

# Developmentally regulated expression of a chicken muscle-specific gene in stably transfected rat myogenic cells

(gene transfer/sequence conservation/actin gene/globin gene/myogenesis)

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**ABSTRACT** To test the evolutionary conservation of DNA sequences specifying the developmentally regulated expression of the skeletal muscle actin gene, a recombinant plasmid containing the chicken skeletal muscle actin gene was introduced into rat myogenic cells. In a significant number of isolated clones, the accumulation of chicken actin mRNA increased greatly during differentiation. To test the expression in myogenic cells of a gene that is normally expressed during terminal differentiation of another tissue, rat myogenic cells were transfected with a mouse/human  $\beta$ -globin chimeric gene. A decrease by a factor of 2–3 in the amount of globin mRNA during differentiation was observed in most clones in which the gene was expressed. The results indicate the conservation of the muscle-specific regulatory DNA sequences for more than 300 Myr. Comparison of DNA sequences of the 5' flanking regions of rat and chicken skeletal muscle actin highly conserved sequences in the region of the "TATA" box and 180 base pairs upstream.

The accumulation of skeletal muscle actin mRNA is correlated with the differentiation of mononucleated myogenic cells into multinucleated fibers through cell fusion (1). In proliferating mononucleated cells of the myogenic cell line L8, the skeletal muscle actin gene is not preferentially sensitive to DNase I but becomes sensitive during the transition to the stage of cell fusion (2). These data indicate that the activation of the transcription of this gene occurs during the terminal differentiation process.

In a previous communication (3), the introduction of chimeric genes containing the promoter region of the rat skeletal muscle actin gene into rat myogenic cells was reported. In a significant number of the isolated clones carrying these genes, the expression of the chimeric genes greatly increased during differentiation. The temporal relation between differentiation of the cultures and accumulation of the transcripts of the transferred genes was very similar to that of the native skeletal muscle actin gene, suggesting a similar mechanism of regulation. The expression of chimeric genes containing the 5' region of the cytoplasmic  $\beta$ -actin gene did not increase during the differentiation of the transfected muscle cells (3). The experiments indicated that DNA sequences in the 5' region of the skeletal muscle actin gene are involved in the developmentally regulated expression of this gene. In the present study we asked whether the regulatory DNA sequences have been conserved during evolution. To this end we transfected rat myogenic cells with the chicken skeletal muscle actin gene. In a significant number of clones of transfected rat myogenic cells, the expression of the chicken skeletal muscle actin gene was developmentally regulated. These results indicate that DNA sequences determining the tissue-

specific expression of the skeletal muscle actin gene have been conserved for more than 300 Myr.

## MATERIALS AND METHODS

**Cell Cultures.** Mononucleated cells of the rat myogenic line L8 (4) were grown in Waymouth medium supplemented with 15% fetal calf serum, which promotes cell proliferation without cell fusion. To induce cell fusion the medium was changed, when the cells reached confluency, to Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 0.1  $\mu$ g of insulin per ml (2HI medium; ref. 4). This procedure induces a phase of rapid cell fusion, which started approximately 30 hr after the change of medium. The transfection and isolation of neomycin-resistant clones was done as described (3), with the exception that, 3–4 days before isolation of the clones, the growth medium was changed to the fusion-inducing medium 2HI, and only clones containing multinucleated fibers were marked and isolated.

**Recombinant DNA Procedures.** Recombinant bacteriophages and plasmids were grown and purified as described (5). Restriction endonucleases were used under conditions specified by the manufacturers (New England Biolabs).

**S1 Nuclease Analysis.** RNA was extracted from cultures of L8 cells by the lithium chloride/urea extraction procedure described by Auffray and Rougeon (6). Probes were labeled at their 3' end by filling in with reverse transcriptase or at their 5' end by successive treatment with bovine intestine alkaline phosphatase and polynucleotide kinase. Hybridization, treatment with S1 nuclease, and electrophoresis of the products were done as described by Weaver and Weissmann (7). In order to quantitate the protected probe, the fluorograms were scanned by a Beckman spectrophotometer and the areas of the peaks were calculated.

