain permissive for gene expression.

It should be noted that in most myogenic clones which expressed the transfected gene, small amounts of transcripts were detected even in RNA extracted from cultures of proliferating mononucleated cells. At that stage no native muscle actin mRNA was detected. Possibly the control of the transfected gene is more relaxed than that of the native gene. This is in agreement with several other observations suggesting that cloned genes introduced into host cells behave differently from the corresponding native genes. For example, α2u globulin is normally expressed in the liver and is induced by glucocorticoids. When an α2u globulin gene cloned in pBR322 was introduced into Ltk$^-$ cells, its expression could be induced by glucocorticoids. However, the native L cell α2u globulin gene remained inactive (Kurtz, 1981). Likewise, globin genes introduced into L cells were expressed while the endogenous globin genes were not (Mantei *et al.*, 1979; Wold *et al.*, 1979). It is probable that the establishment of additional control mechanisms which stabilize the program of gene expression (e.g., via methylation of DNA) is circumvented by the introduction of the genes into somatic cells, after early embryonic development (Jahner *et al.*, 1982; Stewart *et al.*, 1982; Jaenisch *et al.*, 1981; Lacy *et al.*, 1983).

The experiments presented here indicate correct initiation of transcription and polyadenylation of the actin/globin chimeric gene products. However, the endonuclease S1

analysis experiment, using the p749 probe, showed that in all clones in which transcripts of the donor gene were detected, smaller DNA fragments were also protected indicating that a fraction of the transcripts was not properly processed. These fragments must have resulted from hybridization between the probe and the chimeric gene transcripts, since they were never detected when RNA from myogenic cultures not carrying the actin/globin gene was examined. It also seems unlikely that the presence of the smaller protected fragments was a result of over-production of transcripts, which could not be handled by the processing system, since such fragments were observed even when the expression of the actin/globin gene was barely detectable (Figures 3 and 6a). These results may be attributed to transcription from transferred genes which underwent rearrangements. However, they may also indicate that, by replacing the 3′ region of the actin gene with a 3′ region of a globin gene, some information related to the proper processing was lost. Comparison of actin genes from different organisms reveals that while the 3′ untranslated regions of mRNAs of different actins (e.g., β cytoplasmic, skeletal muscle, and cardiac actins) share very limited sequence homology, there is a very impressive conservation of sequences of parts of the 3′ untranslated regions of genes coding for homologous actins in different organisms. Thus, a great part of the 3′ untranslated region of human and rat cardiac actin genes share homology of >92% (Mayer *et al.*, 1984). A great sequence homology is found also between parts of the 3′ untranslated regions of rat and human β actin genes (Nudel *et al.*, 1983; Hanukoglu *et al.*, 1983). Furthermore, there are also conserved sequences between the 3′ untranslated regions of rat and chicken skeletal muscle actin genes. This sequence conservation strongly suggests a functional role for this region of the gene or mRNA.

Comparison of the sequence of the rat and chicken skeletal muscle actin genes (Zakut *et al.*, 1982; Fornwald *et al.*, 1982) which separated at least 250 million years ago, reveals no considerable homology between the 5′ untranslated regions of the two genes. However, the regions between the CAAT and TATA box are quite conserved. Of particular interest is an almost identical sequence of 20 nucleotides which includes the CAAT box (Nudel *et al.*, 1984; Ordahl and Cooper, 1983). Several additional short conserved sequences are located 100–230 bp upstream from the CAP site (unpublished). The involvement of these sequences in the control of the developmentally regulated expression of the actin gene is now amenable to experimental analysis.

Materials and methods

Cell cultures

Mononucleated cells of the rat myogenic line L8 (Yaffe and Saxel, 1977) were grown in Weymouth medium supplemented with 15% FCS, which promotes proliferation without cell fusion. To induce cell fusion the medium was changed, when the cells reached confluency, to Dulbecco's modified Eagle medium supplemented with 2% horse serum and 0.1 μg/ml insulin (2HI medium; Yaffe and Saxel, 1977). This procedure induced a phase of rapid cell fusion which started ~30 h after the change of medium.

Construction of the plasmids containing the chimeric genes

Plasmid pCV. The 1.27-kb DNA fragment extending from the *Bam*HI site in the third exon of human ε-globin gene to the *Eco*RI site 172 nucleotides downstream from the poly(A) addition site (Baralle *et al.*, 1980), was spliced at the *Bam*HI site to the 3-kb DNA fragment extending from the *Eco*RI site 730 nucleotides upstream from the cap site of the rat skeletal muscle actin gene to the *Bam*HI site in the sixth exon of this gene (Zakut *et al.*, 1982). The chimeric actin/globin gene was then inserted into the *Eco*RI site of the plasmid pWR1 (Guo and Wu, 1983).