**Dot-Blot Hybridization.** Total RNA (3.5  $\mu$ g) was dissolved in 10  $\mu$ l of 5 mM methylmercuric hydroxide and was applied to a nitrocellulose sheet that had been washed in H<sub>2</sub>O overnight at 65°C and equilibrated with 10 $\times$  NaCl/Cit (1 $\times$  NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7). The nitrocellulose sheet was then washed with 10 $\times$  NaCl/Cit, dried at room temperature, and then baked for 2 hr at 80°C. It was then hybridized with a nick-translated *EcoRI*-*Bgl* II DNA fragment from the mouse-human  $\beta$ -globin gene (8). The blot was washed in 0.1 $\times$  NaCl/Cit and 0.1% NaDodSO<sub>4</sub> at 50°C.

**Southern Blot Analysis.** DNA was isolated from transfected cells as described (9). After digestion with *Hind*III, the DNA was electrophoresed on 1% agarose gel, blotted to nitrocellulose paper (10), and hybridized with nick-translated probe (specific activity, ca.  $4 \times 10^8$  cpm/ $\mu$ g of DNA).

## RESULTS AND DISCUSSION

**Expression of the Chicken Skeletal Muscle Actin Gene in Rat Myogenic Cells.** Cultures of mononucleated L8 cells were cotransfected with the recombinant plasmid pG $\alpha$ actin-

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1, containing a 6.2-kilobase chicken DNA insert, including the skeletal muscle actin gene plus 2 kilobases of the 5' flanking region and 2 kilobases of the 3' flanking sequence (11), and with a plasmid containing the neomycin resistance gene, pIPB1. Eleven neomycin-resistant muscle-forming clones were isolated and grown into mass cultures. Southern blot analysis showed that all clones contained the chicken DNA. The estimated gene copy number varied between 1 and 400.

Cell cultures of all clones were grown under conditions that favored cell proliferation without cell fusion, and RNA was extracted. In parallel, cells were induced to differentiate as described in *Materials and Methods* and were harvested after the formation of multinucleated fibers. The presence of the chicken actin gene transcripts was determined by S1 analysis as described in Fig. 1. In two clones no chicken actin mRNA was detected. Several clones expressed the gene in similar amounts prior to and after cell fusion. However, in at least six clones there was clearly a greater amount of chicken actin mRNA in RNA preparations from differentiated cultures. In clones 1a and 7a, the increase was 21.5- and 12.7-fold, respectively (Fig. 2; Table 1). A variability in the expression of the transferred gene in myogenic clones containing chimeric rat skeletal muscle actin genes and in cloned mouse erythroleukemia cells transfected with mouse/human  $\beta$ -globin chimeric gene or with human  $\beta$ -globin gene have been described and discussed elsewhere (3, 8, 13). As has been observed in other studies, there was no direct correlation between the number of integrated copies of the transferred gene and the expression of the gene (3, 8).

In the previous study in which myogenic cells were transfected with the rat skeletal muscle actin/human globin chi-

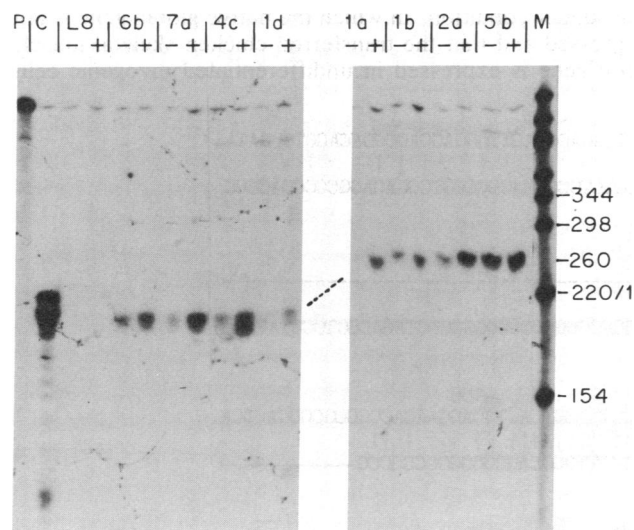


Fig. 1. S1 endonuclease analysis of RNA from myogenic clones containing the chicken skeletal muscle actin gene. The probe used was a 950-base-pair-long DNA fragment extending from the *Bcl* I site in the 3' untranslated region of the chicken skeletal muscle actin gene to the *Pvu* I site of pBR322 (12), labeled at the *Bcl* I site. Samples containing 20  $\mu$ g of total RNA from undifferentiated (lanes designated -) and differentiated (lanes designated +) cultures of the indicated clones were hybridized with approximately 20 ng of <sup>32</sup>P-labeled probe at 54°C. After the hybridization, the samples were treated with 1500 units of endonuclease S1 (30 min at 37°C), electrophoresed on an acrylamide/urea sequencing gel, and fluorographed. The probe protected a 260-nucleotide fragment of the 3' untranslated end of the chicken actin mRNA. Lanes: L8, RNA from untransfected L8 cells; C, RNA from chicken skeletal muscle; M, size markers (in nucleotides) of pBR322 digested with *Hinf*I; others, RNA from designated clones. The data for clones 3a, 3b, and 6a (in Table 1) were obtained from another experiment and are not shown in this figure.

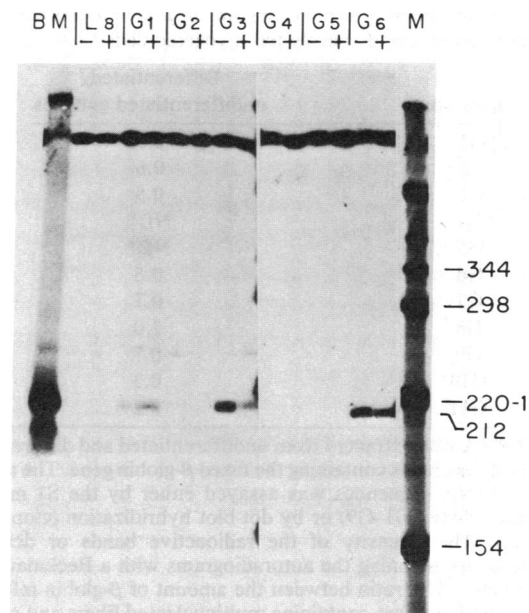


Fig. 2. S1 endonuclease analysis of RNA from myogenic clones containing the chimeric mouse-human  $\beta$ -globin gene. The probe used was a 750-base-pair-long DNA fragment extending from the *Eco*RI site in the third exon of the human  $\beta$ -globin gene to the *Pst* I site downstream from the gene (8) labeled at the *Eco*RI site. Samples containing 15  $\mu$ g of total RNA extracted from undifferentiated (lanes -) and differentiated (lanes +) cultures of the various clones (G1-G6) were hybridized to approximately 20 ng of labeled probe at 52°C. [In lane G1(-) (undifferentiated cultures), only 3  $\mu$ g of RNA was hybridized.] After the hybridization, the samples were treated with 1000 units of endonuclease S1 (30 min at 37°C), electrophoresed on an acrylamide/urea sequencing gel, and fluorographed. The analysis was also performed with RNA from untransfected L8 cells (lanes L8) and RNA from human bone marrow cells (lane BM). Lane M shows size markers (in nucleotides) of pBR322 digested with *Hinf*I.

meric gene, some clones showed a greater differentiation-associated increase (up to 50-fold) in the amount of transcripts of the transferred gene than was observed in the present study with the chicken skeletal muscle actin gene (3). It seems also that the proportion of clones showing constitutive expression of the transfected gene is higher in the

Table 1. Expression of the chicken skeletal muscle actin gene in transfected rat myogenic cells

Clone no.	Gene copy no.	Induction, -fold
1a	200-400	21.5
7a	3-6	12.7
4c	4-8	7.3
1d	1-3	4.5
2a	40-80	4.4
6b	150-300	3.4
6a	10-20	1.81
1b	4-8	1.44
5b	30-60	1.29
3a	2-4	ND*
3b	2-4	ND*

The copy number per genome of the integrated chicken actin gene was estimated from Southern blot analysis of *Hind*III-cut DNA by comparing the intensity of the bands produced on the fluorogram with those produced on tracks containing known amounts of *Hind*III-digested plasmid DNA mixed with carrier. The fold of induction was calculated by scanning the S1 fluorogram with a Beckman DU-8 spectrophotometer.