Plasmids pα-CAT and pβ-CAT. The recombinant plasmid PSVO-CAT (Gorman *et al.*, 1982) containing the bacterial CAT structural gene, the origin of replication and the ampicillin resistance marker of pBR322, and an early transcription unit from the SV40 genome, was used as a recipient DNA for the insertion of the promoter containing DNA fragments of rat actin genes. For the construction of pα-CAT, an 810-bp DNA fragment, extending from the *Eco*RI site 730 bp upstream from the cap site to the *Bst*EII site near the 5′ end of the intron in the 5′ untranslated region of the rat skeletal muscle actin gene, was used (Figure 7; Zakut *et al.*, 1982). For the construction of the β-CAT plasmid, an 1.8-kb DNA fragment, extending from the *Xba*I site 1.6 kb upstream from the cap site to the *Hind*III site near the 5′ end of the intron in the 5′ untranslated region of the rat β-actin gene, was used (Figure 7; Nudel *et al.*, 1983). The cohesive ends produced by the restriction enzymes were converted to blunt ends by filling in with *Escherichia coli* DNA polymerase large fragment (Klenow enzyme; Boehringer). The appropriate fragments were eluted from an agarose gel, and ligated into the *Hind*III site of pSVO-CAT which had been converted to blunt end sites. After ligation and transformation, positive colonies were identified by hybridization to the radiolabeled promoter containing fragments. The orientation of the inserted DNA fragments and the structure of the plasmids were verified by restriction endonuclease mapping.

Transfection of L8 myogenic cells with chimeric genes, and isolation of transformed cell lines

Transfection of L8 myoblasts with a mixture of the plasmids pCV and $pIPB_1$ (containing the neomycin resistant marker of Tn5 spliced to the promoter region of *Herpes simplex* thymidine kinase gene) was done essentially as described by Wigler *et al.* (1978) with minor modifications. 10 μg of plasmid DNA were used/10 cm plate containing 1.5–3 million cells. No carrier DNA was added and pCV was at 10–20-fold excess over $pIPB_1$. The $CaPO_4$-DNA precipitate was allowed to form for 30 min at room temperature and then diluted 1:4 in culture medium. The cells were incubated for 6 h in the precipitate containing medium, followed by a glycerol shock (incubation for 3 min at room temperature in 25% glycerol). The cells were subsequently washed several times with PBS, and then once with 5 mM EDTA. After growing for 18 h, the cells were diluted and transferred to several plates. 24 h later the medium was changed to the medium containing 300 μg/ml G418 (a gift from the Scherring Co.). After 8–10 days, a single clone was isolated from every dish and amplified. Transfection of L8 cells with the CAT-gene containing plasmids, was done as described by Maroteaux *et al.* (1983) with the addition of a glycerol shock step (incubation for 1 min in 10% glycerol). The efficiency of transfection was 1 stable transformation/5 x 10^5–5 x 10^6 cells.

Preparation of RNA

Total cellular RNA was prepared from undifferentiated and differentiated muscle cells by the lithium chloride/urea extraction method (Auffray *et al.*, 1980).

S1 endonuclease mapping analysis of RNA

The appropriate DNA fragments were end-labeled either by filling in with reverse transcriptase or by treatment with alkaline phosphatase and labeling with polynucleotide kinase. The Tm of each fragment (in the hybridization buffer) was determined. The hybridization with RNA (20–40 μg/sample) was carried out at temperatures ~2°C above the Tm of DNA/DNA hybrids. Digestion with endonuclease S1 was carried out as described by Berk and Sharp (1977). The sizes and amounts of protected DNA fragments were determined by electrophoresis on polyacrylamide/urea sequencing gels (Maxam and Gilbert, 1977) with subsequent fluorography and quantitation by scanning the fluorograms with a Beckman Du8 spectrophotometer.

Assay of CAT activity in cell extracts

CAT activity was measured according to Gorman *et al.* (1982). Aliquots of the cell extracts were incubated in 145 μl with 0.4 μCi of [^{14}C]chloramphenicol (sp. act. 50 μCi/μmol) in the presence of 4 mM acetyl CoA and 250 mM Tris-HCl pH 7.8. The reaction was optimized for each clone by adjusting the amount of cell extracts used. After 20–40 min of incubation at 37°C, the reaction was stopped by the addition of 1 ml ethyl acetate used to extract the chloramphenicol and its acetylated forms. The extracted material was concentrated and then spotted on thin layer chromatography plates (aluminium sheets silica 60, Merck). After chromatographing in chloroform:methanol (95:5) the plates were autoradiographed. Quantitation of the radioactivity of each spot was done by cutting the spot, and extracting the radioactive material (>90% efficiency) into tissue solubilizer (Eastman) which was then counted in a scintillation counter. The percentage conversion of chloramphenicol to the acetylated forms was calculated by dividing the radioactivity in the spots of the acetylated forms by the total radioactivity in the acetylated and non-acetylated chloramphenicol.

Preparation of plasmids and restricted DNA fragments

Plasmid DNA was prepared by the alkaline extraction method (Birnboim and Doly, 1979) and purified by centrifugation to equilibrium in CsCl/EtBr gradients. Restricted DNA fragments were purified by electrophoresis on agarose or acrylamide gels, followed by electroelution.

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