\*Chicken actin mRNA was not detectable in undifferentiated and differentiated cultures.



that do not express the native skeletal muscle actin gene suggests that the control of expression of the transferred genes is less stringent than the control of expression of the native genes. Transferred genes may escape some negative control mechanisms that suppress the expression of the native genes in nonexpressing tissues.

**The 5' Flanking Region of the Skeletal Muscle Actin Gene Is Highly Conserved.** We have reported earlier that while there is little homology in the DNA sequence between the 5' untranslated region of rat and chicken skeletal muscle actin genes, there is a considerable sequence homology in the "CAAT" box region (14, 15). Further analysis has shown the existence of four blocks of highly conserved sequences in the region extending from nucleotide -80 to nucleotide -230 (Fig. 3). The existence of such sequence homology between the rat and chicken skeletal muscle actin genes, which separated 300 Myr ago, indicates a very strong constraint to conserve this sequence. The regulated expression of the chicken skeletal muscle actin gene in the rat myogenic cells supports the suggestion that this conservation is associated with the regulation of expression of this gene. Sequence conservation in a similar region has been observed in the 5' flanking DNA of several other genes (e.g., human and rat growth hormone genes; ref. 16). The sequences of the rat fast muscle myosin light chain 2 gene and the 5' flanking region of the human myosin light chain 2 gene have been determined and compared (17). The region between the cap site and *ca.* 200 base pairs upstream is highly conserved in the two genes (>80% homology). Interestingly, no obvious sequence homology has been found in this region between the rat skeletal muscle actin gene and the myosin light chain 2 gene (except for a 100% homology in a sequence of 12 nucleotides that included the "TATAAA" box and 6 nucleotides upstream; ref. 17).

The present results do not exclude the possibility that additional regions of the gene are also involved in the transcriptional or post-transcriptional control of the tissue-specific expression of this gene. It should be mentioned here that there is also a great sequence conservation in the 3' untranslated region among genes coding for homologous (isotypic) proteins in different species—e.g., the rat and chicken skeletal muscle actin gene (15); the rat and human cardiac actin (18); and the rat, human, and chicken  $\beta$ -cytoplasmic-actin genes (refs. 19–21, 24).

It has been reported that the expression of the chicken skeletal muscle actin gene introduced into the mouse myogenic cell line C2 did not increase during the differentiation of the cultures (22). The restriction map indicates that the same gene was used in both investigations. One possible explanation for the difference in the results is the nature of the myogenic cells. We used a rat myogenic cell line in which the time of transition from cell proliferation to cell fusion was easily controlled; therefore, we were able to obtain sufficient quantities of mononucleated cells of a stage at which mRNA for the native skeletal muscle actin and other muscle-specific mRNAs is undetectable. In contrast, in our hands, the mouse C2 cells (23) tended to fuse even in sparse cultures, and it was not easy to obtain large quantities of proliferating mononucleated cells free of fusing cells. In fact, contrary to our results with the L8 line (1, 3), in the experiments reported by Eiler-Tuyns *et al.* (22), significant amounts of the native skeletal muscle actin mRNA were detected in RNA from cultures harvested on the day of plating of the mononucleated cells (0 time). Thus, it could be that the transferred chicken muscle actin gene already was activated at that

stage, and no further increase in activity could be detected. It also should be noted that, while the L8 line originated from newborn rat myoblasts (4), the C2 line originated from adult muscle satellite cells (23). Another important difference between our experiments and those described by Eiler-Tuyns *et al.* is that, while we examined the expression of the chicken actin gene in clonal cell populations originating from single transformed myogenic cells, they pooled and amplified the entire population of neomycin-resistant cells without cloning. Thus, perhaps the increased expression during differentiation in a fraction of the cell population was more difficult to detect on a background of the transcripts from cells in which the transferred gene was expressed constitutively.

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